Smolec, Jo Marie Ellen

THE TRANSCRIPTION OF THE CYTOMEGALOVIRUS GENOME

The University of Arizona PH.D. 1982

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THE TRANSCRIPTION OF THE CYTOMEGALOVIRUS GENOME

by

Jo Marie Ellen Smolec

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF MICROBIOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Jo Marie Ellen Smolec

entitled THE TRANSCRIPTION OF THE CYTOMEGALOVIRUS GENOME

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of DOCTOR OF PHILOSOPHY.

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SIGNED: Jo Marie Ellen Smalec

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to Dr. Randall Pritchett for his support, encouragement, and never ending patience.

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ABSTRACT

The replication of cytomegalovirus (CMV), Towne strain, in permissively infected cells is characterized by a long eclipse phase and lengthy growth cycle which may reflect the activity of mechanisms which regulate viral gene expression at the transcriptional level. To correlate the study of transcription with other events occurring during CMV replication, experiments were first conducted to determine the time post-infection for the onset of virus maturation and virus DNA synthesis under defined conditions. The onset of virus maturation was between 2 and 3 days post-infection. The hybridization kinetics of labeled CMV DNA with DNA extracted at different times post-infection indicated that the onset of viral DNA synthesis was between 24 and 36 hours post-infection in human foreskin fibroblast cells permissively infected with CMV. The results of hybridizations of stable viral RNA accumulating at various times post-infection, and at early times in the absence of protein synthesis, with labeled CMV DNA, showed that there is temporal regulation of transcription. The transcription of the genome is restricted in the presence of an inhibitor of protein synthesis to 5-6% of the genome (immediate early RNA). There is a rapid switch of immediate early to the early phase of transcription, which extends to at least 24 hours post-infection, and consists of transcripts homologous to approximately 30% of the viral genome throughout the entire phase. After the onset of viral DNA synthesis, the transcription extends into the late phase during which transcripts

homologous to approximately 42% of the genome are synthesized. Early and late RNA was analyzed for the presence of symmetric transcripts. Transcription was found to be asymmetric at early times post-infection, with only 5% symmetric transcription during the late phase. The extent to which immediate early, early, and late stable RNA is transcribed from the repeat regions of the CMV genome was determined by hybridizations of stable RNA to labeled XbaI restriction fragments Q and M, which comprise the long repeat regions of the CMV genome. The hybridization of the probes indicated the presence of RNA transcripts at immediate early times that were homologous to 15% of the repeat sequences. Early RNA contained transcripts homologous to 18.5% of the repeat regions, and late RNA was homologous to 34% of the long repeat sequences.

INTRODUCTION

Human cytomegalovirus (CMV), a member of the herpesvirus group, is a ubiquitous pathogen associated with a wide range of clinical manifestations, including congenital defects, prematurity, intrauterine death, cytomegalic inclusion disease, heterophile negative mononucleosis, interstitial pneumonia, post-perfusion syndrome, and hepatitis (32, 35, 37, 62). It is a common pathogen in subjects with impaired immune defenses (13, 27). CMV is capable of infecting the developing fetus. The majority of newborns with congenital infections are asymptomatic at birth and remain so throughout infancy (45, 51). These asymptomatic infections may have central nervous system sequelae of mild to moderate auditory and mental dysfunction detected later in life (25, 45).

CMV has been implicated as a possible etiologic factor of Kaposi's sarcoma, a human tumor. This implication is based on serological studies (23, 24) and on the detection of the CMV genome in Kaposi's sarcoma tissue (7). In addition, UV-irradiated CMV was found to transform hamster cells which, when injected into hamsters, produce malignant fibrosarcomas (2).

CMV resembles other members of the herpesvirus group morphologically. It has an outer lipid-glycoprotein envelope surrounding a nucleocapsid which is composed of 162 capsomeres arranged in icosahedral symmetry. Internal to the nucleocapsid is a DNA containing core (38). Also found in virus preparations are spherical

particles containing amorphous granular material surrounded by a membrane similar to the viral envelope. These particles, devoid of DNA, are called dense bodies (47). The buoyant density in cesium chloride of enveloped virions and nucleocapsids purified from extracellular fluid was shown to be 1.219 g/cm^3 and 1.263 g/cm^3, respectively (28). CMV is composed of approximately twenty to thirty structural polypeptides which range in molecular weight from 22,000 to 230,000 daltons (14, 47, 53).

The genome of CMV is a linear double-stranded DNA. The DNA extracted from purified non-defective extracellular virus (strains Towne, AD169, and Davis) was found to have an average molecular weight of approximately 140-150 x 10^6 daltons as determined by sedimentation in neutral sucrose gradients and by contour length measurements (11, 21, 31, 34, 55, 64). The density of CMV DNA, as determined by analytical centrifugation in cesium chloride, is reported to be 1.716 g/cm^3 (28).

The structure of the CMV genome has been shown to be similar to that of the herpes simplex virus (HSV) genome, which consists of long unique and short unique regions, U_L and U_S, respectively, flanked by different repeat sequences that are inverted relative to one another TR_L/IR_L and IR_S/TR_S,(Fig. 6) (26, 33, 49, 64). Four genome arrangements, resulting from the combinations of inversions of the two unique regions, are present in virion DNA preparations in approximately equal amounts (12, 31, 64). Partial denaturation maps of CMV Towne DNA have

shown distinctive adenine plus thymine rich and guanine plus cytosine rich localizations (31).

The replication of CMV in permissively infected cells is characterized by a long eclipse phase and a lengthy growth cycle. In cells infected with laboratory strains of CMV, the onset of viral DNA synthesis occurs between 24 and 36 hours post-infection and progeny virus may not appear until 48 to 72 hours post-infection (16, 39, 53, 56). Besides productive infections, CMV may undergo an abortive type infection in non-permissive cells, where the virus adsorbs, penetrates, and produces early antigen, but no detectable viral progeny is produced (3, 15, 17). It may also enter into a persistent infection which appears to be due to an equilibrium between the release of virus by infected cells and the growth of uninfected cells (19). Latent infections, characterized by inability to detect infectious virions between intervals of virion production, have been described both in vivo (human host) and in vitro (cell culture) (38, 44). Also CMV infection may result in cellular transformation (2, 20, 36).

The lengthy replication cycle of CMV may reflect the activity of mechanisms which regulate viral gene expression. Such mechanisms may also influence persistent, latent, and transforming infections. There are several levels in the replication process at which expression of the viral genome may be regulated. The first level could involve preferential transcription of portions of the viral genome either by cellular or viral coded DNA dependent RNA polymerases. It is conceivable, for example, that a cellular polymerase is responsible for

transcribing a portion of the genome and that a viral coded or modified enzyme transcribes the remainder.

There are several other steps in the replication process at which expression of the viral genome may be regulated. One of these steps may be in the post-transcriptional processing of the primary RNA transcripts, which may include the action of endonucleases and exonucleases which alter the size of the transcript (1), the addition of a 5' cap structure (48), polyadenylation of the 3' end (8), internal methylation (48), and splicing (the removal of intervening sequences from the primary transcript) (1, 6). Another level of regulation may occur during the translation of immediate early, early, or late messenger RNA (59). Translation products may consist of proteins capable of regulating transcription or viral DNA replication.

In general, the viral RNA transcripts accumulating during herpesvirus infection of permissive cells can be divided into three phases: 1) immediate-early (IE), that RNA which is transcribed soon after infection in the presence of a protein synthesis inhibitor; 2) early, that RNA which is transcribed prior to the onset of viral DNA synthesis if de novo protein synthesis occurs; and 3) late, that RNA which is transcribed after the onset of viral DNA synthesis. This implies that the expression of the viral genome is sequential or temporal. The basic assumption is that viral expression is in someway regulated (12, 53, 54, 59, 60).

Demarchi, Schmidt, and Kaplan (12), Wathen, Thomsen, and Stinski (59), and Wathen and Stinski (60) recently published data

indicating that transcriptional controls, at least in part, regulate the infectious cycle. In their studies, patterns of transcription were established by hybridizing ^{32}P-radiolabeled RNA extracted at various times post-infection to restriction endonuclease digested CMV DNA bound to nitrocellulose filters. Their results indicated that: 1) IE RNA hybridized predominantly to a very restricted region of the genome between map units 0.66 to 0.77; 2) early RNA (synthesized up to 24 hours post-infection) hybridized to most regions of the genome, but in greater abundance to certain fragments; and 3) late RNA hybridized in approximately equal abundance to most regions of the genome.

The experiments reported here were designed to obtain additional information regarding the regulation of transcription in CMV infected cells. It was anticipated that there is preferential transcription of certain regions of the CMV genome at various stages of the replication cycle. To establish this, total cell RNA extracted from infected cells at various times post-infection was analyzed by liquid DNA-RNA hybridization, with RNA in excess. The percentage of ^{32}P-labeled DNA in hybrid form is indicative of the proportion of the genome which has been transcribed. Experiments were also conducted to determine the extent, if any, to which the CMV genome is transcribed symmetrically (transcription from both strands of DNA in a homologous region).

In addition, experiments were conducted to characterize transcription from the repeat and adjacent regions of the CMV genome. An analysis of the transcription of these regions is of interest due to

the findings that these are the regions where DNA nucleotide sequence heterogeneity between CMV strains (AD169 and Towne) has been located (42). Also, structural alterations in the DNA of CMV obtained from human osteogenic sarcoma cells persistently infected with CMV Towne have been recently observed. These alterations, which appear to be sequence deletions, map in the repeat-unique-joint regions of the CMV Towne genome (19). Furthermore, it has been recently shown that immediate early messenger RNA transcribed from the junction of unique and repeat regions of the herpes simplex virus type 1 genome are spliced (9, 61). Recent findings of other investigators have shown that RNA homologous to DNA restriction fragments containing repeat and unique-repeat joint sequences were present primarily in the early and late phase of CMV infection (59, 60). The experiments reported here were designed to determine the extent to which these regions are transcribed and at what time post-infection transcription occurs.

MATERIALS AND METHODS

Virus, cell culture, and virus propagation

The Towne strain of CMV isolated from the urine of an infant with microcephaly and hepatosplenomegaly (40) was obtained from M. F. Stinski (University of Iowa) and plaque assayed using methyl cellulose overlays (63).

Primary human fibroblast (HFF) cells were prepared from human foreskins. The cells were cultivated in 260 mm glass roller bottles (Bellco Glass, Inc., Vineland, N. J.) and 32 oz glass prescription bottles with Eagle's minimal essential medium (EMEM, GIBCO Laboratories, Grand Island, N. Y.) supplemented with 10% fetal calf serum (GIBCO), 2 mM L-glutamine, 0.075% $NaHCO_3$, 0.01 M Tricine, and antibiotics (100 U of penicillin and 100 μg of streptomycin per ml). Cells were subcultured at a 1:3 split ratio once per week. Cells between passages 5 and 19 were used for virus propagation and for infected cell nucleic acid studies.

Virus was propagated by infecting roller bottle cultures of HFF cells 2 days after subculturing with 0.1 plaque forming units (PFU) per cell. Virus adsorption was for 90 minutes at 37 C, after which 80 ml of EMEM containing 2% fetal calf serum, 0.075% $NaHCO_3$, 0.01 M Tricine, antibiotics, and 2 mM L-glutamine was added. The infected cell cultures were incubated at 37 C and rolled at 0.25 rpm. The culture medium was replaced at 4 days post-infection and the L-arginine

concentration was increased to 1.2 mM. Virus was harvested from the extracellular culture fluid at 9 days post-infection.

Virus purification

Extracellular culture fluid harvested 9 days post-infection served as the source of virus. All steps of the purification procedure were conducted on ice or at 4C. Cellular debris was removed from the extracellular fluid by centrifugation at 5,900 X g for 20 minutes in a GSA Sorvall (DuPont Instruments, Newtown, Conn.) rotor. Virus was then pelleted from the supernatant by centrifugation at 27,000 X g for 90 minutes in an SS-34 Sorvall rotor. Virus pellets were resuspended in 3 ml of 0.5 mM sodium phosphate (pH 7.4), homogenized with 25 strokes of a Dounce homogenizer, clarified by centrifugation at 2,000 X g in an SS-34 rotor for 10 minutes, layered onto a 10% to 60% (wt/wt, in Tris-buffered saline (TBS), 0.15 M NaCl-50 mM Tris-hydrochloride pH 7.2) sucrose gradient, and centrifuged at 82,000 X g for 1 hour in an AH627 Sorvall rotor. Two light-scattering bands were present: an upper, distinct band of particles containing DNA (virions) and a lower, more diffuse band of particles devoid of DNA (dense bodies) (47). The material in both bands was removed from the gradient, diluted with TBS, and pelleted by centrifugation in an AH627 Sorvall rotor for 90 minutes at 116,000 X g.

Extraction and purification of viral DNA

Virus pellets (including dense bodies) were resuspended in a solution containing 0.1 M NaCl, 0.01 M EDTA, and 0.05 M

Tris-hydrochloride (pH 8.0). SDS (Sigma Chemical Co., St. Louis, Mo.) and Sarkosyl (Geigy Pharmaceutical, Ardsley, N. Y.) were added to final concentrations of 0.5% (wt/vol) and 1% (wt/vol), respectively, and the mixture was incubated at 60 C for 2 minutes. The mixture was then incubated for 2 hours at 37 C with 1 mg of pronase (Calbiochem-Behring, La Jolla, Calif.; pretreated by incubation for 2 hours at 37 C and 10 minutes at 80 C) per ml. The DNA solution was then extracted with equal volumes of neutralized redistilled phenol and chloroform containing 2% (vol/vol) isoamyl alcohol and then exhaustively dialyzed against 0.3 M NaCl-0.01 M EDTA-0.05 M Tris-hydrochloride (pH 7.5). The DNA was then dialyzed into a cesium chloride (CsCl) solution containing 2 mM EDTA and 50 mM Tris-hydrochloride (pH 7.5). Dialysis was continued and saturated CsCl added until the density of CsCl outside the dialysis bag remained constant at 1.717 g/cm^3 (refractive index of 1.4010 at 25C). The DNA solution was then centrifuged in 12.5 ml tubes, using a T-865.1 Sorvall rotor at 61,000 X g for 66 hours at 22 C. Fractions (0.25 ml) of the gradients were collected and the refractive index determined using a refractometer (Bausch & Lomb Corp., Rochester, N. Y.). Those fractions having a density between 1.710 and 1.725 g/cm^3 were combined and stored at -20 C.

Infection and harvesting of cells for nucleic acid isolation

Roller bottle cultures of HFF cells were infected with CMV at a multiplicity of 10 PFU/cell 2-3 days after subculturing. The cell cultures were first rinsed with approximately 20 ml of EMEM containing

0.075% $NaHCO_3$. The virus was added, in a volume of 10 ml, and allowed to adsorb for 90 minutes at 37 C. After adsorption, 80 ml of EMEM containing 2% fetal calf serum, 0.075% $NaHCO_3$, 0.01 M Tricine, antibiotics, 2 mM L-glutamine, and 1.2 mM L-arginine was added. The infected cell cultures were incubated at 37 C and rolled at 0.25 rpm. At the indicated times post-infection, cells were scraped off into the culture fluid, pelleted by centrifugation, and used for the isolation of cellular nucleic acid.

To obtain immediate early RNA (RNA synthesized in the absence of protein synthesis), 32 oz prescription bottle cultures of HFF cells were incubated with 20 ml of EMEM containing 0.075% $NaHCO_3$ and 200 µg/ml cycloheximide (Sigma Chemical Co., St. Louis, Mo.) for 2 hours prior to infection. The cells were then infected with CMV at a multiplicity of 10 PFU/cell. Virus adsorption was allowed to proceed in the presence of 200 µg/ml cycloheximide at 37 C for 90 minutes, after which time 30 ml of EMEM containing 2% fetal calf serum, 0.075% $NaHCO_3$, 0.01 M Tricine, antibiotics, 2 mM L-glutamine, 1.2 mM L-arginine, and 200 µg/ml cycloheximide was added. At the indicated times post-infection, cells were scraped off into the culture fluid, pelleted by centrifugation, and used for the isolation of cellular nucleic acid.

Isolation of nucleic acid from infected cells

Cells harvested at various times post-infection were washed twice in TBS, resuspended in 2.0 ml of TBS, and 20 µl of 0.1 M EDTA was immediately added (41). A 1/10 volume of 20% SDS was then added. The mixture was shaken, heated to 55 C for 5 minutes and a 1/10 volume of

20% Sarkosyl added. After 5 minutes of heating at 55 C, 20 µl of 25 mg/ml Proteinase K (Calbiochem-Behring, La Jolla, Calif.) was added, and the mixture was incubated at 37 C for 1 hour. An equal volume of 8 M guanidine hydrochloride, (Sigma Chemical Co.), prepared in 0.001 M EDTA and 0.01 M Tris-hydrochloride (pH 7.5) was added and the mixture heated to 65 C with vigorous shaking. CsCl was then added at 0.5 g/ml of cell extract and the mixture homogenized with a Dounce homogenizer. The homogenate was then layered over a 3 ml cushion of CsCl (1.735 g/cm^3 in 10 mM EDTA and 10 mM Tris-hydrochloride pH 7.5) in a 5/8 X 4 inch centrifuge tube, overlaid with mineral oil and centrifuged for 18 hours at 74,000 X g at 20 C in an AH627 (small bucket) rotor. The mineral oil was removed and the supernatant aspirated (saved for the extraction of DNA) leaving the RNA as a small pellet on the tube bottom. The pellet was resuspended in 0.01 M Tris-hydrochloride (pH 7.5). NaCl was added to 0.1 M, and the RNA precipitated overnight at -20 C after the addition of an equal volume of isopropanol. The precipitate was collected by centrifugation at 8,700 X g for 5 minutes in a Microfuge B (Beckman Instruments, Inc., Palo Alto, Calif.) centrifuge. The precipitate was vacuum evaporated and the pellet dissolved in approximately 250 µl of water. The RNA concentration was determined from the absorbance at 260 nm.

The aspirated supernatant, from the CsCl cushion, was dialyzed for 24 hours against 0.01 M Tris-hydrochloride (pH 7.5), extracted three times with equal volumes of neutralized redistilled phenol and chloroform containing 2% (vol/vol) isoamyl alcohol, and the DNA

precipitated overnight at -20 C after the addition of a 1/10 volume of 1 M NaCl and an equal volume of isopropanol.

Extraction of DNA from CMV infected cells

Cells remaining on the glass after removal of extracellular culture fluid at 9 days post-infection were scraped off into TBS, pelleted by centrifugation at 1,500 X g in a GSA Sorvall rotor at 4 C and resuspended in 2 ml of TBS. After the addition of a 1/10 volume of 0.1 M EDTA and 10% (wt/vol) SDS, the mixture was incubated at 60 C for 2 minutes. A 1/10 volume of 20% (wt/vol) Sarkosyl was added, and incubation at 60 C continued for another 2 minutes before the addition of 1 mg of pretreated pronase per ml. The mixture was incubated at 37 C for 12 hours. The nucleic acid was then extracted twice at 60 C with equal volumes of phenol and chloroform containing 2% (vol/vol) isoamyl alcohol and precipitated for 24 hours with 2 volumes of ethanol at -20 C. The precipitated nucleic acid was pelleted by centrifugation in an SS-34 Sorvall rotor at 9,800 X g for 30 minutes at 4 C. The pellet was dissolved in 0.5 M KOH, incubated at 37 C for 18 hours, neutralized with HCl, and the DNA precipitated with 2 volumes of ethanol at -20 C.

In vitro radioisotope labeling of viral DNA

The DNA extracted from sucrose density gradient banded virus and purified by equilibrium CsCl buoyant density centrifugation was dialyzed at 4 C against four 1 - liter changes of deionized glass-distilled water over a 48 hour time period. It was then labeled in

vitro with α^{32}P(dTTP) (400 Ci/mmol; New England Nuclear, Boston, Mass.) by nick translation (30) with Escherichia coli DNA polymerase I (Boehringer Mannheim Corp., Indianapolis, Ind.) as described in detail by Rigby et al. (46). Approximately 1 µg of viral DNA was added to a reaction mixture of 50 mM Tris-hydrochloride (pH 7.5), 10 mM 2-mercaptoethanol, 10 mM $MgCl_2$, and 4 ng of activated DNAase (to activate the DNAase, 50 µl of 1 mg/ml stock solution of DNAase in 0.01 M HCl was mixed with 450 µl of activation buffer consisting of 10 mM Tris-hydrochloride (pH 7.5), 5mM $MgCl_2$, and 1 mg/ml bovine serum albumin and incubated at 4 C for 2 hours). The reaction mixture was incubated at 37 C for 15 minutes. Three unlabeled deoxynucleotides (dCTP, dATP, and dGTP) were then added to a final concentration for each of 0.02 mM and the reaction mixture transferred to a tube containing α^{32}P(dTTP). The tube was placed in a 15 C water bath, 5 units of polymerase I were added, and the reaction allowed to proceed for 2 hours. The reaction was stopped by the addition of 20 µl of 0.1 M EDTA and the unreacted deoxynucleotides were removed by passing the reaction mixture over a column of Sephadex G50 (fine) equilibrated with 0.01 M Tris-hydrochloride (pH 7.5), 0.001 M EDTA, and 0.1% Sarkosyl. The fractions containing DNA, detected by counting 5 µl portions of each, were pooled and stored at -20 C. The specific activity of labeled DNA was between $1x10^7$ and $3x10^7$ cpm/ g.

To select specifically for single-stranded DNA, the labeled DNA was mixed with 100 µg of calf thymus DNA, denatured with 0.2 N NaOH, diluted 15-fold in water and neutralized with HCl. The denatured DNA

was then bound to a 1 g column of hydroxyapatite (Bio-Gel HTP; Bio-Rad Laboratories, Richmond, Calif.), which had been equilibrated overnight in 0.04 M $NaPO_4$ buffer plus 0.1% SDS and boiled before being placed into the column. The single-stranded DNA was eluted in 0.16 M $NaPO_4$ (pH 6.8) buffer at 60 C and the fractions containing DNA were pooled.

Nucleic acid liquid hybridization and reassociation kinetics

For DNA-RNA hybridizations, ^{32}P-labeled CMV DNA ($2x10^{-4}$ µg) or ^{32}P-labeled fragments of CMV DNA, labeled in vitro by nick translation, were mixed with the RNA (3,000 µg/ml: immediate early, 2 hour, and 4 hour; and 750 µg/ml: 12 hour, 24 hour, 36 hour, and 48 hour) extracted from cells at various times post-infection in a 100 µl reaction mixture containing 0.5 mg/ml yeast RNA, 0.3 M NaCl, 0.005 M Tris-hydrochloride (pH 7.5), 0.005 M EDTA, and 5% formamide. Equal portions of the mixtures were then sealed into 10 µl micropipettes, heated at 110 C for 5 minutes, and incubated at 68 C for variable intervals of up to 24 hours. The samples were stored in ethanol at -20 C until all were collected. Controls consisted of reaction mixtures containing an additional 0.5 mg/ml of yeast RNA in place of the infected cell RNA.

Controls were conducted to prove that the RNA samples were not contaminated with DNA. An aliquot of each RNA was treated with 0.5 M KOH for 18 hours at 37 C and neutralized with HCl. This RNA was then mixed with the labeled CMV DNA in a 100 µl reaction mixture, sealed into micropipettes, denatured, and incubated at 68 C for various times.

No hybridization in excess of self-hybridization of the labeled CMV DNA was observed.

For DNA-DNA hybridizations, ^{32}P-labeled CMV DNA was mixed with the DNA to be analyzed (200 μg/ml) in a 0.5 ml reaction mixture containing 0.5 mg/ml salmon DNA, 0.9 M NaCl, 0.01 M Tris-hydrochloride (pH 7.5), and 0.003 M EDTA. Equal portions of the mixtures were then sealed into 50 μl micropipettes, heated at 110 C for 5 minutes, and incubated at 67 C. Samples were removed and stored in ethanol at -20 C after various times of incubation. Controls consisted of reaction mixtures containing an additional 0.5 mg/ml of salmon DNA in place of the test DNA.

The single-strand-specific S1 nuclease (Miles Laboratories, Inc., Elkhart, Ind.) was used to distinguish DNA-DNA and DNA-RNA hybrids from single-stranded DNA (43). Each hybridization sample was mixed with 1.7 ml of S1 buffer (0.1 mM $ZnSO_4$, 0.25 M KAc (pH 4.5), and 0.3 M NaCl, containing 100 μg/ml denatured and 100 μg/ml native calf thymus DNA) and then divided into 3 tubes (0.5 ml each). Two of the tubes received 100 units of S1 nuclease and were incubated for 90 minutes at 45 C. The third tube served as the control for total counts. One drop of 0.5 mg/ml calf thymus DNA was added to the digested samples and the undigested nucleic acid precipitated with 5% TCA (trichloroacetic acid). The precipitate was collected on nitrocellulose filters and prepared for scintillation counting.

The percentage of ^{32}P-labeled viral DNA reassociated at various times was plotted as a function of $R_o t$ for total RNA (29). R_o is the

starting concentration of total RNA in moles of nucleotides per liter and t is the time of incubation in seconds. The percentage of reassociation = $(cpm_r - cpm_d)/(cpm_t - cpm_d) \times 100$, where cpm_r is the S1-resistant radioactivity of the sample at a given time, cpm_d is the S1-resistant radioactivity of the denatured DNA, and cpm_t is the total radioactivity in the sample not digested with S1 nuclease.

The DNA driven reactions were plotted as C_o/C versus time of hybridization in hours, where C_o and C are single-stranded DNA concentrations at time t_o and t respectively (55). C_o and C were also corrected for S1-resistant radioactivity of denatured DNA. Thus the data were corrected for the efficiency with which S1 nuclease digested denatured DNA, but no corrections were made for the percentage of native DNA sensitive to S1.

Restriction enzyme digestion

A 10 μg amount of unlabeled purified viral DNA was incubated for 2 hours at 37 C in 0.2 ml of a solution consisting of 1.0 mM NaCl, 6 mM $MgCl_2$, 6 mM Tris-hydrochloride (pH 7.4), and 30 units of restriction endonuclease XbaI (Bethesda Research Laboratories, Rockville, Md.). The restriction enzyme digestion was terminated by the addition of a 1/10 volume of a solution consisting of 60% (wt/wt) sucrose, 0.25% (wt/vol) bromphenol blue, and 0.1 M EDTA. The DNA fragments were separated by electrophoresis in a cylindrical (1.2 x 18 cm) 0.5% (wt/vol) agarose (Seakem; Microbiological Associates, Bethesda, Md.) gel when used for Southern blots, and in 0.6% (wt/vol) Seaplaque agarose (Seakem) gel when being used for the extraction of

specific fragments from the gel. The separated DNA fragments were stained by placing the gels in electrophoresis buffer containing 0.5 µg of ethidium bromide per ml for 0.5 hour at room temperature. The stained DNA fragments were visualized and photographed under short-wavelength UV light.

Extraction of restriction fragments from an agarose gel

Upon visualization of the separated DNA fragments in the ethidium bromide stained 0.6% Seaplaque agarose gel, the desired restriction fragments were carefully sliced out of the gel, placed in a small culture tube, and frozen at -20 C. To the frozen agarose was added 0.5 ml of 100 mM NaCl-5 mM EDTA and the agarose was melted by heating to 65 C. The mixture was placed on ice for 30 minutes. The polymerized agarose was then re-melted at 65 C and extracted twice with phenol-chloroform at 55 C. After the last extraction, the aqueous phase was placed on ice for 15 minutes and then centrifuged at 8,400 X g for 5 minutes in a Microfuge B centrifuge. The supernatant was extracted three times with butanol and the DNA precipitated with 0.6 volume of isopropanol for 18 hours at -20 C. The DNA precipitate was pelleted by centrifugation as described above, resuspended in 50 µl of water, and labeled by nick translation.

Southern blot hybridization

DNA fragments were transferred to nitrocellulose sheets according to the Southern blotting technique (50). The strips of nitrocellulose to which the DNA had been transferred (blots) were cut into

0.5 mm strips, rinsed in 2X SSC (0.15 M NaCl, 0.015 M sodium citrate) and dried at 60 C for 12 hours.

Blots of restriction enzyme fragments were pretreated by incubation at 67 C in a solution of 6X SSC containing 0.02% (vol/vol) polyvinylpyrrolidone (Sigma Chemical Co.), 0.02% (vol/vol) Ficoll (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), and 10 µg of sonicated denatured salmon testes DNA per ml for 8 hours. The pretreated blots of DNA fragments were incubated at 67 C for 20 hours with denatured ^{32}P-labeled CMV DNA or ^{32}P-labeled restriction enzyme fragments of CMV DNA in 2 ml of hybridization buffer consisting of 0.9 M NaCl, 0.003 M EDTA, 0.02 M Tris-hydrochloride (pH 7.5), and 1 mg of sonicated denatured salmon testes DNA. The blots and hybridization mixture were placed inside screw-cap culture tubes (16 x 150 mm) which were heat sealed in plastic packages (Packaging Aids Corp., San Francisco, Calif.) and submerged lengthwise in a water bath during incubation. After incubation, the blots were washed at 67 C with four changes of 4X SSC containing 0.1% (wt/vol) SDS over a 1 hour time period and then with two changes of 3X SSC at room temperature over a 30 minute time period. The blots were allowed to dry and exposed at -70 C to X-Omat R film (Eastman Kodak Corp., Rochester, N. Y.) using Cronex lightning plus screens (DuPont Corp., Wilmington, Del.).

Detection of symmetric transcripts

The RNA extracted from infected cells at various times post-infection (3,000 µg/ml 4 hour, 750 µg/ml 12 and 48 hour) was added to two 100 µl reaction mixtures, each consisting of 5% formamide, 0.3 M

NaCl, 0.005 M EDTA, 0.005 M Tris-hydrochloride (pH 7.5), and sealed in 10 µl micropipettes, and heated at 110 C for 5 minutes. These hybridization mixtures were then allowed to prehybridize at 68 C for 18 hours. The mixtures were then divided in half. One-half of the prehybridized RNA mixture received 30,000 cpm of denatured ^{32}P-labeled DNA probe. Aliquots of the mixture were then sealed into 10 µl micropipettes and incubated at 68 C for variable intervals of up to 24 hours. The other half of the prehybridized RNA mixtures received 30,000 cpm of denatured ^{32}P-labeled DNA probe. Aliquots were sealed into 10 µl micropipettes, heated at 110 C for 5 minutes, and incubated at 68 C for variable intervals of up to 24 hours. The extent of DNA-RNA hybridization was then determined by S1 nuclease digestion.

RESULTS

Determination of onset of progeny CMV maturation

The onset of progeny CMV maturation in HFF cells infected at multiplicities of infection (MOI) of 1 and 10 was determined by first freezing cultures of infected cells at daily intervals post-infection. The infectious virus was then titrated by plaque assay. As is indicated in Table 1, the onset of progeny CMV maturation was between 2 and 3 days post-infection, regardless of the MOI used.

Determination of when viral DNA synthesis begins

A preliminary reconstruction experiment was performed assuming a molecular weight of 1.42×10^8 daltons for the CMV genome (11, 21, 31, 34, 55, 64) and 4×10^{12} daltons for the host cell genome (22). Known amounts of unlabeled CMV DNA, corresponding to 10 (0.03 μg/ml), 50 (0.15 μg/ml), and 100 (0.3 μg/ml) genome equivalents per cell were mixed with 250 μg of uninfected cell DNA and 2.5 ng of ^{32}P-labeled viral DNA probe, denatured, and allowed to hybridize. The rate of reassociation was directly proportional to the amount of CMV DNA added. Therefore there was a linear relationship between the rate of reassociation and the genome equivalents of viral DNA per cell.

To determine when viral DNA synthesis begins, DNA extracted from infected cells at various times post-infection was hybridized to ^{32}P-labeled CMV DNA. Fig. 1 represents the reassociation of 50 copies

Table 1. Onset of progeny CMV maturation in HFF cells infected at MOIs of 1 and 10[a]

Time post-infection (days)	PFU/ml[b]	
	MOI= 1	MOI = 10
1	5.5×10^2	4.5×10^3
2	2.5×10^2	8.3×10^3
3	1.5×10^4	1.3×10^5
4	7.2×10^5	6.3×10^6
5	7.4×10^5	6.5×10^6
6	2.3×10^6	1.0×10^6

[a]Human foreskin fibroblast (HFF) cells infected at multiplicities of infection (MOI) of 1 and 10 were frozen at daily intervals post-infection. Infectious virus was titrated by plaque assay.

[b]The virus titer is expressed in plaque forming units (PFU) per ml of culture fluid.

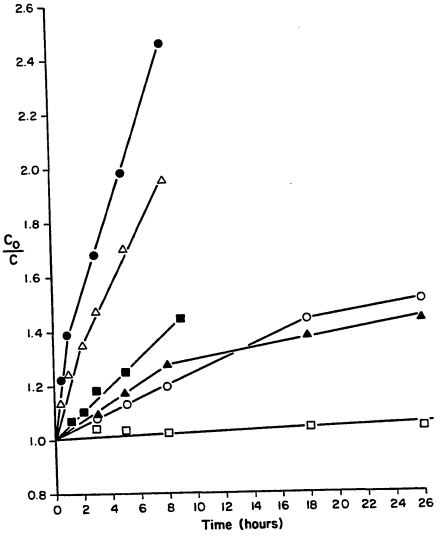

Fig. 1. Time of onset of viral DNA synthesis--Reassociation of ^{32}P-labeled CMV Towne DNA (0.03 µg/ml, 2 x 10^7 cpm/µg, 10 genome equivalents) in the presence of 200 µg/ml of DNA extracted from CMV infected cells at 12 hours (▲), 24 hours (○), 36 hours (△), and 48 hours (●) post-infection was measured. The reassociation of ^{32}P-labeled CMV Towne DNA in the presence of salmon DNA (1 mg/ml, □) and 50 genome equivalents of CMV DNA (0.15 µg/ml, ■) was also measured. C_o is the initial concentration of labeled DNA and C is the concentration of residual single-stranded DNA.

of the genome. Using the relationship between the rate of reassociation and genome equivalents per cell, cells at 12 and 24 hours post-infection contained approximately 30 genome equivalents of CMV DNA per diploid complement of cell DNA, while at 36 and 48 hours, cells contained 150 and 200 genome equivalents, respectively. Therefore, viral DNA synthesis begins between 24 and 36 hours post-infection.

Percentage of the CMV genome transcribed at early and late times post-infection

Stable RNA collected at various times post-infection was hybridized with ^{32}P-labeled CMV DNA. That RNA synthesized prior to DNA synthesis, at 2 hours, 4 hours, 12 hours, and 24 hours post-infection was designated as early RNA. RNA accumulating after DNA synthesis has begun, 36 hours and 48 hours post-infection, is referred to as late RNA.

Fig. 2 represents the fraction of DNA driven into a DNA-RNA hybrid as a function of RNA concentration and time for the 2, 4, and 12 hour RNA. Fig. 3 illustrates the reassociation of the 24, 36, and 48 hour RNA. From the data the following observations are noted.

(i) RNA present at 2 hours post-infection is homologous to 13% of the viral genome; stable RNA collected at 4 and 12 hours post-infection is homologous to 27% of the viral genome, and at 24 hours post-infection there is approximately 30% hybridization with the labeled viral DNA.

(ii) After the onset of viral DNA synthesis, a transition to relatively extensive transcription occurs. At 36 hours and 48 hours

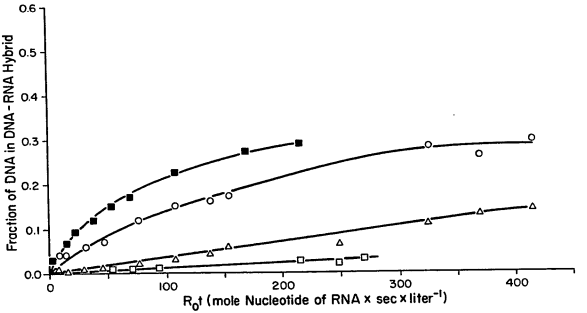

Fig. 2. Hybridization of ^{32}P-labeled CMV DNA with unlabeled RNA extracted from infected cells at 2, 4, and 12 hours post-infection--Hybridization of ^{32}P-labeled CMV DNA (0.03 µg/ml, 2 x 10^7 cpm/µg, 10 genome equivalents) with unlabeled RNA extracted from infected cells at 2 hours (3.0 mg/ml, △), 4 hours (3.0 mg/ml, ○), and 12 hours (.750 mg/ml, ■) post-infection. The control consisted of yeast RNA (1 mg/ml, □).

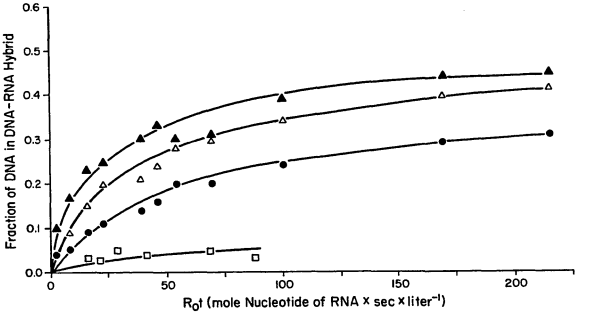

Fig. 3. Hybridization of ^{32}P-labeled CMV DNA with unlabeled RNA extracted from infected cells at 24, 36, and 48 hours post-infection--Hybridization of ^{32}P-labeled CMV DNA (0.03 µg/ml, 2 x 10^7 cpm/µg, 10 genome equivalents) with unlabeled RNA extracted from infected cells at 24 hours (.750 mg/ml, ●), 36 hours (.750 mg/ml, Δ), and 48 hours (.750 mg/ml, ▲) post-infection. The control consisted of yeast RNA (1 mg/ml, □).

post-infection there is 42% and 45% hybridization, respectively, to the viral genome.

Preliminary experiments were conducted to determine optimum RNA concentrations to allow reassociation kinetics to reach a plateau. In each of three independent experiments, plateau values did not exceed those percentages indicated.

To establish that the hybridization percentages obtained were not due to contaminating viral DNA, a portion of each RNA sample was alkali digested, mixed with ^{32}P-labeled CMV DNA and hybridized. No hybridization in excess of self-hybridization of the DNA probe was observed. Thus, RNA samples were not contaminated with viral DNA.

Viral RNA in total infected cell extracts in the presence and absence of protein synthesis

To determine the effect of de novo protein synthesis on early virus specific RNA synthesis, viral RNA accumulating in the presence and absence of cycloheximide was analyzed. The hybridization of RNA extracted from cells at 4 hours post-infection with ^{32}P-labeled CMV DNA (Fig. 4) indicated the presence of transcripts from approximately 27% of the genome. However, when cycloheximide was present before infection, during adsorption, and up to 4 hours post-infection at the time RNA was extracted from the cells, RNA homologous to only 5-6% of the CMV genome was detected (Fig. 4).

These data provide evidence that in the absence of de novo protein synthesis post-infection, transcription is restricted to

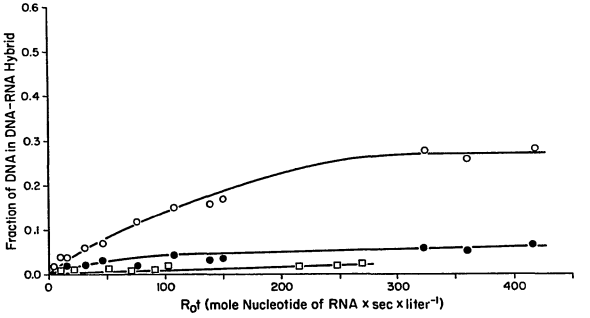

Fig. 4. Hybridization of ^{32}P-labeled CMV DNA with unlabeled RNA extracted from infected cells at 4 hours post-infection and at 4 hours post-infection in the presence of cycloheximide--Hybridization of ^{32}P-labeled CMV DNA (0.03 µg/ml, 2 x 10^7 cpm/µg, 10 genome equivalents) with unlabeled RNA extracted from infected cells at 4 hours post-infection (3 mg/ml, ○) and at 4 hours post-infection in the presence of cycloheximide (3 mg/ml, ●). The control consisted of yeast RNA (1 mg/ml, □).

approximately 5-6% of the genome. Thus, that region of the CMV genome encoding immediate early RNA is approximately 5-6%.

In addition, viral RNA transcripts accumulating at 2 hours post-infection in the presence and absence of cycloheximide were analyzed. However, at 2 hours post-infection in the presence of cycloheximide, there was insufficient viral specific RNA (even at 3 mg/ml) to accelerate the reassociation rate in excess of background hybridization of the ^{32}P-labeled CMV DNA (data not shown). The reassociation of RNA extracted at 2 hours post-infection in the absence of cycloheximide (Fig. 2) indicates the presence of transcripts to at least 15% of the genome, which is in excess of the 5-6% found for immediate early RNA. Thus, transcription at 2 hours post-infection is less restrictive than in cycloheximide treated cells.

Detection of symmetric transcripts

An analysis fundamental to understanding the regulation of viral genome expression was to determine whether or not symmetric transcription occurs, and if so, the extent to which the viral genome is transcribed symmetrically. Since the analysis of late RNA indicated the presence of RNA homologous to less than 50% of the CMV genome, the extent to which the genome is transcribed symmetrically was expected to be limited. To obtain more direct evidence, RNA extracted from infected cells at 4 hours, 12 hours, and 48 hours post-infection were analyzed for the presence of symmetric transcripts. The difference in the percentage of hybridization observed for pre-hybridized RNA denatured after the time of adding single-stranded DNA probe as

compared to that observed for pre-hybridized RNA not denatured at the time of adding single-stranded DNA probe represents the percentage of DNA transcribed symmetrically.

Fig. 5 illustrates the results obtained for the analysis of symmetric transcripts present in early and late RNA. For the 4 hour RNA (4% hybridization) and 12 hour RNA (15% hybridization) there was no difference observed for hybridization of ^{32}P-labeled CMV DNA in the presence of pre-hybridized, non-denatured RNA and pre-hybridized, denatured RNA. For the 48 hour RNA, 37% hybridization of pre-hybridized, denatured RNA and 33% hybridization of pre-hybridized, non-denatured RNA was observed. This indicates that approximately 4-5% of the genome is transcribed symmetrically late in infection. For the most part, transcription of the CMV genome appears to be asymmetric.

The plateau levels of hybridization of ^{32}P-labeled CMV DNA to pre-hybridized RNA were less than those observed in previous experiments (Figs. 2 and 3), in which the RNA had not been pre-hybridized. These decreased levels of hybridization are probably the result of degradation of labeled CMV DNA or RNA over the extended period of hybridization.

Detection of transcripts from the repeat regions of the CMV genome

To determine to what extent immediate early, early, and late stable RNA is transcribed from the repeat regions of the CMV genome, ^{32}P-labeled XbaI restriction fragments Q and M were hybridized to RNA

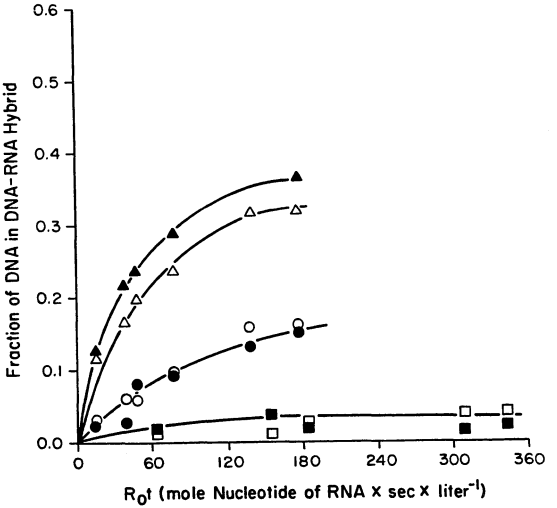

Fig. 5. Hybridization of RNA extracted from infected cells at indicated times post-infection with ^{32}P-labeled CMV DNA for the detection of symmetric transcripts--Hybridization of RNA extracted from infected cells at indicated times post-infection with ^{32}P-labeled CMV DNA (0.03 µg/ml, 2 x 10^7 cpm/µg, 10 genome equivalents). 4 hour RNA (3 mg/ml, □), 12 hour RNA (.750 mg/ml, ○), and 48 hour RNA (.750 mg/ml, △) were pre-hybridized, mixed with the denatured CMV DNA, and hybridized for various time intervals. 4 hour RNA (■), 12 hour RNA (●), and 48 hour RNA (▲) were pre-hybridized, mixed with the denatured CMV DNA, heat denatured, and hybridized for various time intervals.

extracted from infected cells at 4 hours post-infection in the presence of cycloheximide, 12 hours post-infection, and 48 hours post-infection.

The linkage map of CMV DNA fragments produced by cleavage with the restriction endonuclease XbaI is shown in Fig. 6. Restriction fragments Q and M are located in the repeat regions, which are found on either end of the long unique sequences. Fig. 7 shows the autoradiographs of Southern blots of the XbaI digested total CMV DNA, which were incubated in the presence of ^{32}P-labeled total CMV probe DNA, ^{32}P-labeled XbaI fragment M probe, or ^{32}P-labeled XbaI fragment Q probe. Hybridization of the ^{32}P-labeled probes to DNA bound to the blots demonstrated their specificity. The fragment M probe was found to hybridize to M and a fragment of the DEF complex. There was also a minor amount of hybridization to fragment L. The fragment Q probe was found to hybridize to Q and fragment O. The hybridization of specific fragment probes to more than one fragment indicates that these fragments contain repeat sequences and confirms the location of these fragments in the linkage map shown in Fig. 6. The minor amount of hybridization to fragment L, which is in the middle of the unique region, could be due to contamination of fragment M as a result of partial degradation of the DNA. This minor amount of contamination would not be expected to contribute significantly to values obtained for the hybridization of restriction fragment probe M in the presence of infected cell RNA.

Preliminary experiments were conducted to determine the extent of hybridization of the ^{32}P-labeled fragments in the presence of excess

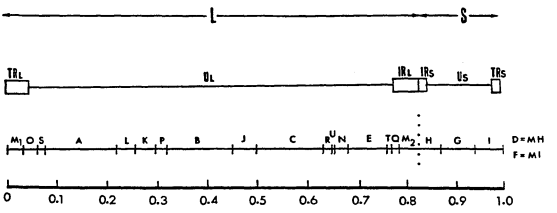

Fig. 6. Physical map and linkage map of XbaI cleavage sites of the CMV (Towne) genome--The genome consists of unique (U) sequences in a long (L) and short (S) section that are bounded by terminal repeats (TR) and inverted internal repeats (IR). The bottom line represents map units. The XbaI restriction enzyme linkage map was determined by LaFemina and Hayward (personal communication).

Fig. 7. Demonstration of specificity of hybridization probes--XbaI fragments of CMV DNA were electrophoresed in a 0.6% Seaplaque agarose gel, stained with ethidium bromide and photographed under short-wavelength UV light (lane 1). The DNA was then blotted onto a nitrocellulose filter and cut into 0.5 mm strips. Each strip was then incubated with the ^{32}P-labeled CMV DNA (100,000 cpm, lane 2), ^{32}P-labeled fragment M probe DNA (50,000 cpm, lane 3), or ^{32}P-labeled fragment Q probe DNA (50,000 cpm, lane 4). To detect hybridization to fragments, the blots were autoradiographed by exposure to X-ray film with a Cronex intensifying screen for 10 hours. The XbaI fragments of CMV Towne DNA are designated by capital letters of the alphabet in order of increasing electrophoretic mobility. The subscript "m" refers to a minor fragment.

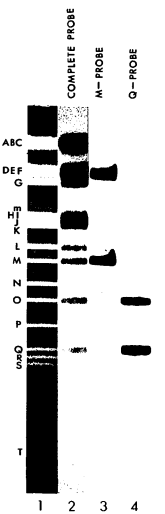

Fig. 7. Demonstration of Specificity of Hybridization Probes

homologous DNA. Fig. 8 illustrates the hybridization of ^{32}P-labeled restriction fragments Q and M with CMV infected cell DNA. The hybridization rate of probe M was found to be linear to a C_o/C value of greater than 9.4 (89.4%). The hybridization rate of probe Q was found to be linear to a C_o/C value of 5.8 (83%), at which point hybridization continued at a slower rate to a value of 7.1 (86%).

The extent of self-hybridization of probe DNA was also examined (Fig. 9). Probe M hybridized to a C_o/C value of 1.38 (27.5%) in 20 hours. Probe Q hybridized to a C_o/C value of 1.41 (29.1%). The percent hybridization for each time point was subtracted as background from the hybridization of ^{32}P-labeled probes in the presence of RNA extracted from infected cells.

The hybridization of M and Q probes with immediate early RNA (Fig. 10) indicated the presence of RNA transcripts homologous to 7% of fragment M and 15% of fragment Q.

Fig. 11 illustrates the hybridization of fragment Q to early (12 hour) and late (48 hour) RNA. Early RNA contained transcripts homologous to 16% of fragment Q, while late RNA was homologous to 40% of fragment Q.

The hybridization of fragment M to the early (12 hour) and late (48 hour) RNA is shown in Fig. 12. The early RNA contained transcripts homologous to 20% of fragment M, and the late RNA was homologous to 32% of fragment M.

These data suggest that immediate early transcription of the repeat regions is limited, but there is more extensive transcription from these regions at later times post-infection.

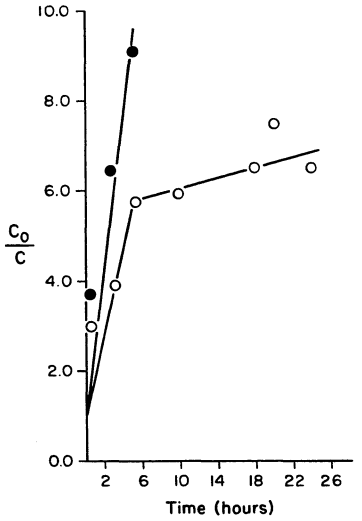

Fig. 8. Extent of hybridization of ^{32}P-labeled restriction fragments in the presence of excess homologous DNA--Reassociation of ^{32}P-labeled XbaI restriction fragment Q (0.03 µg/ml, 2 x 10^7 cpm/µg, O) and ^{32}P-labeled XbaI restriction fragment M (●) with 0.1 mg/ml CMV infected cell DNA was measured. C_o is the initial concentration of labeled DNA and C is the concentration of residual single-stranded DNA.

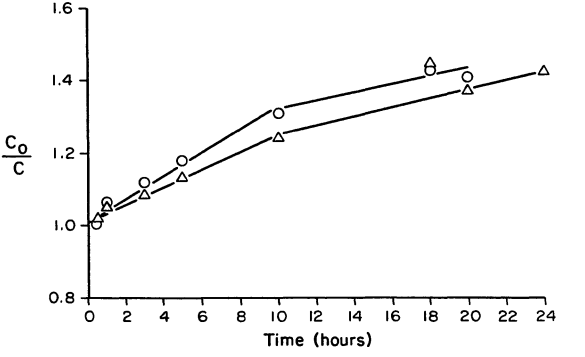

Fig. 9. Extent of self-hybridization of ^{32}P-labeled restriction fragment probes--Reassociation of ^{32}P-labeled XbaI restriction fragment Q (0.03 μg/ml, 2 x 10^7 cpm/μg, O) and ^{32}P-labeled XbaI restriction fragment M (Δ) with 1 mg/ml salmon DNA was measured. C_o is the initial concentration of labeled DNA and C is the concentration of residual single-stranded DNA.

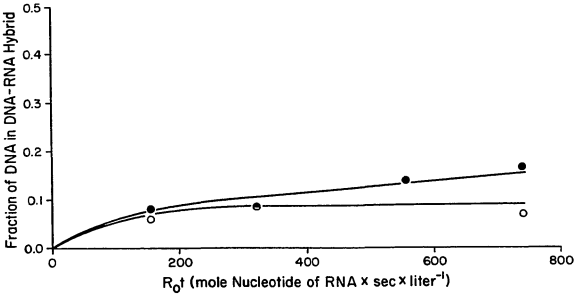

Fig. 10. Hybridization of ^{32}P-labeled XbaI restriction fragments Q and M with unlabeled RNA extracted from infected cells at 4 hours post-infection in the presence of cycloheximide--Hybridization of ^{32}P-labeled XbaI restriction fragment Q (0.03 μg/ml, 2 x 10^7 cpm/μg, ●) and ^{32}P-labeled XbaI restriction fragment M (○) with unlabeled RNA extracted from infected cells at 4 hours post-infection in the presence of cycloheximide (3 mg/ml).

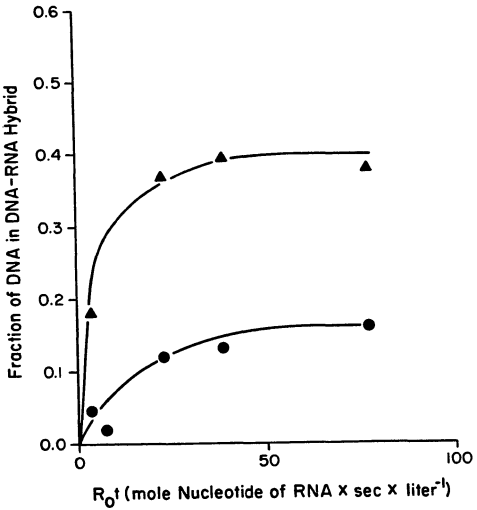

Fig. 11. Hybridization of ^{32}P-labeled XbaI restriction fragment Q with unlabeled RNA extracted from infected cells at 12 and 48 hours post-infection--Hybridization of ^{32}P-labeled XbaI restriction fragment Q (0.03 μg/ml, 2 x 10^7 cpm/μg), with unlabeled RNA extracted from infected cells at 12 hours (.750 mg/ml, ●) and 48 hours (.750 mg/ml, ▲) post-infection.

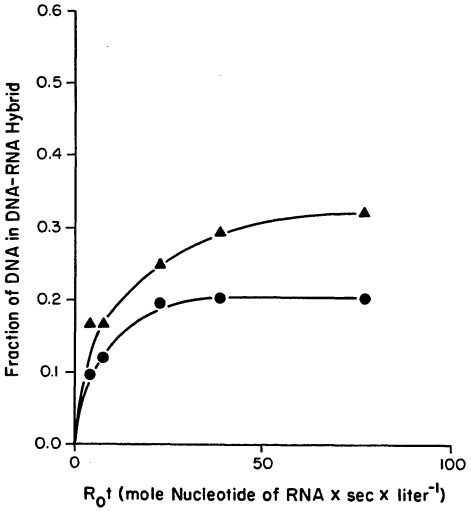

Fig. 12. Hybridization of ^{32}P-labeled XbaI restriction fragment M with unlabeled RNA extracted from infected cells at 12 and 48 hours post-infection--Hybridization of ^{32}P-labeled XbaI restriction fragment M (0.03 μg/ml, 2 x 10^7 cpm/μg) with unlabeled RNA extracted from infected cells at 12 hours (.750 mg/ml, ●) and 48 hours (.750 mg/ml, ▲) post-infection.

DISCUSSION

The major events in the replication cycle of CMV have been characterized previously (10, 28, 54). However, controversy existed with respect to the time for the onset of viral DNA synthesis, which ranged from 12 hours to after 24 hours post-infection. The methods of determination, the virus strains employed, and the multiplicity of infections were different for each report in the literature. Thus, to correlate the study of transcription with other events occurring during CMV replication, experiments were first conducted to determine the time post-infection for the onset of virus maturation and virus DNA synthesis under defined conditions. Irrespective of whether the multiplicity of infection was 1 or 10, viral maturation did not occur until between 2-3 days post-infection. The onset of CMV DNA synthesis, determined by hybridization of viral DNA with DNA extracted from cells at various times post-infection, was found to occur between 24 and 36 hours post-infection.

The results of experiments to determine the fraction of the CMV genome transcribed at various periods of time post-infection provide evidence for temporal regulation of transcription. The transcription of the CMV genome was restricted in the presence of an inhibitor of protein synthesis to 5-6% of the genome (immediate early phase of transcription). Viral transcripts accumulating at 2 hours post-infection (homologous to at least 15% of the genome) suggests that there is a rapid switch of immediate early to the early phase of

transcription, which extends to at least 24 hours post-infection. Stable RNA homologous to approximately 30% of the viral genome was found throughout most of the early phase of transcription. This switch from immediate early to early was also reported by Wathen et al. (59, 60) and Demarchi et al. (12) using different technology. The CMV Towne (59, 60) and CMV Davis (12) immediate early and early RNA were analyzed by hybridization of ^{32}P-labeled RNA extracted from cells, with viral DNA restriction enzyme fragments that were bound to nitrocellulose filters. As suggested by Wathen et al. the switch may involve either the synthesis of a viral polymerase, the synthesis of a protein that modifies the existing host DNA-dependent RNA polymerase, or the template activity of the viral DNA may be modified by an immediate early virus-induced protein(s).

After the onset of viral DNA synthesis, the transcription extends into the late phase during which RNA is homologous to approximately 42% of the viral genome. Thus, qualitative differences in CMV RNA transcripts were detectable between early and late times. This is unique to CMV. No qualitative difference in RNA transcripts was detectable in HSV type 1, other than in the immediate early RNA (9, 29). The difference in the extent to which the CMV genome was found to be transcribed at late and early times post-infection is approximately 10-15%. Since asymmetric transcription predominates, this would require approximately 20-30% of the double-stranded viral DNA molecule. An estimate of the portion of the viral genome required to encode the information for structural polypeptides (30 polypeptides with an

average molecular weight of 50,000 daltons), assuming asymmetric transcription and the absence of multiple splicing events, is approximately 21%. Thus, the increase in the extent to which the genome is transcribed late is sufficient to provide messenger RNA for most, if not all, of the structural polypeptides.

The early and late RNA was also analyzed for the presence of symmetric transcripts. Transcription was found to be asymmetric during the early phase, while 5% of the RNA present late was transcribed symmetrically. Since only stable RNA was studied, it is possible that symmetric transcription is more extensive, but that one strand of the symmetric transcript may be rapidly degraded.

Other investigators have indicated that one of the major differences between HSV and CMV is that immediate early transcription of HSV originates from the long and short repeat sequences and adjacent sequences (61), whereas that of CMV originates predominantly from the long unique region of the genome (0.660 to 0.770 map units) (60). In the studies reported here, it was found that immediate early RNA is transcribed from 10% of the long repeat region (combined 15% of fragment Q, 2.7 X 10^6 daltons and 7% of fragment M, 5.4 X 10^6 daltons). In contrast, at early times after infection there is 18.5% transcription of the long repeat region (combined 16% of fragment Q and 20% of fragment M), and at late times there is approximately 34% transcription (combined 40% of fragment Q and 32% of fragment M). The transcription of early and late RNA from the long repeat regions is extensive and of interest because 1) these regions are where nucleotide sequence

heterogeneity between CMV strains has been located (42), 2) alterations in the DNA of CMV obtained from human osteogenic sarcoma cells persistently infected with CMV Towne map in these regions (19) and 3) by analogy to HSV, these regions may be transcribed into RNA that undergoes splicing (9, 61).

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