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Molecular Cloning and Analysis of Three cDNA Clones Homologous to Human Cytomegalovirus RNAs Present During Late Infection

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Three virus-specific clones were isolated from a cDNA library synthesized from human cytomegalovirus (AD169)-infected cell RNA and cloned into the expression vector lambda gt11. These clones, designated C3, D10, and H10 were each found to express a human cytomegalovirus/beta-galactosidase fusion protein that was reactive with antibody prepared against purified virions. By using the cloned cDNA, we were able to identify the transcripts that code for each gene product and study the kinetics of expression during permissive infection. Our results suggest that at least two of the RNAs undergo posttranscriptional processing and appear in infected cells at immediate-early times. The authentic C3 gene product was identified by probing Western blots with antibody prepared against fusion protein fpC3.

Human cytomegalovirus (HCMV), a ubiquitous member of the herpesvirus family, is an opportunistic pathogen that is the leading cause of death in immunocompromised individuals such as acquired immunodeficiency syndrome and bone marrow transplant patients (20, 27). Concern for the association of HCMV with mental retardation caused by prenatal infection has led to the trial development of a vaccine with attenuated virus (30). However, the implication that HCMV may cause oncogenic transformation (1) makes the use of an attenuated virus vaccine questionable. The development of a subunit vaccine is a logical alternative. Encouraging results have been obtained with herpes simplex types 1 and 2 by using synthetic polypeptides that mimic antigenic determinants that play an important role in neutralization (7). Detailed knowledge of HCMV late antigens is incomplete, necessitating further study if a similar approach is to be used in analyzing virus structural proteins.

The HCMV virion consists of an icosahedral capsid surrounded by a lipid envelope that contains approximately 35 proteins with molecular sizes ranging from 15 to 230 kilodaltons (kDa) (13, 17). Of these virion-associated proteins, at least five have been identified as glycoproteins (12, 35). Studies with infected cell proteins indicate that these may be subdivided into four antigenically distinct classes based on their reactivity with human immune sera (29). Additionally, proteins that are likely to be important in virus neutralization have been identified with monoclonal and polyclonal sera known to have HCMV neutralizing antibodies (4, 26, 31, 32). Identification of the genes coding for these proteins has been more difficult; however, several have been identified. The coding sequence for the major 64-kDa structural protein has been localized to an *Eco*RI fragment (map coordinates 0.55 to 0.57) in the long unique region of the HCMV Towne genome. By using information from the partial amino acid sequence, oligonucleotide probes were synthesized and used to identify the corresponding gene (28). The mapping of a 67-kDa viral protein has also been accomplished. It has been found to originate in the long unique region between map coordinates 0.37 and 0.39 (8, 9). The map locations of several virus proteins were also identified by Nowak et. al. (25) by hybrid selection of mRNA from infected cells and subsequent in vitro translation.

The approach we used to identify virion protein coding sequences involves the cloning of a cDNA library into lambda gt11. This expression vector allows selection of recombinants either by conventional DNA hybridization methods or with antibody probes. The library screened in these studies was synthesized from poly(A)$^+$ RNA isolated from HCMV-infected cells at 62 h postinfection (p.i.). Therefore, the RNA template was enriched for messages transcribed late in the infectious cycle, which resulted in a library enriched with late cDNA clones. Recombinants were selected by induction of the HCMV/beta-galactosidase fusion protein and screened with antibody prepared against virions. Antiserum was prepared against one of the fusion proteins and subsequently was used to identify the authentic virus polypeptide. Consequently, we were able to isolate virus-specific coding sequences and correlate them with particular regions on the HCMV genome.

MATERIALS AND METHODS

Cells and virus. Human embryonic lung (HEL) cells were grown in monolayers and maintained on Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. HCMV (AD169), used exclusively in these studies, was propagated as previously described (5).

Enzymes. All restriction enzymes and RNase H, T4 ligase, and T4 kinase were purchased from Bethesda Research Laboratories, Inc., and used according to the instructions of the manufacturer. *Escherichia coli* ligase, *Eco*RI methylase, and Klenow polymerase were purchased from New England BioLabs, Inc. DNA polymerase I (endonuclease free) was purchased from Boehringer Mannheim Biochemicals, and reverse transcriptase was purchased from Life Sciences, Inc.

Antiserum and antibody preparation. Antiserum to HCMV virions was prepared by immunizing a goat with extracellular virions purified by differential and velocity sedimentation by using a modification of the procedure described by Huang et. al. (19). Subscapular and intramuscular injections of purified

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virus mixed with adjuvant were given at 1-week intervals. Blood was drawn 10 days after the final inoculation and assayed for anti-HCMV antibody with dot blots and plaque reduction assay. Human convalescent serum obtained from a patient who had experienced a prolonged HCMV infection was tested by both complement fixation and immunofluorescence and found to have antibody directed against virus-specific proteins.

Antiserum to HCMV/beta-galactosidase fusion proteins was made by immunizing rabbits with proteins purified from *E. coli* lysates. *E. coli* Y1089 lysogenized with a recombinant bacteriophage (40) was grown at 30°C to an optical density at 600 nm of 0.3. The cultures were induced for protein production by heating to 42°C for 15 min and by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. Incubation was continued for an additional hour at 37°C with vigorous shaking before the bacteria were collected by centrifugation. The bacterial pellets were washed twice and suspended in phosphate-buffered saline (PBS; 150 mM NaCl, 12 mM Na_2HPO_4, 1 mM KH_2PO_4 [pH 7.6]). The suspension was then frozen at −20°C and thawed, and bacterial cells were disrupted by sonication with a sonifier (Branson Sonic Power Co., Danbury, Conn.). Cellular debris was removed by centrifugation at 54,000 × *g* for 1 h at 5°C. Proteins with a beta-galactosidase moiety were isolated by passing the supernatant over Sepharose 4B coupled with anti-beta-galactosidase antibody (Cooper Biomedical, Inc., West Chester, Pa.). Fusion proteins were eluted with 3 M potassium thiocyanate (pH 7.4) and dialyzed against three changes of PBS (pH 7.6). The dilute fusion proteins were lyophilized and suspended in 1 ml of PBS and size fractionated on a Sephacryl S200 column. Fusion proteins were eluted in the void volume as a single peak. For initial injection, column fractions containing purified fusion proteins were mixed with an equal volume of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). Preparations for subsequent injections were mixed with an equal volume of Freund incomplete adjuvant (Difco). Rabbits previously tested and found to have low anti-*E. coli* antibody titers were immunized essentially as described by Eberle and Courtney (11). At 10 days after the final injection, immune rabbit serum was tested for the presence of anti-HCMV antibody by probing filters spotted with uninfected cell proteins, cells infected for 72 h with strain AD169, and *E. coli* proteins. The protocol used was the same as that described for protein blotting.

RNA preparations. RNA used for hybridizations and cloning was prepared by infecting confluent monolayers of HEL cells with HCMV (AD169) at a multiplicity of infection of 10 PFU per cell. At 62 h p.i., the cells were removed by trypsinizing and washed two times with ice-cold PBS supplemented with 10% glucose. Total cell RNA was isolated by using the guanidinium-cesium chloride method described by Maniatis et al. (22). Chromatography on oligo(dT) cellulose (Collaborative Research, Inc., Waltham, Mass.) was used to isolate poly(A)$^+$ RNA.

cDNA cloning. First-strand DNA synthesis from poly(A)$^+$ RNA was carried out as described in detail by Maniatis et al. (22). After incubation at 42°C for 2.5 to 3 h the first-strand reaction was terminated and applied to a Sephadex G-50 spin column to separate the cDNA from unincorporated nucleotides. After precipitation the cDNA pellet was suspended in 40 μl of H_2O and second-strand synthesis was done as described by Gubler and Hoffman (15) by using RNase H, DNA polymerase I, and *E. coli* ligase to replace the RNA strand with deoxynucleotides. After methylation and *Eco*RI linker addition the cDNA was cloned into *Eco*RI-digested lambda gt11. The ligated DNA was packaged by using a commercial packaging reaction purchased from Promega Biotec. The library was initially screened with nick-translated HCMV DNA to isolate recombinants containing HCMV inserts as described by Benton and Davis (2). Positive plaques were then screened with goat antibody as described by Young and Davis (40, 41) to identify recombinants synthesizing fusion proteins capable of being detected with goat anti-HCMV antiserum.

DNA and RNA hybridizations. DNA was either fractionated on agarose gels and transferred to nitrocellulose by the method of Southern (33) or spotted onto Gene Screen Plus (New England Nuclear Corp., Boston, Mass.) after first being denatured with 1 M NaOH and then neutralized with 1 M HCl. Filters were prehybridized in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1× Denhardt solution–0.1% sodium dodecyl sulfate (SDS)–25 mM EDTA–20 mM $NaPO_4$ (pH 7.0)–50 μg of salmon sperm DNA per ml at 42°C. HCMV DNA, labeled with [α-^{32}P]dCTP by nick translation, was hybridized to the filters in the same solution.

RNA was fractionated on denaturing agarose gels containing formaldehyde (21), transferred to Gene Screen Plus, and hybridized with labeled DNA probes as recommended by the manufacturer. RNA dot blots were conducted by suspending equal amounts of RNA test samples in 1× SSC and by applying these directly to the filter without prior treatment. Filters were air dried and subsequently hybridized to probes as recommended by the manufacturer.

Protein blotting. Protein samples were size fractionated on either 7.5% single concentration or 5 to 20% gradient SDS-polyacrylamide gel electrophoresis (PAGE) gels formulated according to Hames (18). Proteins were transferred to nitrocellulose filters (38), and nonspecific binding activity of the filters was blocked with 10 mM Tris (pH 7.4)–150 mM NaCl–3% bovine serum albumin. Primary antisera were diluted 1/200 in 1× NETG (1× NETG is 150 mM NaCl, 1 mM EDTA, 10 mM Tris [pH 7.4], 10% gelatin, 0.05% Nonidet P-40) and agitated with filters for 1 h at room temperature. Filters were washed five times for 10 min with 200 ml of NETG–0.01% SDS–0.05% Triton X-100 and then briefly rinsed in distilled water. The primary antibody was detected with affinity purified anti-goat, -human or -rabbit antibody (Cappel) labeled with ^{125}I by the chloramine-T method (23). The secondary antibody (10^9 cpm/mg), 10^6 cpm/ml in 1× NETG, was incubated with the filters for 1 h at room temperature with constant agitation and then washed as described above. Autoradiography (X-Omat AR film; Eastman Kodak Co., Rochester, N.Y.) was done at −70°C overnight with intensifying screens.

RESULTS

Antibody characterization. This study depended on the ability of our antibody probes to react with virus-specific protein domains expressed from HCMV-specific recombinants. Therefore, initial experiments were directed toward determining which virion proteins were identified by goat anti-HCMV and human convalescent sera. In addition, it was essential to determine if antibody against uninfected cell proteins was present in the test serum since the specificity of the antisera would directly affect whether virus or host recombinants were isolated from the library. Proteins detectable by the antibodies were identified by probing Western blots with goat anti-virion serum and human convalescent

serum preadsorbed with *E. coli* and uninfected HEL cell proteins. Additionally, the goat anti-HCMV serum was characterized by plaque reduction assay and found to have significant complement-independent virus neutralizing activity (data not shown).

Blots probed with goat anti-virion serum consistently detected at least 9 of the 32 proteins reported to constitute the HCMV virion (Fig. 1). These ranged in size from ca. 19 to 118 kDa. Additional proteins (not visible in Fig. 1), several of which are more prominent when human antibody is used, were also faintly detected. In addition, dot blots probed with preadsorbed goat anti-HCMV serum indicated that antibodies to *E. coli* and uninfected cell proteins were not present (data not shown). Duplicate blots probed with human convalescent serum showed a banding pattern similar to that demonstrated by the goat serum, i.e., five of the HCMV proteins were readily detected by both sera. The total number of proteins detected by both antisera was 13 or approximately one-third of the total virion proteins (13, 17).

Cloning and screening. Poly(A)$^+$ RNA isolated from HCMV (AD169)-infected HEL cells 62 h p.i. was used to synthesize a cDNA library. At this time virus DNA replication is occurring at an accelerated rate, and structural proteins are synthesized in abundance (34, 36). Consequently, this starting RNA should contain an abundance of messages coding for HCMV proteins found in the virion.

Lambda gt11 recombinants containing HCMV inserts were initially isolated by plaque hybridization with ^{32}P-labeled HCMV DNA. There were 300 positive clones identified, which represented 4% of the total recombinants. This value agreed with previously published values for the fraction of total cell RNA represented by virus-specific RNA (5). Recombinants containing HCMV inserts were transferred to microtiter trays and subsequently grown on bacterial lawns

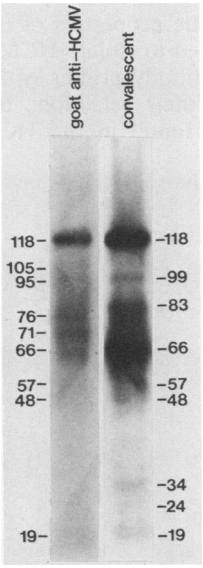

FIG. 1. Characterization of antibody probes by Western blot analysis of HCMV proteins from purified virions. HCMV (AD169) virions were isolated from infected cell supernatants by differential centrifugation and sedimentation through 10 to 60% sucrose gradients. Virions representing a total of 25 μg of protein were solubilized in SDS sample buffer and loaded onto a 5 to 20% gradient SDS-PAGE gel. After electrophoresis, proteins were transferred to nitrocellulose and probed with either human anti-HCMV antiserum (convalescent) or goat anti-HCMV antiserum. The molecular sizes of proteins detected by each antiserum are indicated in kilodaltons.

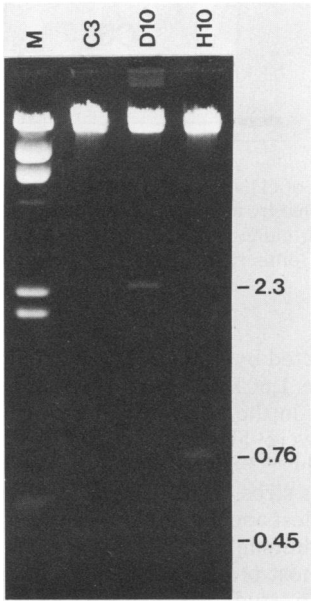

FIG. 2. Analysis of cDNA recombinants by agarose gel electrophoresis. Plaque-purified lambda gt11 clones identified by their ability to produce an HCMV/beta-galactosidase fusion protein detected by goat anti-virion antibody were grown in large amounts and DNA was isolated from the phage. Five micrograms of each DNA sample was digested with *Eco*RI and electrophoresed in a 1% agarose gel with lambda *Hin*dIII markers (lane M). Bands were visualized by ethidium bromide staining. Sizes of the cDNA inserts are given in kilobase pairs.

of *E. coli* Y1090. Production of fusion proteins was induced by placing isopropyl-β-D-thiogalactopyranoside-saturated filters on the plates and incubating for 1 h at 37°C. The filters were removed from the plates and probed with the anti-virion serum prepared in goats. Three clones designated C3, D10, and H10 were found to express fusion proteins with epitopes detectable by the goat anti-HCMV serum. The recombinants were subjected to three rounds of plaque purification before extraction of DNA from phage grown in large quantities. DNA from each recombinant was digested with *Eco*RI and fractionated on a 1% agarose gel. Clones C3, D10, and H10 each contained a single insert with apparent sizes of 0.45, 2.3, and 0.76 kilobases (kb), respectively (Fig. 2).

Mapping cDNA clones. After isolation the clones were mapped to the HCMV (AD169) genome. Initial localization of the cDNA clones was accomplished by hybridizing nick-translated inserts from clones C3, D10, and H10 to dot blots containing cloned AD169 *Hin*dIII fragments (provided by Jon Oram, Portdown, United Kingdom). Each cDNA clone hybridized to a single *Hin*dIII fragment. The results diagramed in Fig. 3 indicate that all three recombinants originated from the long unique region of the prototype arrangement described by Greenaway et al. (14) and were widely distributed on the virus genome.

Size of original transcript and kinetics of appearance. Identification of the original transcript that gave rise to each clone was desired to determine the size of the HCMV RNAs homologous to a particular cDNA clone. This information was used to predict the molecular size of the protein product. Each clone was hybridized to Northern blots prepared with total cell RNA isolated 62 h p.i. As can be seen in the autoradiogram (Fig. 4), at least one RNA of major abun-

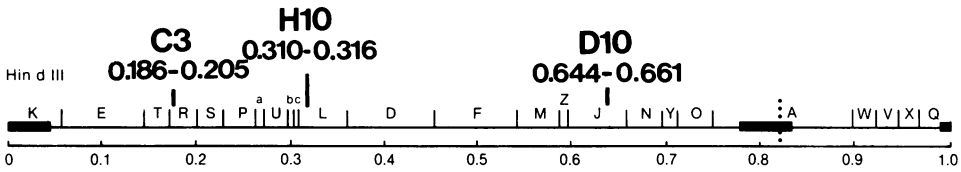

FIG. 3. Origin of cDNA coding sequences on the virus genome. Inserts from cDNA clones were purified from low-melting-temperature agarose gels, labeled by nick translation, and hybridized to dot blots of cloned *Hin*dIII fragments of HCMV (AD169) DNA. The location of the origin of each clone is identified on a schematic representation of a *Hin*dIII map of the AD169 genome. Detailed localization was accomplished by Southern blot hybridization of appropriate fragments digested with several restriction enzymes. Locations are given in map unit coordinates.

dance was detected by each recombinant. Two small RNAs calculated to be 1,600 and 1,300 nucleotides in length are most prominent in the C3 lane. Additionally, 10-, 8.0-, and 3.7-kb RNAs were also seen and have been repeatedly detected with the C3 probe. Clone D10 also hybridized to RNAs of various sizes. An RNA with a calculated size of 2.2 kb was the smallest and most abundant RNA detected by the D10 clone. Additional RNAs at 2.4 and 5.4 kb were also detected. The most prominent RNA detected by clone D10 was 5.4 kb. To study the appearance of clone-specific transcripts in infected cells, total RNA was isolated at different times p.i. (as described in Materials and Methods) and spotted onto Gene Screen Plus (New England Nuclear Corp.). These dot blots were probed with gel-purified cDNA inserts labeled by nick translation, and densitometer scans were made of the resulting autoradiograms (Fig. 5). Transcripts homologous to all three clones were detectable at low levels in cyclohexamide-blocked cells and as early as 2 h p.i. in normal infected cells. In general, it can be seen from Fig. 5 that transcripts detected by the probes tended to increase in abundance with time as infection continued; however, transcripts homologous to clone H10 demonstrated a modified pattern of appearance in the infected cell. A major increase in abundance was observed at 12 h p.i. However, this was followed by a decrease in abundance of approximately 75% at 24 h p.i. Several experiments were conducted to obviate trivial explanations of these results such as might be observed because of pipetting errors. In all experiments similar results were attained.

Additionally, it was found that the D10 transcripts declined in abundance between the 48- to 62-h points. The levels of D10 transcripts reached an apparent maximum in the cell at 48 h p.i. under the conditions used in these experiments. RNA samples from time points later than 62 h p.i. were not tested.

Protein characterizations. Preliminary characterizations of the protein products synthesized by the three clones consisted of isolating fusion proteins and determining their affinity to goat and human antisera. Clones lysogenized in appropriate bacterial hosts were induced to synthesize fusion proteins as described in Materials and Methods. Affinity-purified fusion proteins were fractionated on a 7.5% SDS-PAGE gel and electroblotted onto nitrocellulose filters. Duplicate blots were probed with anti-beta-galactosidase, goat anti-HCMV virion antiserum, or human convalescent serum (Fig. 6). All affinity-purified fusion proteins display some heterogeneity, with several bands appearing in the blots probed with anti-beta-galactosidase antibody. A number of bands are also visible in Western blots probed with goat anti-HCMV antibody. The largest fusion proteins fpC3, fpD10, and fpH10 were calculated to have molecular sizes of 129, 145, and 159 kDA, respectively. These values correspond to the projected size for each recombinant fusion protein if one assumes that beta-galactosidase is 116 kDa and 1,000 base pairs of DNA translate into 37,000 daltons of protein (10). When human convalescent serum was used to probe Western blots containing the three fusion proteins, only fpC3 was readily detected, whereas fpD10 and fpH10 were only faintly visible.

Because human convalescent serum reacted most readily with the fpC3 fusion protein, we felt that the authentic virion protein might be of clinical significance in the pathogenesis of HCMV infections. To examine the virion protein in greater detail, antibody against purified fpC3 was prepared in rabbits. This antiserum was used to probe Western blots of purified virion proteins size fractionated on 5 to 20% gradient SDS-PAGE gels. The results of these experiments are shown (Fig. 7). When the anti-fpC3 serum was used to probe Western blots of purified HCMV virion proteins, only one band representing a protein with a calculated molecular size of 19 kDa was detected. A band with a similar molecular weight was also observed in lanes probed with goat anti-virion serum and human convalescent serum.

DISCUSSION

In these studies, the properties of the expression vector lambda gt11 were used to isolate HCMV genes present late during infection. Antibody probes with known characteristics were used for their selection. Because of the large number of proteins found in the HCMV virion, detailed

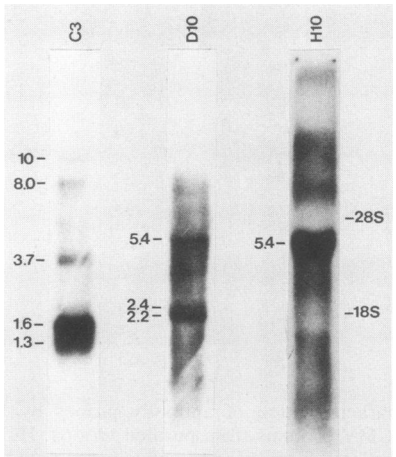

FIG. 4. Identification of transcripts homologous to C3, D10, and H10 in infected cell RNA. Total cell RNA was isolated from infected cells and fractionated on 1% agarose gels containing formaldehyde. Gels were blotted onto nitrocellulose and probed with gel-purified, nick-translated inserts for each clone. 28S and 18S, rRNA markers. Bands are marked with the calculated sizes of the RNAs listed in kilobase pairs.

studies of individual polypeptides have proven difficult except when the protein is purified in amounts large enough and of sufficient purity to enable amino acid sequencing (6). The lambda gt11 system offers several advantages in studying the HCMV proteins. By using the appropriate antisera, coding sequences for any protein may be isolated, allowing subsequent mapping and sequencing. In addition, the fusion protein expressed by the recombinants may be used to generate a polyclonal antibody specific for a single protein.

Mocarski et al. (24), with a similar approach, have identified the coding sequences for a member of a family of proteins by using a library made from random genomic fragments. The strategy taken in this communication uses lambda gt11 and is centered on the synthesis of a cDNA library. By using RNA isolated from cells late during infection we were able to enrich the library for clones that contained sequences of greatest interest. Isolated HCMV cDNA clones were used as probes to study the appearance of homologous RNAs in infected cells and to locate the corresponding coding sequences on the virus genome. In addition antibody was prepared against the HCMV/β-galactosidase fusion protein synthesized by clone C3 and used to identify the authentic virion protein. Thus, we identified a gene coding for a virus-specific protein and were able to correlate it with a polypeptide found in the virion.

The cDNA clones characterized in these studies were isolated with an antibody probe that was prepared by immunization with purified HCMV virions. This antivirion serum detected at least 9 of the 32 reported virion proteins with molecular sizes similar to those previously reported (17) and did not have reactivity with proteins from uninfected HEL cells when preadsorbed with uninfected cell proteins. Therefore, it was concluded that clones C3, D10, and H10 were recombinants containing cDNA copies of RNA messages translated at late times p.i. Each clone was found to originate from a different area in the long unique region of the AD169 genome. Detailed mapping localized the clones to sites within the appropriate *Hin*dIII fragments (J. Martinez, R. Lahijani, and S. St. Jeor, manuscript in preparation). Also clones D10 and H10 originate from areas on the genomic map that have previously been shown to give rise to HCMV late antigens (24, 25).

When the fusion proteins synthesized by the recombinants were characterized on Western blots with human convalescent serum it was evident that only fpC3 was readily detectable (Fig. 6). This may signify immunodominance of the authentic C3 virion protein or simply inability of the human convalescent serum to react with the denatured fusion proteins fpD10 and fpH10. To identify the authentic virus gene product represented by clone C3, antibody was prepared against purified fpC3 and used to probe Western blots of virion proteins. A single polypeptide with a calculated molecular size of 19 kDa was detected. This value is considerably lower than would be expected for a protein translated from even the shortest 1,300-nucleotide-long C3 transcript. There is enough coding capacity in this transcript to synthesize a polypeptide of approximately 52 kDa if one assumes an average molecular size of 120 daltons per amino acid (10).

At least two mechanisms could explain the smaller than expected authentic C3 protein. First, if the mature C3 message was only partially translated as with the small t antigen of simian virus 40 (3) then a much smaller-sized protein would result from the RNA. In this case antibody could not be generated against amino acids that might be coded by the 3′ end of the message since this region would

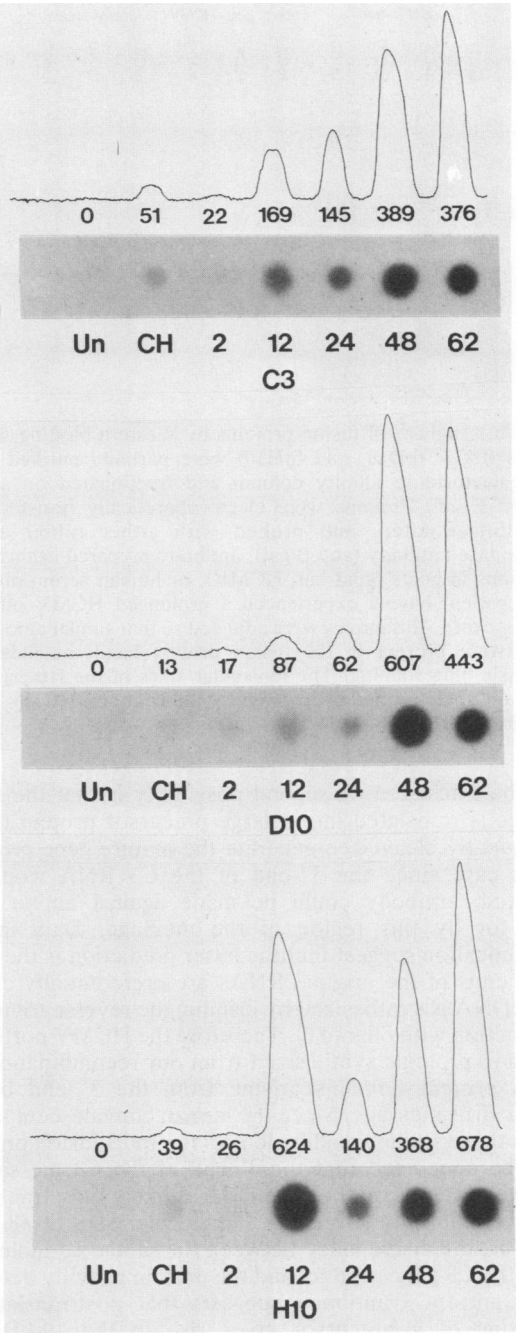

FIG. 5. Appearance of transcripts homologous to cDNA clones during HCMV infection. Confluent monolayers of HEL cells were infected at a multiplicity of infection of 10 PFU per cell with strain AD169. Total cell RNA was isolated from uninfected cells (Un), infected cells blocked with cyclohexamide (CH), and infected cells at 2, 12, 24, 48, and 62 h p.i. Immediate-early RNA was obtained by infecting cells under the influence of 150 μg of cyclohexamide per ml. Cells were incubated for an additional 12 h with cyclohexamide before harvesting total cell RNA. Then 10-μg quantities of RNA from each time point were blotted onto Gene Screen Plus and probed with gel-purified inserts labeled by nick translation. The counts per minute hybridized to each dot was determined by scintillation counting and densitometry of the finished autoradiograms. Information is arranged from top to bottom in each panel as follows: densitometer tracing of dot blot autoradiogram, ^{32}P counts per minute hybridized per spot, autoradiogram of dot blot, time point in hours p.i. represented by the RNA sample and clone hybridized to dot blot.

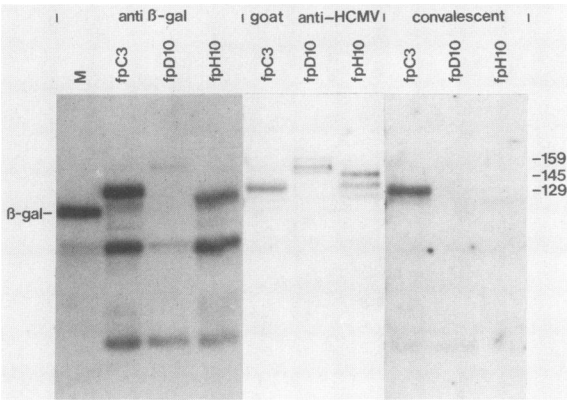

FIG. 6. Analysis of fusion proteins by Western blotting. Fusion proteins (fpC3, fpD10, and fpH10) were partially purified on an anti-β-galactosidase affinity column and fractionated on a 7.5% SDS-PAGE gel. Proteins were electrophoretically transferred to nitrocellulose filters and probed with either rabbit anti-β-galactosidase antibody (anti β-gal), antibody prepared against purified virions in goats (goat anti-HCMV), or human serum obtained from a patient having experienced a prolonged HCMV infection (convalescent). All samples were adjusted so that similar amounts of protein were present in the fusion protein bands as judged by Coomassie blue staining. The molecular sizes of the largest fpC3, fpD10, and fpH10 proteins detected by the goat anti-HCMV serum are indicated in kilodaltons.

never be translated. A second possibility is that the entire message is translated into a large precursor protein that is subsequently cleaved to generate the mature gene product. In this case since the 3′ end of the C3 RNA would be translated, antibody could be made against amino acids coded for by this region of the message. Data in this communication suggest that the latter prediction is the case. The 3′ ends of the original RNAs are preferentially cloned when cDNA is synthesized by priming the reverse transcriptase reaction with oligo(dT). Therefore the HCMV portion of the fusion proteins synthesized from our recombinants represents protein domains arising from the 3′ end of the original virus message. Since the human convalescent serum used in these studies readily detects the fpC3 fusion protein, it can be concluded that the 3′ end of the C3 message is translated. Therefore the C3 mRNA is likely fully translated into a protein with a predicted molecular size of 48 kDa. However, the discrepancy between the predicted molecular size of the C3 gene product and the protein actually detected by the anti-fpC3 antibody suggests that posttranslational processing of a precursor gives rise to the 19-kDa C3 polypeptide. Additionally, we found that Western blots of infected cell proteins probed with anti-fpC3 serum revealed two proteins with molecular sizes calculated to be 50 and 19 kDa.

The results presented in Fig. 5 demonstrate that transcripts homologous to all three clones could be detected in infected cells at immediate-early times and in cells blocked with cyclohexamide. This suggests that the transcription of at least some late genes is not strictly dependent on replication of the virus genome (37). In addition it was observed that the levels of transcripts detected by at least one of the clones was not constant but instead varied in what appeared to be a cyclic manner. In particular, transcripts homologous to clone H10 underwent a dramatic decrease in abundance from 12 to 24 h p.i. Each clone, however, was found to have its own characteristic pattern of expression during infection. D10 for example, hybridized to transcripts that apparently peaked in abundance at 48 h p.i. and declined at 62 hours p.i. This maximum observed at 48 h may indicate that the D10 messages become less abundant after this time point. Alternatively, a cyclic pattern of expression that continues throughout late infection could account for these results and could be determined by examining later time points (16).

Northern analysis of total cell RNA isolated at 62 h p.i. demonstrated that a number of RNA species were detected by the clones (Fig. 4). These additional bands were observed consistently and imply that precursors for these transcripts undergo posttranscriptional processing to generate mature messages. Northern blots were used to analyze RNAs obtained from cells at the same time points as those shown in Fig. 5. This data suggests that the transcripts homologous to C3 present in the cell at 12 h p.i. consist primarily of unprocessed precursors and little or no mature message (unpublished data). Previous reports by Wathen and Stinski (39) have demonstrated that transcription from the long unique region, 0.075 to 0.325 on the Towne strain orientation, occurs early in infection but that little or no RNA is found in the cytoplasm at this time. Therefore, the transcripts coding for late gene products appear early during infection but are not translated until about 48 hours p.i.

As with herpes simplex virus, gene expression in HCMV is temporally controlled and has been divided into immediate-early, early, and late stages based upon the onset of the regulatory landmarks of virus-specific protein synthesis and DNA replication (5, 37). This has led to the assumption that genes whose protein products to not appear until late infection are not transcribed until after virus DNA replication has begun. These experiments suggest that this is not the case for the RNAs homologous to the three cDNA clones isolated here and that these genes may be transcribed at early times also. Whereas the transcripts homologous to C3 accumulate in infected cells at 12 hours p.i., preliminary evidence suggests that they are not processed into a mature message until later. Examination of these early time points with goat

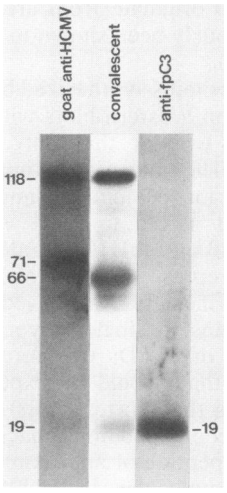

FIG. 7. Identification of the authentic C3 virion protein. Twenty-five micrograms of protein from HCMV virions purified by sedimentation through sucrose gradients was fractionated on a 5 to 20% gradient SDS-PAGE gel. Proteins were then transferred to nitrocellulose, and duplicate lanes were probed with either goat anti-virion serum (goat anti-HCMV), human anti-HCMV serum (convalescent), or antibody prepared in rabbits against purified fpC3 (anti-fpC3). The molecular sizes of apparent protein bands are marked in kilodaltons.

anti-virion serum does not detect virus proteins in the infected cells, suggesting that these first transcripts are not translated. The authentic gene product detected by anti-fpC3 serum is a small polypeptide in purified virions that appears to be processed from a larger precursor found in infected cells. This suggests that at least the authentic C3 protein is either posttranslationally modified or cleaved during virion assembly. We are currently preparing antibodies to fusion proteins fpD10 and fpH10 to examine more closely the corresponding HCMV proteins.

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