Temporal Regulation of Human Cytomegalovirus Transcription at Immediate Early and Early Times After Infection

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The immediate early transcripts of human cytomegalovirus originated from restricted regions of the viral genome. In contrast, transcription at early times was complementary to all regions of the viral genome that were fractionated by restriction endonuclease treatment followed by agarose gel electrophoresis. The viral genome was also extensively transcribed when 2 h of protein synthesis or longer was permitted after infection in permissive cells treated with an inhibitor of viral DNA replication or in nonpermissive cells of animal origin that permit little or no viral DNA replication. The size and *in vitro* translation products of the cytomegalovirus-specified mRNA's at immediate early and early times after infection were determined. Discrete size classes of virus-specified polyadenylated RNA accumulated on the polyribosomes of cells infected in the presence of an inhibitor of protein synthesis. When 2 or 24 h of protein synthesis occurred after infection, there were changes in the relative abundance of the virus-specified RNAs that accumulated on polyribosomes. Treatment of infected cells with an inhibitor of viral DNA replication or infection of nonpermissive cells had little effect on the size classes of viral RNA found associated with the polyribosomes at early times after infection. These viral mRNA's were assumed to represent early viral gene expression. *In vitro* translation of the viral mRNA isolated from polyribosomes at immediate early and early times after infection identified many of the virus-specified gene products and demonstrated (i) a switch from immediate early to early viral gene expression and (ii) a prolonged phase of early viral gene expression. The data also indicated that the initiation of viral RNA synthesis does not depend on the formation of viral protein, but that *de novo* viral protein synthesis may influence the extent of transcription of the viral genome.

In cytomegalovirus (CMV)-infected cells, there is a sequential synthesis of viral RNA (12) and proteins (37). Viral RNAs synthesized in the presence of an inhibitor of protein synthesis are referred to as immediate early, and the proteins synthesized immediately after the removal of the inhibitor are also referred to as immediate early. Those proteins synthesized after at least 2 h of protein synthesis are referred to as early. Both immediate early and early CMV-induced proteins are detectable in infected cells before viral DNA replication or in the absence of DNA replication (37). However, late virus-induced proteins are not detectable in the absence of viral DNA replication (36, 37). Since late viral protein synthesis proceeds at a slow rate (37), it is assumed that the rate of CMV DNA replication influences the relatively slow production of infectious virus. CMV DNA replication for the Towne strain is initiated at approximately 12 h

after a high-multiplicity infection, but does not reach maximal rates until 72 to 96 h postinfection (p.i.) (33, 37). Thus, this herpesvirus replicates slowly, with infectious virus production usually not detectable until 48 to 72 h p.i. (15, 36).

The slow replication of human CMV is presumably related to the propensity of this virus to establish persistent infection in the host (24, 30, 31). The nature of this infection may reflect the activity of regulatory mechanisms influencing latency or productive infection. We have recently isolated cells carrying CMV genomes either in a latent-like state or held in the early stage of infection (26). In latently infected cells containing approximately 45 genome equivalents of CMV DNA per cell, there is relatively little immediate early antigen and no infectious virus. In cells held in the early stage of infection, viral early antigens are detectable, but late an-

tigens and virions are not. The above observations suggest a regulation in the expression of the CMV genome.

To study the relationship between transcription of the CMV genome and protein synthesis, virus-specified RNA synthesized at various times after infection was characterized according to location of origin on the physical map, size class, relative abundance, and in vitro translation product. Virus-specified RNA synthesized in the presence or absence of an inhibitor of viral DNA replication or in nonpermissive cells was also analyzed. This report demonstrates that CMV transcription is restricted to defined regions of the viral genome in the absence of de novo protein synthesis and that de novo protein synthesis influences changes in the extent of transcription, the size classes of the viral mRNA's, and the relative abundance of the viral mRNA's associated with polyribosomes. Many of the viral gene products coded for by immediate early and early viral mRNA were characterized.

MATERIALS AND METHODS

Virus and tissue culture. Plaque purification of human CMV (Towne strain) was previously described (37). The amount of infectious virus was determined by assays for plaques (42) or tissue culture infective doses (15). Permissive human fibroblast (HF) cells and nonpermissive guinea pig embryo fibroblast (GPEF) cells were prepared as previously described (37).

Viral infection, incubation with radioactive precursors, and definition of immediate early and early RNA. Cells were infected with CMV at a multiplicity of 10 to 20 PFU/cell. To radioactively label immediate early RNA, cells were treated with 100 or 200 μ g of cycloheximide (CH) per ml 1 h before infection, during the 1-h viral adsorption period, and during pulse-labeling with either phosphate-free Eagle medium containing 2% dialyzed fetal calf serum and 1 to 2 mCi of 32 P_i per ml or regular Eagle medium containing 10% fetal calf serum and 15 μ Ci of [3 H]-uridine per ml. Since HF cells contact inhibit, it was assumed that the majority of the cells used in an experiment were in the G₀ phase of the cell cycle and that 100 or 200 μ g of CH per ml should be sufficient to completely inhibit protein synthesis. Under these conditions, 99% or more of the incorporation of [35 S]-methionine into acid-insoluble radioactivity was inhibited. To label early RNA, 2 or 4 h of protein synthesis was permitted after infection, and then the cells were pulsed with either 32 P or [3 H]uridine in the presence of CH as described above. This RNA is also referred to as 2- or 4-h p.i. RNA. Early viral RNA was also obtained by pulsing at 24 h p.i. as described above, but in the absence of CH. This RNA is referred to as 24-h p.i. RNA.

Isolation of RNA. All reagents and glassware were treated with 0.1% diethyl pyrocarbonate (Sigma Chemical Co., St. Louis, Mo.) and autoclaved before

use. RNA was isolated from whole cells or from polyosomal fractions prepared by the method of Palmiter (28). Several different procedures were used. (i) When 8-h pulses with 32 P were used for blot hybridization, the cells were treated with 1% sodium dodecyl sulfate (SDS) and 100 μ g of proteinase K per ml (preheated to 37°C for 30 min; Beckman Instruments, Inc., Palo Alto, Calif.) at 37°C for 30 min. The nucleic acids were extracted with phenol at 55°C. Finally, the nucleic acids were extracted several times with chloroform. After dialysis against 0.02 M Tris-hydrochloride (pH 7.4) containing 0.01 M MgCl₂, the DNA was degraded by treatment with 20 μ g of RNase-free DNase per ml (Sigma) for 2 h at room temperature. The remaining RNA was extracted with phenol and chloroform-isoamyl alcohol and dialyzed briefly against 4 $\times$ SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate) and then 0.1 $\times$ SSC containing 0.01 M EDTA. The RNA was heated to 100°C for 1 min and then added to hybridization buffer prepared as described by Jeffreys and Flavell (20). (ii) When 2-h pulses with 32 P were used for blot hybridization, the cells were lysed in 5.8 M guanidine-hydrochloride containing 0.05 M lithium citrate, 0.45% Sarkosyl, and 0.45% β -mercaptoethanol. The cell lysate was layered onto 1.745 g of CsCl per ml containing 50 μ g of spermine per ml and centrifuged in an SW27.1 rotor at 100,000 $\times g$ for approximately 24 h. The translucent RNA-containing pellet had less than 0.6% DNA contamination when tested by the diphenylamine reaction (17). The RNA was extracted three times with phenol and twice with chloroform-isoamyl alcohol and then precipitated with ethanol. The second procedure, referred to as the guanidine-hydrochloride-CsCl gradient method, yielded approximately twofold more RNA. Since DNase treatment was not used, this procedure was used only for pulses before the initiation of viral DNA replication. The RNA was suspended in water containing 0.5% SDS, heated to 100°C for 1 min, and added to hybridization buffer as described above. (iii) Polyribosome-associated RNA was isolated by the magnesium precipitation method of Palmiter (28) as described by Stringer et al. (39).

Viral DNA blots and RNA hybridization. Viral DNA was purified as previously described (38) and cleaved by treatment with restriction enzyme *Xba*I or *Hind*III (2.5 U/ μ g; New England Biolabs, Beverly, Maine). The resulting DNA fragments were fractionated by electrophoresis in 0.5% agarose gels by the method of Bachi and Arber (4) and immobilized onto nitrocellulose filters by the method of Southern (35). Filters containing immobilized viral DNA were pre-treated at 68°C with hybridization buffer prepared as described by Jeffreys and Flavell (20). After addition of 32 P-labeled RNA, the filters were incubated at 68°C for 48 h. The DNA concentration on the blot strip was approximately 10-fold higher than the concentration of the CMV RNA, and consequently the counts hybridized were proportional to the concentration of the input RNA (data not shown). After hybridization, the filters were washed with 2 $\times$ SSC, treated with 20 μ g of RNase A per ml (heated to 80°C for 10 min; Sigma) in 2 $\times$ SSC for 30 min at room temperature, and then washed six times for 15-min periods with hybridization

buffer at 68°C. Hybridization of virus-specific [³²P]-RNA was detected by autoradiography for 24- or 48-h periods. All immobilized viral DNA fragments were detected by hybridizing ³²P-labeled single-stranded viral DNA labeled by nick translation as described by Rigby et al. (32).

Gel electrophoresis of denatured poly(A) RNA. Polyadenylated [poly(A)] RNA was selected from the total polysome-associated RNA by using oligodeoxythymidylic acid-cellulose (Collaborative Research, Inc., Waltham, Mass.). After the poly(A) RNA was bound to cellulose in 0.01 M Tris-hydrochloride (pH 7.5) containing 0.5 M NaCl, the poly(A) RNA was eluted in water. Approximately 5% of the total RNA was recovered as poly(A) RNA. The poly(A) RNA was fractionated by electrophoresis in cylindrical or slab 1.0% agarose gels containing 10 mM methylmercury hydroxide (Alpha, Danvers, Mass.) as described by Bailey and Davidson (5). Electrophoresis was at 11.5 mA/cm² at 20°C. Molecular weight standards for slab gels were 23S (3.3 kilobases [kb]) and 16 S (1.7 kb) *Escherichia coli* rRNA (7) and 28S (5.3 kb) and 18S (2.0 kb) human cell rRNA (3). Slab gels were stained in a solution containing 1 µg of ethidium bromide per ml and 0.5 M ammonium acetate. For cylindrical gels, ³²P-labeled 28S and 18S human cell rRNA's were used as molecular weight standards. Cylindrical gels were sliced into 1-mm fractions and solubilized with 7% perchloric acid at 78°C. After addition of Aquasol-2, radioactivity in the gels was counted in a liquid scintillation spectrometer. The ³²P counts occurring in the channel set for ³H counting, which were approximately 20% of the total counts per minute, were subtracted from the total counts.

Viral RNA blots and DNA hybridization. After fractionation of the denatured poly(A) RNA in an agarose gel, the gel was treated with a solution containing 50 mM NaOH and 5 mM 2-mercaptoethanol for 40 min at room temperature. The gel was washed three times in 200 mM potassium phosphate buffer (pH 6.5) containing 7 mM iodoacetic acid for 5-min periods and then twice in 0.2 M sodium acetate (pH 4.0) for 5-min periods. The RNA was transferred to activated diazobenzyloxymethyl (DBM)-paper with 0.2 M sodium acetate, pH 4.0, as described by Alwine et al. (1). The DBM-paper containing the transferred RNA was incubated for 24 h at 42°C in hybridization buffer containing 50% formamide, 0.75 M NaCl, 0.075 M sodium citrate, 0.02% (wt/vol) each bovine serum albumin, Ficoll, and polyvinylpyrrolidone, 2.5 mg of sonicated calf thymus DNA per ml, and 1% (wt/vol) glycine. [³²P]CMV DNA was prepared as described above and heated to 100°C for 2 min. Hybridization was for 36 to 48 h at 42°C with approximately 900,000 cpm of [³²P]CMV DNA per cm² in hybridization buffer minus glycine. After hybridization, the DBM-paper containing the RNA:DNA hybrids was washed six times for 45-min periods with a solution containing 50% formamide and 5× SSC and once with 2× SSC. Hybridization of [³²P]CMV DNA was detected by autoradiography.

Hybridization of RNA to DNA bound to cellulose. Total CMV DNA was bound to diazotized cellulose by the method of Noyes and Stark (27) as described by Anderson et al. (3). ³²P-labeled DNA was

included in the reaction so that the efficiency of coupling could be monitored. A total of 65 to 75% of the input radioactivity remained bound to the cellulose after extensive rinsing. Approximately 250 µg of infected-cell ³H-labeled polysome-associated RNA was hybridized to 50 µg of CMV DNA bound to cellulose. Hybridizations were carried out with viral DNA in approximately 50-fold excess by mass of the viral RNA. Hybridization was for 4 h at 57°C in 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 8.0) containing 70% formamide, 0.5 M Na⁺, and 0.005 M EDTA for immediate early and early RNA preparations. The *T_m* of CMV DNA (Towne strain) in the above buffer was determined to be 60.5°C (T. Loepfe and M. F. Stinski, unpublished data). After hybridization, the cellulose was rinsed twice with 2× SSC at room temperature, twice with hybridization buffer at room temperature, and six times with hybridization buffer at 60°C. The hybridized RNA was eluted in 99% formamide containing 10 mM HEPES (pH 8.0) at 60°C. The eluent was diluted to a final formamide concentration of 22% and adjusted to 0.2 M sodium acetate, and then the RNA was ethanol precipitated. The percentage of the input radioactivity recovered for immediate early and early RNA was approximately 4.7%. When ³H-labeled uninfected-cell polysome-associated RNA was added to the CMV DNA cellulose as described above, 0.2% of the input radioactivity was recovered.

The poly(A) RNA was selected from the total hybridized RNA by oligodeoxythymidylic acid-cellulose chromatography as described above. For the immediate early and early hybridized RNA, usually 30 to 50% was recovered as poly(A) RNA. For the uninfected-cell RNA, approximately 10% was recovered. The uninfected-cell poly(A) RNA represented 0.03% of the input radioactivity or approximately 0.1 µg of the 250-µg input of polysome-associated RNA.

In vitro translation of RNA and analysis of polypeptide products. RNA was translated in vitro, using a micrococcal nuclease-treated rabbit reticulocyte lysate system prepared by the method of Pelham and Jackson (29). [³⁵S]methionine was used to label polypeptide translation products. Poly(A) RNA samples in 0.1 M KCl with 10 µg of calf thymus tRNA (Boehringer Mannheim Corp., Indianapolis, Ind.) were precipitated with ice-cold 95% ethanol, and the pellet was rinsed once with ice-cold 70% ethanol containing 0.1 M KCl. Each 25-µl assay contained 10 µl of reticulocyte lysate, 10 to 20 µCi of [³⁵S]methionine (600 to 1,400 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), and 10 µl of mRNA in water. After incubation of the assay at 37°C for 60 min, 5 µl of a 100-µg/ml concentration of pancreatic RNase and 20 U of T₁ RNase per ml in 25 mM EDTA were added, and the mixture was incubated for an additional 10 min at 37°C.

Infected cells were pulse-labeled with [³⁵S]methionine at immediate early and early times as previously described (37).

Polypeptides after in vitro translation or pulse-labeling of infected cells were fractionated by electrophoresis in discontinuous SDS-polyacrylamide slab gels according to the Laemmli (25) method and as previously described (36, 37). Dried gels were autora-

diographed with Kodak Tri-X Pan film. Molecular weights of *in vitro* translation products were determined from their migration relative to unlabeled molecular weight markers as previously described (36, 37) and labeled translation products of RNA from adenovirus type 2-infected cells.

RESULTS

Transcription before and after *de novo* protein synthesis. The CMV immediate early and early RNA was analyzed to determine the effect of protein synthesis on the origin of the transcripts. ^{32}P -labeled RNA was extracted from the cells and hybridized to viral DNA restriction enzyme fragments which had been fixed to nitrocellulose filters as described in Materials and Methods. Approximately 10^6 cpm of ^{32}P -labeled immediate early RNA was used for hybridization to *Xba*I restriction enzyme digests of the viral genome. The physical map of the *Xba*I DNA fragments for the Towne strain of human CMV was determined by R. LaFemina and G. S. Hayward (unpublished data) and is illustrated in Fig. 1. In the absence of protein synthesis, the immediate early transcripts of CMV obtained after 2- or 8-h pulses with ^{32}P , originated from restricted regions of the viral genome (Fig. 2). This immediate early RNA originated primarily from the *Xba*I fragments DEF and N (Fig. 2). Relatively lower amounts of hybridization to *Xba*I fragments ABC, M, and R were detected (Fig. 2). An extremely low amount of hybridization was detected to *Xba*I fragments L, O, and Q when using the RNA from a 2-h pulse which rendered a lower background on the nitrocellulose filter. Hybridization to fragment R was not detected when using RNA from a 2-h pulse. Since hybridization to cloned fragments of A, B, and C was not detected with immediate early RNA (M. W. Wathen and M. F. Stinski, unpublished data), the *Xba*I ABC fragment may have had some incompletely digested viral DNA that hybridized with the immediate early RNA.

The abundant immediate early RNA originated from a region in the large unique section of the viral genome of 0.66 to 0.77 map units (fragments E and N), and the RNA in low abun-

dance originated from fragments containing the large repeat sequences (fragments D, F, M, O, and Q) and two regions in the large unique region of 0.22 to 0.25 (fragment L) and 0.64 to 0.66 (fragment R) map units.

Labeled RNA samples from uninfected cells had no detectable hybridization to any DNA fragment. Last, similar restricted transcription was detected in nonpermissive GPEF cells infected with CMV and maintained in the presence of CH (data not shown).

After infection and 2, 4, or 24 h of protein synthesis, whole-cell virus-specified RNA that accumulated in the presence of CH was complementary to most of the restriction enzyme DNA fragments fractionated by agarose gel electrophoresis (Fig. 2).

The above observations indicated that a switch from restricted to extensive transcription occurred within the first 2 to 4 h p.i., but significant differences between 4 and 24 h were not detected by the Southern blot method.

Transcription in the absence of viral DNA replication. To determine whether extensive transcription of the human CMV genome at early times after infection was affected by viral DNA replication, viral RNA synthesized in the absence of viral DNA replication was analyzed by the Southern blot procedure. We have previously reported that the synthesis of CMV-induced immediate early and early proteins is detectable in nonpermissive GPEF cells but late virus-induced proteins are not detectable (37). In addition, late virus-induced proteins of CMV are not detectable in permissive HF cells treated with phosphonoacetic acid (PAA), an inhibitor of viral DNA replication (36, 37). To inhibit viral DNA synthesis in the permissive cell, 200 μg of PAA per ml was added at 1.5 h p.i. After several hours of protein synthesis, the cells were pulse-labeled with ^{32}P in the presence of CH. Extensive transcription of the CMV genome occurred in both the nonpermissive cells (pulsed 8 to 16 h p.i.) and the permissive cells (pulsed 24 to 32 h p.i.) treated with an inhibitor of viral DNA replication (Fig. 3). RNA synthesized in the absence of viral DNA replication hybridized

FIG. 1. Physical map of the human CMV (Towne strain) genome. The genome consists of unique (U) sequences in a long (L) and short (S) section that are bounded by terminal repeats (TR) and inverted internal repeats (IR). The *Xba*I physical map was determined by LaFemina and Hayward (unpublished data) and represents the prototype arrangement. An inversion of the S section of the viral genome will render an *Xba*I fragment designated as F, which is not shown.

FIG. 2. Autoradiographs of whole-cell viral RNA hybridized to a restriction enzyme digest of CMV DNA. To label virus-specified immediate early RNA, cells were treated with CH (100 $\mu\text{g}/\text{ml}$) before infection, during the viral adsorption period, and during pulse-labeling with ^{32}P from 1 to 9 (track a) or 1 to 3 (track e) h p.i. For early RNA, infected cells were maintained in the absence of CH for various periods of time and then pulse-labeled for 8 (tracks b and c) or 2 (tracks f and g) h in the presence of CH. RNA was isolated from whole cells and hybridized to XbaI restriction enzyme digests of CMV DNA immobilized onto nitrocellulose filters as described in the text. The immobilized viral DNA was detected by hybridization of ^{32}P -labeled viral DNA (track d). All major immobilized viral DNA fragments obtained with the restriction enzyme are alphabetically labeled according to size.

to all DNA restriction fragments fractionated by agarose gel electrophoresis. There were few differences in the relative amounts of transcription from the various regions of the viral genome in the presence (Fig. 3) or absence (see Fig. 2) of viral DNA replication at 24 h p.i. Therefore, inhibition of viral DNA replication did not have a significant effect on the relative amount of transcription from the various regions of the viral genome at an early time of 24 h p.i.

Size classes of CMV-specified poly(A) RNA at various times after infection. To further investigate the relationship between transcription of the CMV genome and protein synthesis, viral poly(A) RNA was isolated from the polysome-associated RNA of infected cells at various times after infection. The RNA was fractionated according to size in gels containing the denaturant methylmercury hydroxide as described in Materials and Methods. Molecular

FIG. 3. Autoradiographs of whole-cell viral RNA synthesized in the absence of viral DNA replication and hybridized to restriction enzyme digests of CMV DNA. Nonpermissive GPEF cells and permissive HF cells treated with PAA (200 µg/ml) were pulse-labeled with ³²P for 8-h periods, and the whole-cell RNA was isolated as described in the text. ³²P-labeled RNA or viral single-stranded DNA was hybridized to either a HindIII or XbaI digest of CMV DNA immobilized onto nitrocellulose filters as described in the text.

weight standards of 5.3, 3.3, 2.0, and 1.7 kb were used. There was a linear relationship between the log of the chain length (in kilobases) of the standard RNAs and the distance migrated. The sizes of the viral mRNA's were interpolated from this type of standard curve, which was prepared for each experiment.

Virus-specified RNA was transferred to DBM-paper and detected by hybridization of a ^{32}P -labeled CMV DNA probe followed by autoradiography as described in Material and Methods. Immediate early RNA, which is the RNA accumulating during a 12-h period in the presence of 100 or 200 μg of CH per ml, consisted of three size classes of 4.8 to 4.6, 2.2, and 1.9 kb (Fig. 4). These virus-specific RNAs represented the abundant and stable mRNA's that accumulated in the infected cells during a 12-h period in the presence of CH. There were also several size classes in low relative abundance, such as the 1.2-kb RNA, that were at the limits of detectability when 5 μg of poly(A) RNA was analyzed (data not shown). The largest size class of virus-specified RNA was occasionally fractionated into a doublet of 4.8 and 4.6 kb (Fig. 4). No RNA was detected when 10 μg of uninfected cell poly(A) RNA was fractionated in methylmercury hydroxide gels and ^{32}P -labeled CMV DNA was used for hybridization (Fig. 4).

Two hours of protein synthesis was permitted, and the RNA size classes accumulating in the infected cell during a 12-h period in the presence of CH were analyzed. The CH treatment was necessary to allow for an accumulation of virus-specific RNA. Under these conditions, the large 4.8- to 4.6-kb species of virus-specified RNA was greatly reduced in relative amount and was only detected when 5 μg of poly(A) RNA was analyzed (data not shown). In contrast, the 2.2- and 1.9-kb size classes of virus-specified RNA were still detected in relatively high concentrations. In addition, viral RNA size classes of 2.7 and 1.2 to 0.85 kb were detected.

Virus-specified RNA associated with polyribosomes was also analyzed at 24 h p.i. without CH treatment of the infected cells. The abundant viral mRNA's were of the 2.7- and 1.2-kb size classes, and those in lower abundance were of the 1.9-, 1.7-, 1.5-, and 0.85-kb size classes (Fig. 4). Viral RNA size classes of 4.8 to 4.6 and 2.2 kb, which were characteristic of immediate early RNA and 2-h p.i. RNA, respectively, were not detected. At 24 h, there were large size classes of mRNA ranging in size from 7.6 to 5.0 kb that were detected only when 5 μg of RNA was analyzed (data not shown). The major differences between 2-h and 24-h virus-specified RNA was a shift in the relative abundance of certain size classes of RNA.

FIG. 4. Autoradiographs of virus-specified poly(A) RNA associated with the polyribosomes at various times after infection. The standard RNAs were 3.3- and 1.7-kb *E. coli* rRNA (7) and 5.3- and 2.0-kb human cell rRNA (3). The RNAs were fractionated in agarose gels containing 10 mM methylmercury hydroxide as described in the text. A standard curve was prepared for each methylmercury hydroxide-gel and used to interpolate the sizes of the virus-specified RNAs. One microgram of immediate early polysome-associated poly(A) RNA or poly(A) RNA associated with the polyribosomes after 2 or 24 h of protein synthesis in infected cells was fractionated according to size in 1.0% agarose gels containing 10 mM methylmercury hydroxide and transferred to DBM-paper as described in the text. Viral RNA was detected by hybridization of ^{32}P -labeled CMV DNA followed by autoradiography. Virus-specified RNA is designated according to kilobase size.

Size classes of CMV-specified poly(A) RNA at 24 h in the presence or absence of viral DNA replication. To determine whether the shift in size classes and relative abundance of viral RNA on polyribosomes at early times after infection was influenced by viral DNA replication, 200 μg of PAA per ml was added at 1 h p.i. to inhibit viral DNA replication. Poly(A)

RNA was isolated from the polyribosomes of PAA-treated and nontreated permissive cells at 24 h p.i. and analyzed as described above. In addition, nonpermissive GPEF cells infected with human CMV were analyzed at 24 h p.i. In the absence or presence of viral DNA replication, the abundant size classes of virus-specific RNA were the 2.7-, 1.9-, 1.7-, 1.5-, and 1.2-kb RNAs (Fig. 5). All virus-specified RNAs that were detected at 24 h p.i. were also detected in the presence of PAA (Fig. 5). When nonper-

Polysome-Associated poly (A) RNA

No
PAA PAA GPEF kb

FIG. 5. Autoradiographs of virus-specified poly(A) RNA associated with the polyribosomes of nonpermissive or permissive cells in the presence or absence of an inhibitor of viral DNA replication. The permissive cells were treated with PAA (200 μ g/ml) or left untreated. Then 1 (permissive cells) or 2 (nonpermissive GPEF cells) μ g of polysome-associated poly(A) RNA was analyzed. The RNA was fractionated in agarose gels containing methylmercury hydroxide and then transferred to DBM-paper as described in the text. Viral RNA was detected by hybridization of 32 P-labeled CMV DNA followed by autoradiography. Virus-specified RNA is designated according to kilobase size.

sive GPEF cells were analyzed, the 2.7-, 1.9-, 1.7-, 1.5-, and 1.2-kb size classes were detected (Fig. 5). In addition, a large size class of 5.4 kb was detected when only 2 μ g of RNA was analyzed. This size class of RNA was present in extremely low amounts in the permissive cells when 5 μ g of RNA was analyzed (data not shown). The relative abundances of the various size classes of virus-specified RNAs at this time after infection were judged empirically to be similar in the presence or absence of viral DNA replication in the permissive cells (Fig. 5). In contrast, there was a considerable reduction in the relative amount of viral RNA in the nonpermissive GPEF cells even though these cells were infected at the same multiplicity as the permissive cells (Fig. 4). Nevertheless, inhibition of viral DNA replication did not cause a significant change in the size classes of virus-specified RNA associated with the polyribosomes at an early time of 24 h p.i. The shift in size classes and relative amounts of virus-specified RNA between 2 and 24 h p.i. are presumably related to the transcription and translation of the viral genes.

Selection of CMV-specified poly(A) RNA synthesized at various times after infection by hybridization to viral DNA bound to cellulose. CMV-specified RNA was separated from host cell RNA by hybridization to viral DNA bound to cellulose. The purpose of this experiment was to confirm the major size classes of virus-specified RNA detected by the Northern blot hybridization procedure and to demonstrate the isolation of viral mRNA for in vitro translation.

By using large quantities of viral DNA (50 μ g) bound to cellulose, even minor species of viral RNA should be selected from the mixture of RNAs by hybridization. CMV-infected cells were pulse-labeled with [3 H]uridine for 8-h periods in the presence of CH as described in Materials and Methods. Polysome-associated RNA was hybridized to CMV DNA-cellulose, the virus-specified RNA was eluted, and the poly(A) RNA was selected by oligodeoxythymidylic acid-cellulose chromatography. The virus-specified poly(A) RNAs were fractionated by electrophoresis in cylindrical gels containing methylmercury hydroxide as described in Materials and Methods. Immediate early virus-specified poly(A) RNAs of 4.8- to 4.6-, 2.2-, and 1.9-kb size classes were isolated (Fig. 6A). In addition, RNA size classes in the range of 1.2 to 0.85 kb were isolated by this procedure.

When 2-h p.i. virus-specified RNA was hybridized to CMV DNA-cellulose, the 2.2- and 1.9-kb size classes were isolated in high relative abundance, and the 2.7-, 1.7-, 1.5-, and 1.2- to

FIG. 6. Electrophoresis of CMV-specified poly(A) RNA selected from total polysome-associated RNA by hybridization to CMV DNA bound to cellulose. Infected cells were pulse-labeled for 8 h with [3 H]uridine as described in the text. RNA that was associated with polyribosomes under immediate early conditions and after 2 h of protein synthesis followed by 8 h of CH treatment was isolated and then hybridized to 50 μ g of CMV DNA bound to cellulose. After elution of virus-specified RNA, poly(A) RNA was selected by oligodeoxythymidylic acid-cellulose chromatography and fractionated in agarose gels containing 10 mM methylmercury hydroxide as described in the text. (A) Immediate early RNA (24,000 cpm; approximately 0.50 μ g); (B) early RNA (39,000 cpm; approximately 0.75 μ g). 32 P-labeled 5.3- and 2.0-kb eucaryotic cell rRNA was used as internal size markers. The kilobase size classes of virus-specified poly(A) RNAs are indicated.

0.85-kb size classes were isolated in lower relative abundance (Fig. 6B).

When RNA from uninfected cells was isolated, incubated with CMV DNA-cellulose under hybridization conditions as described above, eluted, and subjected to oligodeoxythymidylic acid-cellulose chromatography, an insufficient amount of [3 H]RNA was obtained for analysis by methylmercury hydroxide-gel electrophoresis.

In vitro translation of virus-specified immediate early and early mRNA. The Northern blot and the selective hybridization analysis indicated major changes in the relative amounts of the various size classes of viral RNA associ-

ated with the polyribosomes at various times after infection. This was particularly demonstrable with immediate early and 24-h p.i. viral poly(A) RNA. The viral mRNA associated with polyribosomes was isolated by hybridization to CMV DNA bound to cellulose at immediate early times and at two early times after infection. For one of the early times, 6 h of protein synthesis was permitted after infection, the cells were treated for 8 h with CH to allow for an accumulation of virus-specific RNA, and then the polyribosome-associated poly(A) RNA was isolated. For the other early time, polysome-associated poly(A) RNA was isolated at 24 h p.i. from cells not treated with CH. The viral

mRNA's from these various periods after infection were used for *in vitro* translation with [³⁵S]methionine in a rabbit reticulocyte system, and the translation products were fractionated by electrophoresis on denaturing SDS-containing polyacrylamide gels as described in Materials and Methods. Two controls were used to identify polypeptides endogenous to the rabbit reticulocyte system. These were no RNA and uninfected-cell RNA that was incubated with CMV DNA bound to cellulose under the hybridization conditions previously described. Approximately 0.3% of the input uninfected-cell RNA (250 μ g) was eluted from the DNA-cellulose. This RNA was subjected to oligodeoxythymidylic acid-cellulose chromatography, and approximately 0.03% of the original amount of uninfected-cell RNA was recovered. The RNA was precipitated with 10 μ g of calf thymus tRNA and used for *in vitro* translation as described above. *In vitro* translation of approximately 0.5 μ g (estimated from the specific activity of the ³H-labeled viral RNA eluted from the viral DNA-cellulose) of immediate early poly(A) RNA detected nine polypeptides that are designated according to their apparent molecular weights ($\times 10^3$) as 75, 72, 59, 56, 42, 39, 27, 16.5, and 6.7 (Fig. 7A). These polypeptides represented the prominent translation products of immediate early viral mRNA's selected by the hybridization conditions described in Materials and Methods. There were a few polypeptides present in extremely low concentration when immediate early RNA was translated that are indicated but not designated according to apparent molecular weight. Polypeptides below 56 and above 42 may also have been virus specified, but the controls also had some polypeptides with the same electrophoretic mobilities at low relative concentrations. Even though an endogenous polypeptide had the same electrophoretic mobility as the immediate early polypeptide 72, this protein was considered to be virus specified because it had a high relative rate of synthesis when viral mRNA was used, and an infected-cell specific polypeptide of the same electrophoretic mobility was detected in high relative amounts in CMV-infected cells (Fig. 7B). At early times after infection, approximately 0.5 μ g of virus-specified mRNA coded for additional polypeptides that are designated according to their apparent molecular weights ($\times 10^3$) as 82, 64, 44, 41, 27, 20, 16, 11, 8, and 6.9 (Fig. 7A). Additional polypeptides are indicated but are not designated according to apparent molecular weight because of their low relative amounts.

There was a noticeable switch in the *in vitro* translation profile from immediate early to early times. Many of the polypeptides coded for by

immediate early mRNA were greatly reduced in relative amount or were no longer detectable (Fig. 7A). In contrast, there was little to no change in the *in vitro* translation profile when viral mRNA isolated from polyribosomes after 6 or 24 h of protein synthesis were compared (Fig. 7A). Since the apparent molecular weights of the majority of these virus-specified polypeptides were different from those of the majority of the late viral structural polypeptides previously described (37), these proteins were assumed to represent early viral gene products.

Many of the immediate early and early *in vitro*-translated polypeptides had an electrophoretic profile similar to the infected-cell-specific polypeptides (ICSP) detected by pulse-labeling cells with [³⁵S]methionine (Fig. 7B). However, the viral proteins synthesized *in vivo* were presumably subjected to post-translational modifications, such as phosphorylation, glycosylation, and proteolytic cleavage, that could alter the electrophoretic mobility. As previously described (37), the ICSP in the apparent molecular weight range of 59,000 to 53,000 were detected in low relative amounts under immediate early pulse-labeling conditions but in higher relative amounts when 1 to 2 h of protein synthesis was permitted. Although there were few host cell proteins detected by pulse-labeling at immediate early times, host cell proteins were detectable in low relative amounts when pulse-labeling at 6 h p.i., as previously described (37).

Finally, it was estimated that 0.05 to 0.07 μ g of host cell RNA could be contaminating the virus-specified RNA selected by hybridization to CMV DNA bound to cellulose and by oligodeoxythymidylic acid-cellulose chromatography. Whether this RNA represented rRNA or a small amount of uninfected-cell mRNA was not known. Therefore, the polypeptides coded for by early viral mRNA isolated from polyribosomes at 24 h p.i. were compared with the polypeptides coded for by uninfected-cell mRNA that was not selected by DNA-cellulose hybridization. The uninfected-cell mRNA was simply prepared by isolating polysome-associated RNA and then selecting the poly(A) RNA by oligodeoxythymidylic acid-cellulose chromatography as described in Materials and Methods. The major early virus-specified polypeptides coded for by the virus-specified mRNA had electrophoretic mobilities and relative rates of synthesis different from those of the major uninfected-cell polypeptides coded for by uninfected-cell mRNA (Fig. 7C). Therefore, the proteins coded for by mRNA isolated from polyribosomes after 6 or 24 h of protein synthesis represented early virus-specified gene products that appeared after the translation of the viral immediate early mRNA.

A

B

C

DISCUSSION

The genome of human CMV (Towne strain) is a linear, double-stranded DNA molecule of 240 kb (155×10^6 daltons) of which 11% are reiterated twice. The genome is approximately 50% greater in size and molecular complexity than the herpes simplex virus genome (11, 16, 23, 38). The CMV genome, like the herpes simplex virus genome (18, 34), consists of a long unique and a short unique region flanked by different repeat sequences that are inverted relative to each other (43). Four genome arrangements, resulting from the possible combinations of inversions of the two unique regions, are present in DNA preparations in approximately equal amounts as demonstrated by partial denaturation mapping (23) and restriction enzyme analysis (12, 43). Since human CMV DNA is infectious (16), it was assumed that the initial transcription of the viral genome was by host cell-specified RNA polymerase II. Studies using inhibitors have implicated RNA polymerase II in viral transcription (2, 6). However, the presence of a virion-bound DNA-dependent RNA polymerase associated with the CMV genome has not been eliminated.

CMV, like herpes simplex virus (3, 10, 13, 19, 21, 22, 41) and pseudorabies virus (14), may employ a common mechanism for the switch from restricted to extensive transcription. For example, the viruses may code for a protein(s) that is a new RNA polymerase. A system in which the switch from early to late transcription is effected by a RNA polymerase coded for by the early viral genes is the lytic cycle of bacteriophage T7 in *E. coli* (8, 40). Alternatively, the template activity of the viral DNA may be mod-

ified by an immediate early virus-induced protein(s), or the host RNA polymerase may be modified. We have shown here that CMV, like herpes simplex virus (3, 9, 10, 13, 19, 21, 22, 41) and pseudorabies virus (14), has restricted transcription in the presence of an inhibitor of protein synthesis. However, human CMV (Towne strain), unlike herpes simplex virus and pseudorabies virus, has a prominent region of immediate early transcription in the large unique region of 0.66 to 0.77 map units. Similar observations have been made for the AD169 strain (J. L. M. C. Geelen, personal communication) and the Davis strain (J. M. DeMarchi, personal communication).

Northern blots of virus-specified RNA and methylmercury hydroxide-gels of viral RNA selected by hybridization to viral DNA-cellulose demonstrated shifts in size classes of viral RNA and qualitative changes in relative abundance of viral RNA with time. Further investigation is necessary to map the origin of these various RNAs. In general, these data indicate that there is a temporal regulation of human CMV genome transcription at immediate early and early times after infection. The factors that regulate expression of this viral genome are apparently synthesized *de novo* during the first 2 h of infection. Nine predominant proteins were detected by *in vitro* translation of immediate early mRNA. The apparent molecular weights of the *in vitro* translated immediate early polypeptides correlated with the molecular weights of the majority of the virus-induced proteins synthesized in infected cells. However, post-translational modifications of the viral proteins in infected cells could alter electrophoretic mobility and relative amount. At least three immediate early virus-

FIG. 7. Autoradiographs of the *in vitro* translation products of virus-specified immediate early and early mRNA. Virus-specified mRNA was isolated by hybridization to CMV DNA bound to cellulose, and the poly(A) containing RNA was selected by oligodeoxythymidylic acid-cellulose chromatography as described in the text. After *in vitro* translation in a rabbit reticulocyte lysate system, the polypeptides were fractionated by electrophoresis in a denaturing SDS-containing polyacrylamide gel as described in the text. (A) *In vitro* translation with no added RNA (No RNA), adenovirus type 2 cytoplasmic RNA (*Ad*₂), CMV-specified immediate early RNA (IE), CMV-specified early RNA associated with polyribosomes after 6 or 24 h of protein synthesis (6 h, 24 h), and uninfected-cell RNA that was incubated under hybridization conditions with CMV DNA bound to cellulose. Although the majority of the uninfected cell RNA did not hybridize to the CMV DNA cellulose and, consequently, was discarded, the small amount nonspecifically attached to the cellulose was eluted and subjected to oligodeoxythymidylic acid-cellulose chromatography for a control as described in the text (U-Hy). The immediate early polypeptide profile to the far right represents a separate *in vitro* translation and gel analysis. A continuous arrow designates major endogenous proteins; a discontinuous arrow indicates minor endogenous proteins. Proteins in low relative amounts were designated but not according to apparent molecular weight. (B) Comparisons between immediate early and early *in vitro* translation profiles with ICSP from cells pulse-labeled with [³⁵S]methionine under immediate early and early conditions as previously described (37). (C) *In vitro* translation of adenovirus type 2 cytoplasmic RNA (*Ad*₂), virus-specified poly(A) RNA present on polyribosomes after 24 h of protein synthesis (24 h), and uninfected-cell polysome-associated poly(A) RNA not subjected to DNA-cellulose hybridization (U). Apparent molecular weights ($\times 10^3$) of virus-specified polypeptides coded for by immediate early (IE) or early viral mRNA after 6 or 24 h of proteins synthesis in the infected cell are indicated in all panels.

induced proteins were synthesized at relatively high concentrations in infected cells, and they were designated according to apparent molecular weight ($\times 10^3$) as ICSP polypeptides 75, 72, and 68 (37). It was difficult to determine whether ICSP 68 was synthesized *in vitro*. At immediate early times, in infected cells, ICSP 59, 56, and 53 were present in low concentrations, but at 2 h p.i. these ICSP were detected at relatively high concentrations (37). Four proteins at lower concentrations were also detectable and were designated as ICSP 39, 27, 21, and 19 (37). Additional experiments are in progress to characterize the mRNA's and *in vitro* translation products from defined regions of the viral genome. In general, the *in vivo* protein studies are in good agreement with the *in vitro* translation studies indicating that many of the ICSP are virus specified.

A comparison of the *in vitro* translation of immediate early mRNA with early mRNA indicated that the immediate early proteins of CMV are preferentially synthesized within the first few hours after infection and that subsequently their relative rates of synthesis decline. One or more of these virus-specified immediate early proteins may be regulatory proteins that bind to the CMV DNA genome and regulate transcription or influence viral DNA replication. Alternatively, these virus-specified proteins could bind to host chromosomal DNA and have an influence on cellular metabolism as well as viral replication. Once the immediate early viral proteins are synthesized, changes occur in the relative abundance of the viral mRNA size classes present on polyribosomes.

Quantitative differences between the RNA synthesized in the absence or presence of viral DNA replication at an early time of approximately 24 h p.i. were not clearly demonstrable by the Southern or Northern blot procedure when analyzing a complicated genome of 240 kb. Initiation of viral DNA replication for the Towne strain of human CMV occurs at approximately 12 h p.i. when the cells are infected at a high multiplicity (37). The inability to detect major differences at 24 h p.i. between the viral RNA synthesized in the presence or absence of viral DNA replication may be related to the slow rate of viral DNA replication in CMV-infected cells. Viral RNAs that appear in the cells after viral DNA replication would be present in relatively low concentrations, and consequently they would be difficult to detect by either the Southern or Northern blot hybridization procedure. In nonpermissive cells, the RNA size classes characteristic of early viral RNA were demonstrable but at a lower concentration relative to the

permissive cell. Even though extensive transcription of the CMV genome occurred in the absence of viral DNA replication as judged by Southern blot hybridization with infected whole-cell RNA, a definable number of polyribosome-associated RNA size classes were detected by Northern blot hybridization. The majority of the early virus-specified proteins had electrophoretic mobilities different from those of the immediate early proteins and the major late structural proteins of CMV previously described (36, 37). The *in vitro* translation profile of early viral mRNA remained relatively similar when 6 or 24 h of protein synthesis occurred after infection. Even though there appears to be a relatively transient expression of immediate early proteins, the virus-specified early proteins are expressed for a prolonged period of time. The biochemical events of prolonged early viral gene expression and a slow rate of viral DNA replication are presumably related to the human CMV eclipse period of days in infected-cell cultures (15, 36) and to the propensity of this virus to cause persistent infection in the host (24, 30, 31).

The predominant factor(s) influencing transcription of the CMV genome at early times after infection is apparently synthesized within the first 2 h. It is proposed that the immediate early proteins of CMV are virus-specified proteins that control the temporal transcription of the human CMV genome. These regulatory proteins may be crucial for determining whether the viral genome enters latency or productive infection. The option of two possible pathways for the viral genome, latency or productive infection, may be an additional factor that influences the slow replication of the virus and persistent infection in the host.

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