JOURNAL OF VIROLOGY, Jan. 1989, p. 233–241
0022-538X/89/010233-09$02.00/0

Vol. 63, No. 1

Analysis of a Region of the Human Cytomegalovirus (AD169) Genome Coding for a 25-Kilodalton Virion Protein

JESSE MARTINEZ,† ROYA S. LAHIJANI, AND STEPHEN C. ST. JEOR*
Department of Microbiology, University of Nevada-Reno, Reno, Nevada 89557-0046

Received 22 July 1988/Accepted 27 September 1988

In a previous study, we reported the isolation of a lambda gt11 cDNA clone (C3) for a virus message that mapped to the *Hin*dIII R fragment (human cytomegalovirus [AD169]). In this report, we further analyze transcription from this region of the genome. C3 was used to probe Northern (RNA) blots of RNA isolated from infected cells. Two abundant messages, 1.3 and 1.6 kilobases (kb) in size, were detected at 62 h postinfection (p.i.). Examination of different time points determined that the 1.6-kb mRNA accumulated in infected cells between 24 and 48 h p.i. and was classified as a late message. The 1.3-kb message was transcribed early in infection and was initially detected around 12 h p.i. Both transcripts were suppressed when infected cells were treated with inhibitors of DNA synthesis. Sequencing and S1 analysis identified the 5′ ends of these two messages within 240 nucleotides of each other. Two CAAT-TATA motifs were found upstream of the 1.3- and 1.6-kb mRNA initiation sites, which suggested that the promoters were also closely associated. Antisera made to the fusion protein, synthesized from the C3 clone, detected a 25-kilodalton (kDa) virus protein found in infected cells and purified virions. Western blot (immunoblot) analysis of infected-cell proteins at various times after infection demonstrated that the accumulation of the 25-kDA protein coincided with the appearance of the 1.6-kb message. Therefore, we conclude that the 25-kDa virion protein is translated from the 1.6-kb message.

Cytomegalovirus is a ubiquitous member of the herpesvirus family and is associated with a variety of diseases such as cytomegalic inclusion disease, interstitial pneumonia, and human cytomegalovirus (HCMV) mononucleosis. As with other herpesviruses, it exhibits sequential and coordinated gene expression during replication (3, 4, 13, 24, 25; reviewed in reference 21). Virus transcription begins with activation of the immediate-early (alpha) genes (25). These messages are transcribed from isolated regions of the genome, and their protein products bring about the switch from restricted to generalized transcription as infection progresses (4, 13, 24, 25). Early (beta) genes are activated during generalized transcription, and their messages appear before the onset of virus DNA replication. In addition, their activation requires de novo protein synthesis (11, 13). Initiation of late (gamma) gene transcription coincides with the onset of virus DNA replication (3). At this time the structural components of the capsid begin to accumulate, and virions are released shortly afterward (19, 20, 22).

The mechanisms that control transcription in cytomegalovirus immediate-early genes have been investigated in detail. Sequence and biochemical analyses of the HCMV major immediate-early gene demonstrated that a strong enhancer element upstream from the 5′ end stimulates transcription and that the gene product mediates protein levels by regulation of its own message (2, 18). Therefore, the primary regulatory mechanism of the immediate-early virus genes is at the transcriptional level. Studies of several late genes suggest that expression of these genes is fundamentally different from that of the immediate-early genes. Comparison of virus messages found on polysomes of infected cells with total-cell RNA suggests that late RNAs are transcribed early in infection but sequestered in the nucleus until after the onset of virus DNA replication (24, 25). In support of this theory, kinetic studies of ICP27, a structural component of the virion, determined that transcripts are readily detectable in total-cell RNA at early times (5). Studies of late genes in HCMV (Towne) suggest that the sequestering of late transcripts within the nuclei of infected cells is related to posttranscriptional processing (6). A splicing event coincides with the onset of virus DNA synthesis and takes place just before the transcripts are transported into the cytoplasm. Suppression of DNA synthesis prevents splicing of these transcripts and their accumulation in the cytoplasm, which suggests that the primary regulatory mechanism of these late genes is posttranscriptional in nature.

In previous studies, we isolated three cDNA clones of transcripts for virion proteins. One clone (C3) was mapped to the *Hin*dIII R fragment of HCMV (AD169). Human convalescent sera reacted with the C3 fusion protein (fpC3) and antisera made to fpC3 identified a virion protein with a molecular mass of 25 kilodaltons (kDa). In this study, we identified the transcript that codes for this protein and determined the precise location of the gene on the HCMV map. In addition, we characterized transcription from the *Hin*dIII R fragment and determined the temporal characteristics of messages that originate from this area.

MATERIALS AND METHODS

Cells and virus. Human embryonic lung (HEL) cells, NIH 3T3 cells, and Iris monkey lung (IML) cells were grown in monolayers and maintained on Dulbecco modified Eagle medium (DMEM) supplemented with fetal calf serum. HCMV (AD169) was used exclusively in these studies.

Isolation of infected-cell proteins and Western blot (immunoblot) analysis. Confluent monolayers of HEL cells were infected with HCMV (AD169) at a multiplicity of infection of 10 PFU per cell. Infected-cell proteins were harvested at various hours post infection (p.i.) by washing the monolayer three times in ice-cold phosphate-buffered saline (PBS; 150 mM NaCl, 12 mM Na_2HPO_4, 1 mM KH_2PO_4 [pH 7.6]). The cells were lysed directly in sodium dodecyl sulfate (SDS)-

* Corresponding author.
† Present address: Department of Molecular Biology, Princeton University, Princeton, NJ 08544.

polyacrylamide gel electrophoresis (PAGE) sample buffer (0.1 M Tris chloride [pH 6.8], 1% SDS, 0.1 M beta-mercaptoethanol) and frozen at −20°C until needed.

Drug-treated cell proteins were prepared as follows. The zero time point was made by pretreating cells with 150 μg of cycloheximide (CH; Sigma Chemical Co., St. Louis, Mo.) per ml, followed by infection with HCMV (AD169) stock containing 150 μg of CH per ml. These cells were harvested after a 1-h adsorption period as described above. Proteins from CH-blocked cells were made by pretreatment and infection as described for the zero time point. After virus adsorption, medium containing 150 μg of CH per ml was put back on the cells, and the cells were incubated for an additional 12 h. The medium was removed, and the cell layer was washed three times at 37°C with PBS containing 10 μg of actinomycin D (Calbiochem-Behring, La Jolla, Calif.) per ml. Medium supplemented with 10 μg of actinomycin D per ml was added to the cells, and incubation continued for an additional 90 min. The cells were then harvested by lysis in SDS-PAGE sample buffer. Cells treated with 5-fluoro-2′-deoxyuridine (FUdR; Sigma) were infected with AD169 as described above. Medium containing 10^{-6} M FUdR was placed on the cells after a 1-h adsorption period, and incubation continued for 24 h. The proteins were harvested by direct lysis of the monolayer with SDS-PAGE sample buffer. Purified virions were isolated as described by Huang et al. (8). Virion proteins (10 μg) were dissolved in 60 μl of SDS-PAGE sample buffer in preparation for application to the gradient SDS-PAGE gel.

Gradient 7.5 to 20% SDS-PAGE gels were made as described by Hames (7). A 100-μg portion of infected-cell proteins from each sample was applied to the gel and electrophoresed. The proteins were electrophoretically transferred (23) to GeneScreen Plus membranes (Dupont, NEN Research Products, Boston, Mass.) and probed with either human convalescent sera or antisera made against the β-galactosidase fusion protein (fpC3). Affinity-purified anti-immunoglobulin antibody radiolabeled with ^{125}I was used to detect binding of antibodies. Filters were autoradiographed overnight with X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) at −70°C with intensifying screens.

Isolation of RNA samples. At various times p.i., confluent monolayers of cells infected with HCMV (AD169) were harvested by trypsinization and three washes with PBS (pH 7.6). Cells were then solubilized in 6 M guanidinium isothiocyanate–5 mM sodium citrate (pH 7.0)–0.1 M beta-mercaptoethanol–0.5% Sarkosyl (CIBA-GEIGY Corp., Summit, N.J.), and the RNA was isolated by pelleting through a cushion of 5.7 M CsCl (10). All RNA samples were ethanol precipitated twice and suspended in sterile water before determination of optical density at 260 nm.

Cytoplasmic RNA was isolated from cells harvested by trypsinization and washed three times in ice-cold PBS (pH 7.6). After the last centrifugation step (1,500 rpm, 5 min), cell pellets were suspended in 3.74 ml of ice-cold lysis buffer (140 mM NaCl, 10 mM Tris chloride [pH 8.6], 1.5 mM $MgCl_2$, 0.5% Nonidet P-40) for every 10^7 cells and vortexed for 10 s. The lysate was then layered onto an equal volume of ice-cold sucrose solution (lysis buffer supplemented with 1% Nonidet P-40 and 24% sucrose) in Corex tubes and centrifuged at 10,000 × *g* for 20 min at 4°C to remove cellular debris and nuclei. The upper layer, which contained the cytoplasmic lysate, was removed and extracted twice with phenol-chloroform (50:50) and twice with chloroform. The RNA was ethanol precipitated overnight at −20°C and then pelleted at 10,000 rpm for 30 min in a GSA rotor (Ivan Sorvall, Inc., Norwalk, Conn.) The pellet was suspended in 2 ml of 1× PK buffer (0.1 M Tris chloride [pH 7.5], 12 mM EDTA, 150 mM NaCl, 1% SDS), and predigested proteinase K (Sigma) was added to 200 μg/ml (final concentration). The solution was incubated at 37°C for 20 min, extracted twice with phenol-chloroform (50:50) and once with chloroform, and ethanol precipitated. For cytoplasmic poly(A)$^+$ samples, RNA from 62-h-p.i. HEL cells was extracted as described above, and the poly(A)$^+$ fraction was isolated by chromatography on oligo(dT)-cellulose (Collaborative Research, Inc., Waltham, Mass.). A second fractionation on oligo(dT)-cellulose further removed non-poly(A)$^+$ RNA from the samples.

RNA gel electrophoresis and Northern (RNA) hybridizations. All RNA samples were size fractionated on agarose gels containing 2.2 M formaldehyde (9). Gels were transferred to GeneScreen Plus membranes and hybridized to nick-translated probes as previously described (12). For experiments in which oligonucleotides were used as probes, the filters were prehybridized and hybridized in 5× Denhardt buffer (50× Denhardt buffer is 1% Ficoll, 1% polyvinylpyrrolidone, and 1% bovine serum albumin), 5× SSPE (20× SSPE is 3 M NaCl, 0.2 M NaH_2PO_4 [pH 7.4], and 20 mM EDTA), 1% SDS, salmon sperm DNA (100 μg/ml), and 1 mM sodium pyrophosphate. The temperature of prehybridization and hybridization (T_H) for oligomers was calculated by the following formula (1): $T_H = T_D - 5°C$, where T_D = (2°C × the number of A · T base pairs) + (4°C × the number of G · C base pairs). Final concentration of the probe was 5 ng/ml. Blots were washed four times in 200 ml of 6× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS for 10 min each time at room temperature, followed by two washes for 10 min each in 3× SSC–0.1% SDS at 37°C, followed by a 5-min wash in 6× SSC–0.1% SDS at T_H. Filters were air dried and autoradiographed with intensifying screens at −80°C, using X-Omat film.

Nuclease digestion analyses. Cytoplasmic poly(A)$^+$ RNA was used for all S1 nuclease and exonuclease VII (Exo VII) analyses. The procedures used were those described by Sharp et al. (17). Briefly, 10 μg of cytoplasmic poly(A)$^+$ RNA isolated from AD169-infected cells at 62 h p.i. was coprecipitated with approximately 0.10 μg of DNA restriction enzyme fragment end labeled by using T4 kinase and [γ-^{32}P]ATP. After pelleting, the nucleic acids were dissolved in 10 μl of annealing buffer [80% formamide, 40 mM piperazine-*N*,*N*′-bis(ethanesulfonic acid) (PIPES; pH 6.4), 400 mM NaCl, 0.2 mM EDTA] and heated to 85°C for 15 min to denature the probe. The samples were annealed for 3 h at 55°C. For S1 analysis, the annealing reaction was diluted with 300 μl of digestion buffer (280 mM NaCl, 30 mM sodium acetate, 4.5 mM zinc acetate, 20 μg of heat-denatured salmon sperm DNA per ml) containing 500 U of S1 nuclease per ml. Digestions were done at 10°C for 30 min and then terminated with 75 μl of stop buffer (2.5 M ammonium acetate, 50 mM EDTA). The sample was precipitated with 500 μl of isopropanol at −70°C for 15 min, pelleted, and prepared for electrophoresis. Samples digested with Exo VII were annealed in a similar manner but were subsequently diluted with 100 μl of Exo VII digestion buffer (0.03 M KCl, 0.01 M Tris chloride [pH 7.4], 0.01 M EDTA). Five units of Exo VII (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was added to each reaction mixture, and the preparations were incubated for 60 min at 45°C. Reactions were terminated by addition of 10 μl of 1 M NaCl and 2 volumes of ice-cold ethanol. After ethanol precipitation, S1 nuclease and Exo VII samples were prepared for electrophoresis by suspension of the pellets in 20 μl of loading

buffer (80% deionized formamide, 50 mM Tris borate [pH 8.3], 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromphenol blue). The preparations were boiled for 4 min and then quenched on ice until needed. All samples were electrophoresed on a 4% polyacrylamide–7 M urea denaturing gel. After electrophoresis, the gels were fixed in 10% acetic acid and impregnated with En^{3}Hance (Dupont, NEN), dried, and autoradiographed on X-Omat AR film at −80°C.

Sequence analysis. All sequencing was done by the dideoxynucleotide-chain termination method (16). DNA fragments to be sequenced were subcloned into M13, mp18, and mp19 vectors. Single-stranded templates purified from bacteriophage M13 were annealed to the universal primer, synthetic oligonucleotides, or both and extended with either the Klenow fragment of DNA polymerase (Bethesda Research Laboratories) or Sequenase (United States Biochemical Corp., Cleveland, Ohio) in the presence of the appropriate concentration of deoxy- and dideoxynucleotide triphosphates. [α-^{35}S]dCTP was used as the radioactive label in all cases, and the reaction products were analyzed on 6% polyacrylamide sequencing gels. Gels were fixed, dried, and autoradiographed, and the sequence was determined.

Oligonucleotide preparation. All oligonucleotides were made on a Dupont Coder 300, using phosphoramidite chemistry. The nucleic acid was decoupled from the resin substrate as recommended by the manufacturer and ethanol precipitated two times. For experiments in which oligonucleotides were radiolabeled and used as probes, crude preparations from the synthesizer were purified on 7 M urea–17% polyacrylamide denaturing gels. The purified oligomers were end labeled with T4 kinase and [γ-^{32}P]ATP (1). The labeled DNA was separated from unincorporated nucleotides by using Sephadex-G50f (Pharmacia, Inc., Piscataway, N.J.) and then filtered through 0.2-μm-pore-size Acrodisc filters pretreated with 200 μg of tRNA in 6× SSC (Gelman Sciences, Inc., Ann Arbor, Mich.).

RESULTS

vp25 is a late virus gene product. Previously we isolated a cDNA clone (C3) for a late virus message from a lambda gt11 library with antibody prepared against purified virions. C3 was mapped to the *Hin*dIII R fragment in the long unique region of the HCMV (AD169) genome (Fig. 1). A 19-kDa virion protein was detected on Western blots probed with antisera to the C3 β-galactosidase fusion protein (fpC3). We have subsequently recalculated the molecular mass of this protein and found it to be ca. 25 kDa rather than 19 kDa as previously reported (12). With the availability of prestained molecular weight markers, we were able to place size standards more precisely on the immunoblots for a more accurate estimate of the size of the virion protein. Therefore, we will refer to this protein as vp25 in this and subsequent communications.

Kinetic analysis of infected-cell proteins demonstrated that structural proteins of the HCMV virion accumulate late in infection (19). vp25 was previously shown to be part of the virion, which led us to predict that it would be a late virus gene product. To determine this, we conducted Western blot analysis of infected cells. Proteins from HCMV-infected cells were fractionated on 7.5 to 20% gradient SDS-PAGE gels and transferred to GeneScreen Plus membranes. Duplicate protein blots were probed with either human convalescent sera or rabbit fpC3 antisera. Convalescent sera detected numerous virus-specific proteins as infection progressed from 0 to 62 h p.i. (Fig. 2A). The 25-kDa protein was initially

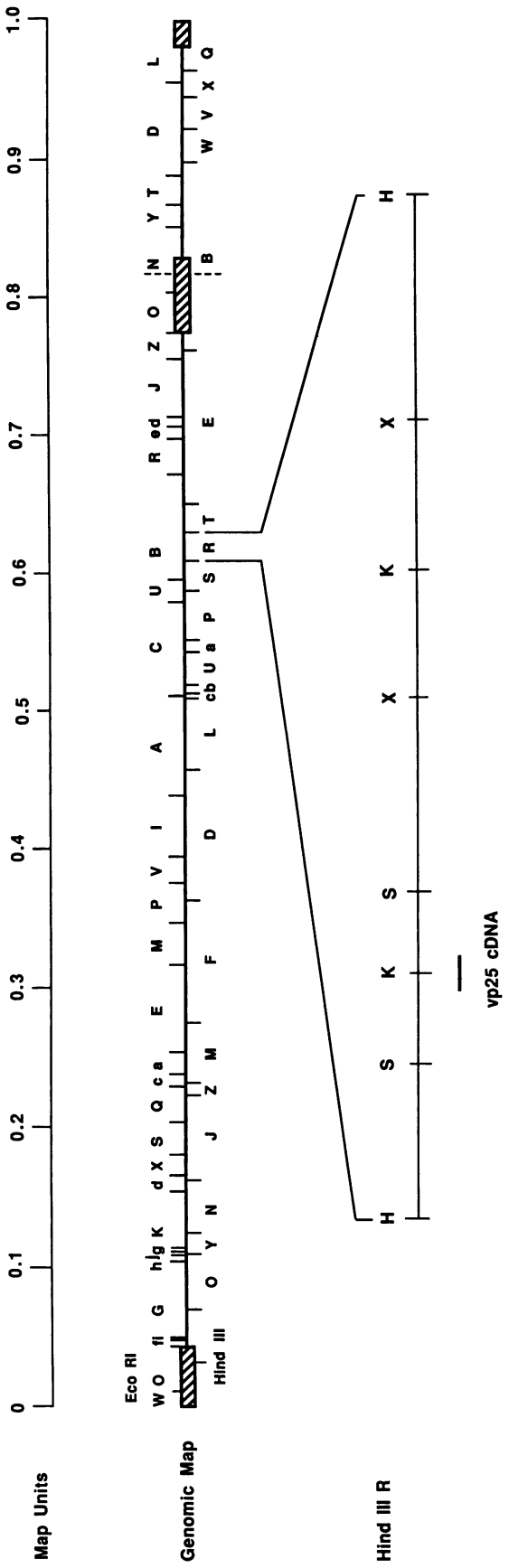

FIG. 1. HCMV (AD169) genomic map and location of the vp25 cDNA clone within the *Hin*dIII R fragment. A schematic of the AD169 genome with locations of sites for the restriction enzymes *Eco*RI and *Hin*dIII is depicted above an expanded map of the *Hin*dIII R fragment. Sites for *Hin*dIII (H), *Sma*I (S), *Kpn*I (K), and *Xba*I (X) within the R fragment are indicated. The line labeled vp25 cDNA indicates homology of the clone with the R fragment.

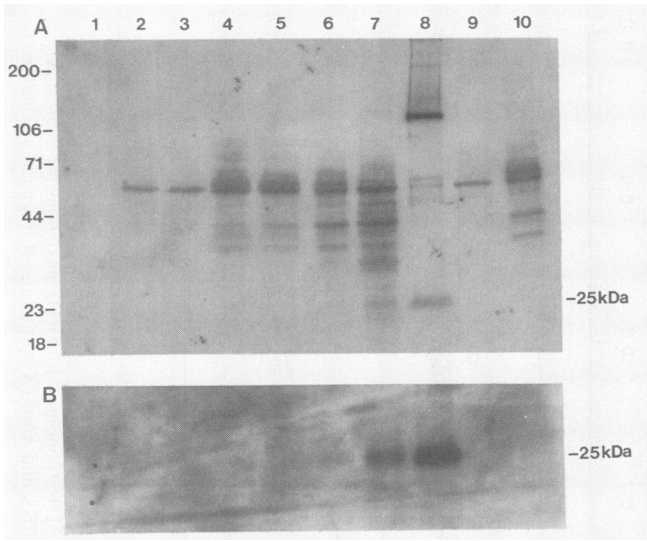

FIG. 2. Production of vp25 in HCMV-infected fibroblasts. Confluent monolayers of HEL cells were infected with HCMV (AD169) as described in Materials and Methods. Proteins were harvested from uninfected cells (lane 1) or from infected cells 2 (lane 3), 12 (lane 4), 24 (lane 5), 48 (lane 6), and 62 (lane 7) h p.i. Lane 2 represents the zero time point, at which cells were pretreated with CH (150 μg/ml) for 1 h and then infected with AD169 stock containing CH at the same concentration. Proteins were harvested immediately after a 1-h adsorption period. The sample in lane 8 contained proteins from virions purified on sucrose gradients. For lane 9, cells were blocked with CH (150 μg/ml) and incubated for 12 h. The CH block was released by washing the cell monolayer with PBS, and incubation continued for 90 min in DMEM supplemented with actinomycin D (10 μg/ml) before harvesting. The protein sample in lane 10 is from infected cells treated with FUdR. Confluent monolayers of cells were infected with HCMV for 1 h. Incubation was continued for an additional 12 h in DMEM with 10^{-6} M FUdR, at which time proteins were harvested. A 100-μg amount of protein from each time point (10 μg of virion proteins) was electrophoresed on a 7.5 to 20% gradient SDS-PAGE gel. Proteins were transferred from duplicate gels to GeneScreen Plus membranes and probed with either human convalescent sera (A) or rabbit fpC3 antisera (B). The sizes (in kilodaltons) of markers are indicated at the left of panel A; positions of the 25-kDa protein are marked at the right of panels A and B.

detected at about 48 h p.i. (Fig. 2A, lane 6) and was most abundant at 62 h p.i. and in purified virions (Fig. 2A, lanes 7 and 8). We were able to detect the 68-kDa major capsid protein (15) of input virus at the zero time point (Fig. 2A, lane 2). As a result, all zero-time-point cell samples were blocked with CH to allow us to identify protein bands contributed by input virus. The human convalescent sera also identified at least six virus-specific proteins in cells treated with 10^{-6} M FUdR. Notably, vp25 was not detected in either CH- or FUdR-treated fibroblasts infected with HCMV (Fig. 2A, lanes 9 and 10). A duplicate blot probed with fpC3 antisera detected a single 25-kDa protein in infected cells and purified virions (Fig. 2B). The accumulation of this protein was first apparent at 48 h p.i. (Fig. 2B, lane 6) in normally infected cells and did not accumulate in cells when DNA synthesis was blocked. Therefore, we conclude that the 25-kDa protein detected by fpC3 antibody is a late virus gene product.

Multiple overlapping transcripts come from the *Hin*dIII R fragment. When hybridized to Northern blots containing 62-h-p.i. RNA, the C3 cDNA identified numerous transcripts of various sizes (12). Since only one time point was analyzed previously, we were unable to determine whether these transcripts represented splicing intermediates of posttranscriptional processing or autonomous messages that coded for other gene products. Analysis of infected-cell proteins identified vp25 as a late gene product, which suggested that its transcript would also appear late in infection. To search for the vp25 message, we identified accumulation of RNAs complementary to C3 cDNA by using Northern blots.

Confluent monolayers of HEL cells were infected with HCMV and harvested from CH-blocked cells at 2, 12, 24, 48, and 62 h p.i. These were the same time points used in the immunoblot experiment and allowed direct comparison of RNA transcripts and proteins present in the infected cell during the same time point. In addition, we sought to correlate transcription with transport into the cytoplasm by comparison of total-cell and cytoplasmic RNAs. Unexpectedly, we found a characteristic rate of accumulation for each of the transcripts (Fig. 3), which suggested that they were not processing intermediates but messages for various gene products. The two most abundant RNAs detected by the C3 cDNA probe in both cytoplasmic and total-cell RNAs were 1.3 and 1.6 kb in size. The 1.3-kb RNA was initially detected in total-cell RNA preparations at 12 h p.i. (Fig. 3A, lane 4) but was most abundant at 62 h p.i. Its appearance in the cytoplasm of infected cells followed similar kinetics (Fig. 3B). A 1.6-kb message complementary to C3 cDNA was also very abundant in infected cells. This transcript was not detectable before 24 h p.i. in either total-cell or cytoplasmic preparations (Fig. 3). The kinetics of appearance in infected cells suggested that the 1.3-kb RNA was an early gene product, whereas the 1.6-kb RNA was classified as a late message. Notably, the 1.6-kb RNA appeared simultaneously in total-cell and cytoplasmic RNA samples, which indicated that accumulation of this RNA in the cytoplasm coincided with its transcription. Therefore, we concluded there was no delay in release of this RNA from the nucleus and that transcription and entry into the cytoplasm were simultaneous.

Several other transcripts were apparently complementary to the C3 cDNA probe. These transcripts began to accumulate at different times, which suggested that they were of different kinetic classes. For example, a 3.7-kb RNA was present in cells blocked with CH (Fig. 3A, lane 2) and was present throughout infection, as is characteristic of an immediate-early gene product. In addition, two large transcripts 8 and 10 kb in length were also complementary to the cDNA probe. The 8-kb transcript first appeared 12 h p.i. and could be detected through the latest time point. The 10-kb RNA apparently was most abundant late in infection.

To further characterize transcription from the *Hin*dIII R fragment, we identified C3 complementary messages in nonpermissive cells and in permissive cells blocked with drugs to prevent protein or DNA synthesis. HEL cells were treated with CH, phosphonoacetic acid (PAA), or FUdR as described in Materials and Methods and infected with HCMV. NIH 3T3 and IML cells, nonpermissive for HCMV replication, were infected, and cytoplasmic RNA was harvested 4 and 62 h p.i. Cytoplasmic RNA was also harvested from untreated, infected HEL cells at 4 and 62 h p.i. for comparative purposes. Northern blots of these RNAs were probed with the nick-translated C3 cDNA insert.

As expected, the 1.3- and 1.6-kb messages were not present in permissive infections at 4 h p.i. but were abundant at 62 h p.i. (Fig. 4, lanes 2 and 3). The 1.3- and 1.6-kb RNAs could not be detected in infected HEL cells when protein

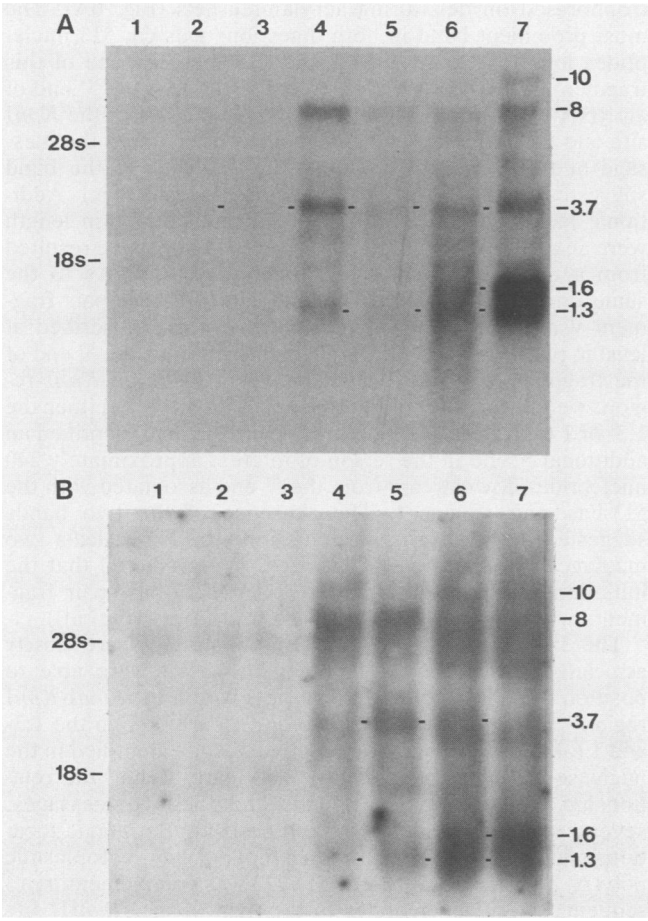

FIG. 3. Kinetics of accumulation of RNAs complementary to C3 cDNA in infected cells. Confluent monolayers of HEL cells were infected with AD169, and either total-cell (A) or cytoplasmic (B) RNA was isolated 2 (lane 3), 12 (lane 4), 24 (lane 5), 48 (lane 6), and 62 (lane 7) h p.i. RNA in lanes 1 was from uninfected cells. Lanes 2 contain RNA isolated from cells preincubated for 1 h with DMEM containing CH (150 μg/ml) and then infected with AD169 stock containing CH at the same concentration. Incubation was continued in DMEM containing CH for an additional 12 h before harvesting. A 10-μg amount of each RNA sample was fractionated on denaturing 1% agarose gels containing formaldehyde. Northern blots were subsequently probed with ^{32}P-labeled C3 cDNA. The sizes (in kilodaltons) of various transcripts are indicated on the right; positions of rRNAs are indicated on the left.

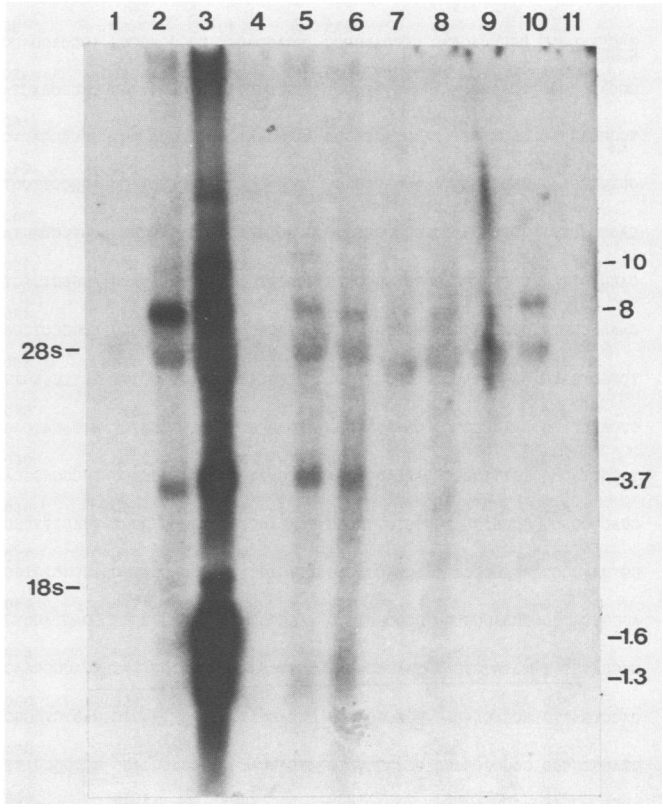

FIG. 4. Accumulation of transcripts complementary to C3 cDNA in permissive and nonpermissive infections. Confluent monolayers of HEL, NIH 3T3, or IML cells were infected with HCMV (AD169). Cytoplasmic RNA was isolated, and 10 μg of each sample was fractionated on denaturing formaldehyde-agarose gels. RNA was transferred to GeneScreen Plus membranes and probed with ^{32}P-labeled C3 cDNA. RNA samples were isolated from uninfected HEL cells (lane 1) and from virus-infected cells 4 (lane 2) and 62 (lane 3) h p.i. Immediate-early RNA (lane 4) was obtained from infected HEL cells incubated for 12 h in DMEM containing CH (150 μg/ml). DNA replication was inhibited in infected HEL cells by incubation in DMEM with PAA (200 μg/ml) (lane 5) or 10^{-6} FUdR (lane 6) for 48 h to obtain early RNA. Nonpermissive-cell RNAs were from uninfected NIH 3T3 cells (lane 9), from infected NIH 3T3 cells 4 (lane 7) and 62 (lane 8) h p.i., or from infected IML cells 4 (lane 10) and 62 (lane 11) h p.i. The sizes (in kilodaltons) of bands are marked on the right; positions of rRNAs are indicated on the left.

synthesis or DNA replication was prevented (Fig. 4, lanes 4 through 6). Neither of these RNAs appeared at any time after infection of nonpermissive NIH 3T3 or IML cells (Fig. 4, lanes 7 through 11). The absence of detectable levels of the 1.6-kb RNA in PAA- and FudR-treated HEL cells in conjunction with the finding that it appeared in permissively infected cells only after the onset of viral DNA replication further supports the conclusion that it is a gene product of the late kinetic class. Interestingly, the 1.3-kb message which appeared early in permissive infection was also eliminated when DNA replication was inhibited. In addition, the 3.7- and 8-kb messages were present early in infection and in cells treated with PAA and FUdR, whereas the 10-kb message was present only in permissive infections at 62 h p.i.

S1 analysis of the C3 cDNA complementary transcripts. Analysis of infected-cell RNA demonstrated the complexity of transcription originating from the *Hin*dIII R fragment. We expected to identify the vp25 message by Northern blotting; however, it became clear that more precise characterizations would be required. Northern blots probed with the adjacent *Hin*dIII S and T fragments (see Fig. 1) did not detect the 1.3- and 1.6-kb messages. Additional hybridizations with various fragments from the left end of the R fragment (Fig. 1) suggested that these two transcripts originated from within the R fragment and that neither extended beyond the *Sma*I site at the left end (data not shown). Therefore, to more precisely analyze the region, we sequenced both the *Hin*dIII-*Kpn*I subfragment of R (Fig. 5) and the C3 cDNA clone. Comparison of the two sequences showed that the 5′ end of the cDNA was located 105 nucleotides 5′ to the *Kpn*I restriction enzyme site. Northern blots had demonstrated that both the 1.3- and 1.6-kb transcripts were complementary to the C3 cDNA. Furthermore, it was known that transcription of the template RNA for the C3 cDNA was carried out from left to right on the HCMV (AD169) map.

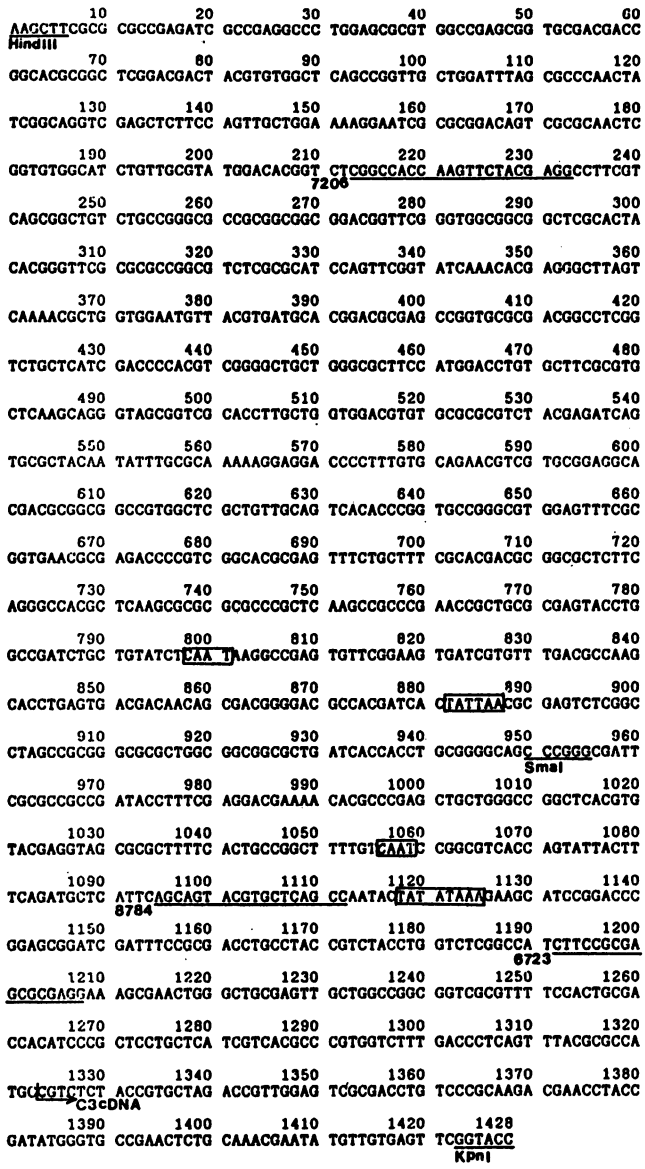

FIG. 5. Sequence of the *Hin*dIII-*Kpn*I subfragment of fragment R. The sequence of a portion of *Hin*dIII-R extending from the *Hin*dIII site separating the R and the adjacent S fragments rightward to the nearest *Kpn*I site is shown. Restriction enzyme sites and positions of oligonucleotides are underlined. The oligonucleotides used for hybridization probes are identified by number. ⟶, 5′ end of the C3 cDNA. Putative CAAT and TATA consensus sequences are shown in boxes.

Collectively, these data suggested that the 5′ ends of the 1.3- and 1.6-kb transcripts were located between the *Sma*I and *Kpn*I sites at the left end of the R fragment (Fig. 1). Therefore, we carried out a detailed analysis of the region between these two restriction enzyme sites.

Nuclease-mapping experiments were carried out by using an end-labeled probe made from the *Hin*dIII-*Kpn*I subfragment of fragment R (Fig. 6B). The restriction enzyme fragment was isolated and labeled at the *Kpn*I site, using T4 kinase as described in Materials and Methods. The probe was annealed to 62-h-p.i. cytoplasmic poly(A)$^+$ RNA and digested with either S1 nuclease or Exo VII. Several nuclease-resistant bands were detected when samples were electrophoresed on denaturing acrylamide gels (Fig. 6A). The most prominent band in both digestions was ca. 513 nucleotides long (Fig. 6A, lanes 1 and 3). The presence of this fragment in the Exo VII digests suggested that the 5′ end of an RNA was located 513 nucleotides to the left of the *Kpn*I site and that no intervening sequences interrupted the message at this point. In addition, the intensity of the band suggested the mRNA was very abundant at 62 h p.i. Additional bands ca. 1,428, 870, and 277 nucleotides in length were also seen. The full-length DNA band may have resulted from a nuclease-resistant hybrid of several RNAs to the same end-labeled DNA fragment. The 870-base-pair fragment was the result of a message that was transcribed at least in part from the *Hin*dIII R fragment. Since the 5′ end of the fragment was not located between the *Sma*I-*Kpn*I region, we felt that it could not be accounted for by either the 1.3- or 1.6-kb RNA. The 277-base-pair fragment identified an additional 5′ end in the region of interest approximately 240 nucleotides downstream from the 5′ end associated with the 513-base-pair fragment. The presence of the two bands suggested that transcription initiation sites for at least two messages were located in this area. We predicted that the initiation sites located by the 513- and 277-base-pair fragments were for the 1.3- and 1.6-kb transcripts (Fig. 6B).

The 5′ ends of the 1.3- and 1.6-kb messages are closely associated. From the S1 mapping data, we were able to position the 5′ ends of two transcripts within the *Sma*I-*Kpn*I region. However, we were unable to clearly assign the 1.3- and 1.6-kb transcripts to the putative 5′ ends identified in the nuclease-mapping experiments. To clearly define the relationship of the putative 5′ ends and the two messages, several oligonucleotides were synthesized at strategic locations and used to probe Northern blots of late, cytoplasmic poly(A)$^+$ RNA. Oligonucleotide 6723 was complementary to sequences 1,192 nucleotides to the right of the *Hin*dIII site (Fig. 5) and was expected to hybridize to both messages, giving rise to 277- and 513-nucleotide-long fragments. Oligonucleotide 8784 was complementary to sequences approximately 100 nucleotides closer to the *Hin*dIII site at 1095 and was expected to hybridize only to the message that gave rise to the 513-nucleotide-long fragment (Fig. 5). A third oligonucleotide, 7026, was complementary to sequences only 212 nucleotides to the right of the *Hin*dIII site and therefore was not expected to hybridize to either of the messages.

The oligonucleotides were end labeled with T4 kinase and [γ-^{32}P]ATP and hybridized to Northern blots of cytoplasmic poly(A)$^+$ RNA extracted from HEL cells at 62 h p.i. For comparison, a fourth lane was hybridized with C3. As in previous experiments, the C3 cDNA hybridized to both the 1.3- and 1.6-kb messages that were present at 62 h p.i. (Fig. 7, lane 1). Oligonucleotide 6723 also hybridized to both of these transcripts (Fig. 7, lane 2), whereas oligonucleotide 8784 hybridized only to the 1.6-kb message. The third oligonucleotide, 7026, did not hybridize to either the 1.3- or the 1.6-kb RNA. From this data, we have concluded that the RNA present at 62 h p.i. that yields the 277-nucleotide-long fragment is the 1.3-kb transcript. The 513-nucleotide-long fragment is generated because of the presence of the 1.6-kb message in late-RNA samples.

DISCUSSION

We have investigated the transcriptional activity of a region of the HCMV genome that contains the gene for a 25-kDa virion protein. Northern blots probed with a cDNA that mapped to the HCMV (AD169) *Hin*dIII R fragment

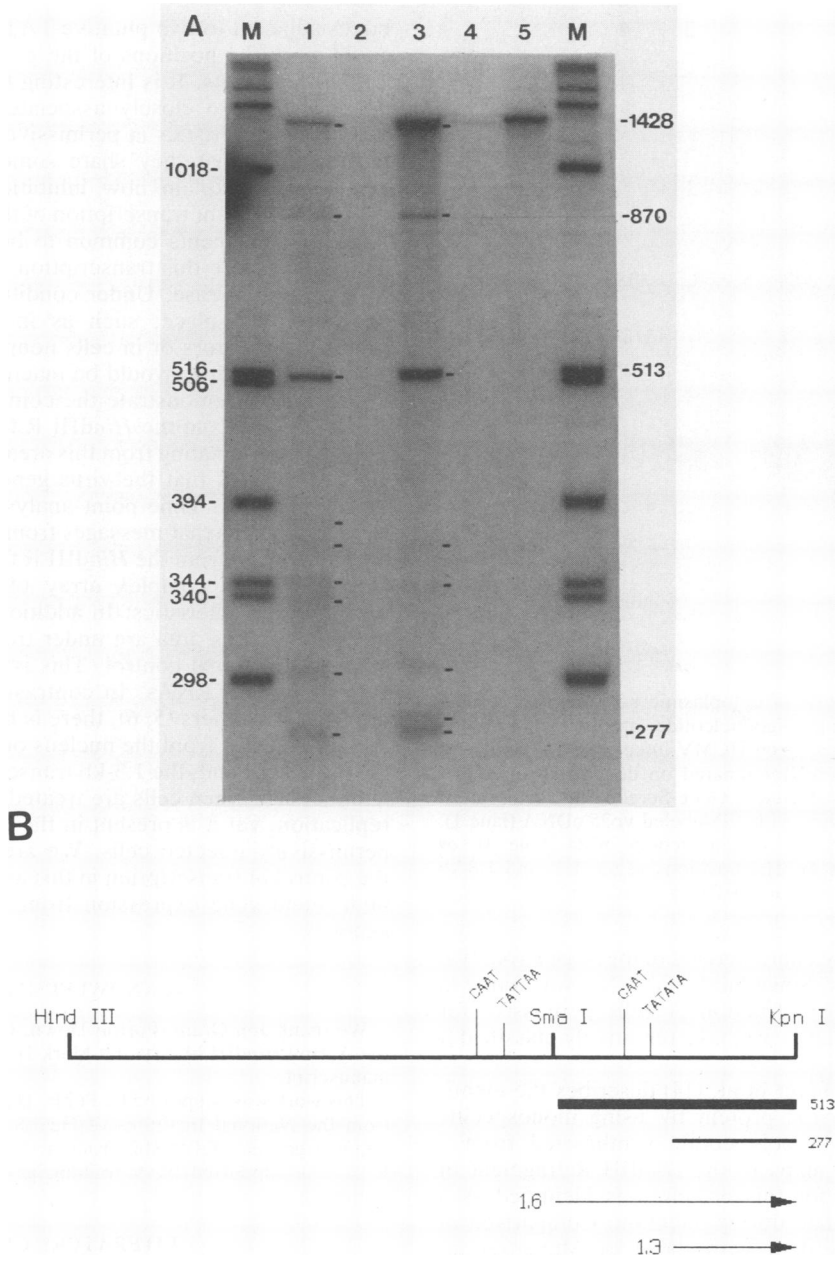

FIG. 6. S1 and Exo VII nuclease analysis of infected-cell RNA. (A) Samples of cytoplasmic poly(A)$^+$ 62-h-p.i. RNA annealed to end-labeled *Hin*dIII-*Kpn*I probes, digested with S1 nuclease or Exo VII as described in Materials and Methods, and analyzed by electrophoresis on denaturing 7 M urea–4% acrylamide gels. Lanes: M, size markers; 1 and 2, 62-h-p.i. RNA and tRNA, respectively, that were hybridized to *Hin*dIII-*Kpn*I probes and digested with S1 nuclease; 3 and 4, same as lanes 1 and 2 except that Exo VII was used instead of S1 nuclease; 5, undigested probe. The size of each marker band is indicated on the left, and significant bands in digestions are indicated on the right. All sizes are given in nucleotides. (B) Depiction of the *Hin*dIII-*Kpn*I probe fragment, with important restriction enzyme sites indicated. Regions protected by the 513- and 277-nucleotide fragments are indicated below the diagram. Heavy lines indicate fragments of greater abundance. The predicted arrangement of the 1.3- and 1.6-kb messages (bottom) and positions of potential TATA and CAAT consensus sequences are shown.

identified 1.3- and 1.6-kb transcripts that were abundant late in infection. Comparison of cytoplasmic and total-cell RNAs from permissive infections showed that there was no delay in accumulation of these messages in the cytoplasm once they appeared in the infected cell. Detection of vp25 was coincident with appearance of the 1.6-kb mRNA, and examination of different time points in permissive and nonpermissive infections suggested that vp25 synthesis was dependent on initiation of virus DNA replication. In addition, vp25 could be detected on Western blots with antisera made by inoculating rabbits with fpC3. We interpret these data to indicate that vp25 is translated from the 1.6-kb mRNA. The 1.3-kb RNA first appeared in infected cells at about 12 h p.i. Although this transcript appeared at early times, inhibition of DNA synthesis with FUdR or PAA also suppressed accumulation of this transcript. These attributes are contra-

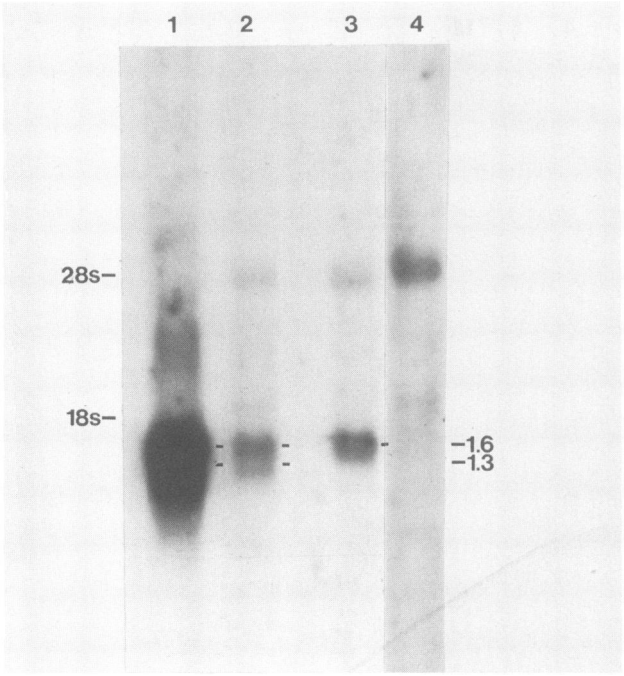

FIG. 7. Northern analysis of cytoplasmic polyadenylated RNA from infected cells, using oligonucleotide probes. Cytoplasmic poly(A)$^+$ RNA was isolated from HCMV-infected HEL cells 62 h p.i. Portions of 10 μg were fractionated on denaturing formaldehyde-agarose gels and transferred to Gene-Screen Plus membranes. Individual lanes were probed with ^{32}P-labeled vp25 cDNA (lane 1), oligonucleotide 6723 (lane 2), oligonucleotide 8784 (lane 3), or oligonucleotide 7206 (lane 4). The positions of the 1.3- and 1.6-kb RNAs (right) and rRNA positions (left) are indicated.

dictory because they are characteristic of messages from two different kinetic classes. Since the 1.3-kb RNA was found in the cytoplasmic poly(A)$^+$ fraction of cells permissive for virus replication at 12 h p.i., we have tentatively classified it as an early message.

A recent report by Meyer et al. (14) describes the identification of a 28-kDa virus protein by using monospecific antibodies made with a polypeptide synthesized from a cDNA clone that also maps to the *Hin*dIII R fragment of HCMV. The message for this protein was identified as a 1.3-kb message found late during infection. Comparison of our Northern analysis data with those of Meyer et al. strongly suggests that the 1.6-kb mRNA we assigned to vp25 is the same as the 1.3-kb message assigned to their 28-kDa protein. We are confident that the 1.3- and 1.6-kb messages we describe are transcribed in the same direction, since both are detected by the same single-stranded oligonucleotide probe (Fig. 7). Therefore, we feel that the 1.6-kb late message is not the same as the 1.5-kb RNA that Meyer et al. found originating from the opposite strand. Meyer et al. do not indicate the size of their smaller transcript, which we found accumulated in infected cells at early times. Therefore, we feel that the 1.3-kb mRNA and the 28-kDa phosphoprotein of Meyer et al. are identical to our 1.6-kb message and vp25. The discrepancy in sizes is undoubtedly due to differences in our calculations.

Sequencing and S1 analysis indicated that the initiation site for transcription of the 1.6-kb message was located approximately 240 nucleotides to the left of the initiation site of the 1.3-kb message. Analysis of sequences 5′ to the initiation sites identified by S1 analysis identified two CAAT boxes adjacent to two putative TATA boxes (Fig. 5). These could mark the positions of the promoters for the 1.3- and 1.6-kb transcripts. It is interesting that the 5′ ends of these messages are so closely associated, given their different kinetic characteristics in permissive infections. This finding may indicate that they share some regulatory sequences, which could explain how inhibition of DNA replication might also prevent transcription of the 1.3-kb early message. Regulatory elements common to both genes could require activation before this transcription unit becomes accessible to RNA polymerase. Under conditions in which activation does not take place, such as in the presence of DNA synthesis inhibitors or in cells nonpermissive for virus replication, both genes would be inactive.

These data demonstrate the complexity of the transcriptional activity from the *Hin*dIII R fragment. The density of transcripts originating from this area seems unusually high in view of the fact that the virus genome has over 240 kb of coding capacity. Time point analysis of infected-cell RNA clearly indicates that messages from different kinetic classes are transcribed from the *Hin*dIII R fragment, which suggests that there is a complex array of genes which differ in temporal characteristics. In addition, our data suggest that the genes in this area are under transcriptional rather than posttranscriptional control. This is most apparent with the 1.3- and 1.6-kb RNAs. In contrast to late virus messages described by others (5, 6), there is no delay in the release of the 1.6-kb RNA from the nucleus once transcription begins. On the other hand, the 1.3-kb transcript behaves much like a late message when cells are treated with inhibitors of DNA replication, yet it is present in the cytoplasm at 12 h p.i. in permissively infected cells. We are currently investigating the control of transcription in this area in order to more fully understand gene expression from this region of the virus genome.

ACKNOWLEDGMENTS

We thank Jon Oram (Porton Down, Great Britain) for providing the AD169 *Hin*dIII library and Mark Hall for critical reading of the manuscript.

This work was supported by Public Health Service grant CA28089 from the National Institutes of Health. J.M. was supported by a minority graduate fellowship from the National Science Foundation. R.L. was supported by a training grant from the Reno Cancer Center.

LITERATURE CITED

1. **Berent, S. L., M. Mahmoudi, R. M. Torczynski, P. W. Bragg, and A. P. Bollon.** 1985. Comparison of oligonucleotide and long DNA fragments as probes in DNA and RNA dot, Southern, Northern, colony and plaque hybridizations. Biotechniques **May/June:**208–220.
2. **Boshart, M., F. Weber, G. Jahn, K. Dorsch-Hsler, B. Fleckenstein, and W. Schaffner.** 1985. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell **41:**521–530.
3. **Chua, C. C., T. H. Carter, and S. C. St. Jeor.** 1981. Transcription of the human cytomegalovirus genome in productively infected cells. J. Gen. Virol. **56:**1–11.
4. **DeMarchi, J. M., C. A. Schmidt, and A. S. Kaplan.** 1980. Patterns of transcription of human cytomegalovirus in permissively infected cells. J. Virol. **35:**277–297.
5. **Geballe, A. P., F. S. Leach, and E. S. Morcarski.** 1986. Regulation of cytomegalovirus late gene expression: gamma genes are controlled by posttranscriptional events. J. Virol. **57:**864–874.
6. **Goins, W. F., and M. F. Stinski.** 1986. Expression of a human cytomegalovirus late gene is posttranscriptionally regulated by a 3′-end-processing event occurring exclusively late after infec-

tion. Mol. Cell. Biol. **6**:4202–4213.
7. **Hames, B. D.** 1981. An introduction to polyacrylamide gel electrophoresis, p. 1–86. *In* B. D. Hames and D. Rickwood (ed.), Gel electrophoresis of proteins: a practical approach. IRL Press, Washington, D.C.
8. **Huang, E. S., S. T. Chen, and J. S. Pagano.** 1973. Human cytomegalovirus. I. Purification and characterization of viral DNA. J. Virol. **12**:1473–1481.
9. **Lehrach, H., D. Diamond, J. M. Wozney, and H. Boedtker.** 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry **16**:4742–4751.
10. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Marks, J. R., J. A. Mercer, and D. H. Spector.** 1983. Transcription in mouse embryo cells permissively infected by murine cytomegalovirus. Virology **131**:247–254.
12. **Martinez, J., and S. C. St. Jeor.** 1986. Molecular cloning and analysis of three cDNA clones homologous to human cytomegalovirus RNAs present during late infection. J. Virol. **60**:531–538.
13. **McDonough, S. H., and D. H. Spector.** 1983. Transcription in human fibroblasts permissively infected by human cytomegalovirus strain AD169. Virology **125**:31–46.
14. **Meyer, H., A. T. Bankier, M. P. Landini, C. M. Brown, B. G. Barrell, B. Ruger, and M. Mach.** 1988. Identification and procaryotic expression of the gene coding for the highly immunogenic 28-kilodalton structural phosphoprotein (pp28) of human cytomegalovirus. J. Virol. **62**:2243–2250.
15. **Nowak, B., A. Gmeiner, P. Sarnow, A. J. Levine, and B. Fleckenstein.** 1984. Physical mapping of human cytomegalovirus genes: identification of DNA sequences coding for a virion phosphoprotein of 71 kDa and a viral 65 kDa polypeptide. Virology **134**:91–102.
16. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
17. **Sharp, P. A., A. J. Berk, and S. M. Berget.** 1980. Transcription maps of adenovirus. Methods Enzymol. **65**:750–768.
18. **Stenberg, R. M., and M. F. Stinski.** 1985. Autoregulation of the human cytomegalovirus major immediate-early gene. J. Virol. **56**:676–682.
19. **Stinski, M. F.** 1977. Synthesis of proteins and glycoproteins in cells infected with human cytomegalovirus. J. Virol. **23**:751–767.
20. **Stinski, M. F.** 1978. Sequence of protein synthesis in cells infected by human cytomegalovirus: early and late virus-induced polypeptides. J. Virol. **26**:686–701.
21. **Stinski, M. F.** 1983. Molecular biology of cytomegaloviruses, p. 67–113. *In* B. Roizman (ed.), The herpesviruses, vol. 2. Plenum Publishing Corp., New York.
22. **St. Jeor, S., and F. Rapp.** 1973. Cytomegalovirus replication in cells pretreated with 5-iodo-2′-deoxyuridine. J. Virol. **11**:986–990.
23. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
24. **Wathen, M. W., and M. F. Stinski.** 1982. Temporal patterns of human cytomegalovirus transcription: mapping the viral RNAs synthesized at immediate early, early, and late times after infection. J. Virol. **41**:462–477.
25. **Wathen, M. W., D. R. Thomsen, and M. F. Stinski.** 1981. Temporal regulation of human cytomegalovirus transcription at immediate early and early times after infection. J. Virol. **38**:446–459.