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## Physical Mapping of Human Cytomegalovirus Genes: Identification of DNA Sequences Coding for a Virion Phosphoprotein of 71 kDa and a Viral 65-kDa Polypeptide

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Polyadenylated RNA was isolated from fibroblast cultures infected with human cytomegalovirus (HCMV) strain AD169 during the late phase of viral replication. The RNA was selected by hybridization to a series of cosmid clones containing the entire viral genome in partially overlapping segments. Translation of this RNA in a reticulocyte cell-free system allowed the mapping of virus specific polypeptides. Nine polypeptides synthesized *in vitro* comigrated with major virion structural proteins. An *in vitro*-translated protein of 71 kDa was precipitated by a monoclonal antibody directed against the phosphorylated internal envelope protein of 71 kDa. The map coordinates of viral DNA coding for this phosphoprotein were localized by hybrid selection with subcloned DNA fragments, and the direction of transcription was determined by hybrid selection with single-stranded DNA cloned in bacteriophage vector M13mp9. An *in vitro* translation with size-fractionated RNA, combined with immunoprecipitation and Northern blot analyses, indicated that an mRNA of 4 kb encodes the 71-kDa phosphoprotein. An mRNA of the same size, map coordinates, and orientation was translated into an abundant 65-kDa polypeptide which had the same size as the major structural phosphoprotein of HCMV.

### INTRODUCTION

The genome of human cytomegalovirus (HCMV) AD169 is a linear, double-stranded DNA molecule of about 235 kb (Geelen *et al.*, 1978; Fleckenstein *et al.*, 1982). Like some other herpesvirus DNA molecules, the HCMV genome consists of a large (L) and a small (S) unique copy segment, which can be oriented in either of two directions. This results in four isomeric conformations of virion DNA. These unique regions are bracketed by a pair of inverted repeats (LaFemina and Hayward, 1980; Ebeling *et al.*, 1983). The HCMV genome has been cloned in plasmid (Thomsen and Stinski, 1981; Oram *et al.*, 1982; Ta-

mashiro *et al.*, 1982) and cosmid vectors (Fleckenstein *et al.*, 1982), providing a basis for the physical and functional mapping of viral DNA. A small, contiguous region of the viral genome is abundantly transcribed into three or four species of RNA during the immediate early phase of viral replication (DeMarchi, 1981; Wathen and Stinski, 1982; McDonough and Spector, 1983; Jahn *et al.*, submitted), and the coding region for one immediate early gene product of 72 kDa has been identified (Stinski *et al.*, 1983). On the other hand, transcripts from most regions of the viral genome are found in infected cells during subsequent phases of viral replication (DeMarchi, 1981; Wathen and Stinski, 1982; McDonough and Spector, 1983), but no correlation has been reported between a defined virion protein and its respective coding sequence. This study describes a procedure which may be

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generally useful for the identification of late viral genes. Sequences of DNA coding for viral proteins were mapped by mRNA hybrid selection, followed by *in vitro* translation of late mRNA. Immunoprecipitation of these *in vitro*-synthesized polypeptides with a monoclonal antibody identified the coding region of a phosphorylated structural protein of 71 K molecular weight (Nowak *et al.*, 1984). Analysis of the transcripts and translated products indicated that the same region of the genome also encoded an abundant viral protein of 65 kDa.

#### MATERIALS AND METHODS

**Virus and cell culture.** HCMV strain AD169 was propagated on human foreskin fibroblasts (HFF) using standard procedures. To obtain pure virus stocks, virion DNA was transfected by the calcium phosphate precipitation method (Graham and van der Eb, 1973; Copeland and Cooper, 1979), and resulting virus was plaque purified. Labeled virus was purified from cultures incubated with 50  $\mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]-methionine (1200 Ci/mmol, Amersham-Buchler, Braunschweig) at 4 days postinfection (Ebeling *et al.*, 1983). Proteins from cell extracts were prepared from [ $^{35}\text{S}$ ]-methionine-labeled, AD169- or mock-infected cells as described (Sarnow *et al.*, 1982). For isolation of immediate early (IE) RNA, cells were infected and maintained in the presence of 0.1 mg/ml cycloheximide, and total cellular RNA was harvested 14 to 16 hr postinfection. Late RNA was isolated after 4 to 6 days of infection. Mock-infected cell RNA was extracted from confluent, uninfected HFF cultures.

**Cloning of DNA in plasmids and bacteriophage M13.** Recombinant clones containing subfragments of cosmid-cloned AD169 DNA (Fleckenstein *et al.* 1982) was constructed by using the plasmid vector pAcyc148 (Chang and Cohen, 1978). Procedures for isolation of cosmid and plasmid DNA and construction of recombinant clones were carried out as described (Fleckenstein *et al.*, 1982; Knust *et al.*, 1983). Each cloned viral DNA fragment was identified by separation on agarose gels after restriction endonuclease digestion and Southern blot hybridization (Southern,

1975). Cloned, single-stranded viral DNA was obtained by using the bacteriophage derivative M13mp9 and the procedure described by Messing *et al.* (1981, 1982).

**Isolation of RNA, size fractionation, and Northern blot analysis.** Cellular RNA was isolated from cultures by a modification of the guanidine hydrochloride method (Liu *et al.*, 1979). HCMV- or mock-infected cells were rinsed twice with ice-cold phosphate-buffered saline (PBS), scraped into PBS, centrifuged at 1500 rpm for 5 min, and washed once more. The cells ( $10^9$  to  $5 \times 10^9$  per pellet) were resuspended in 7 ml of lysis buffer (6 M guanidine hydrochloride, 0.1 M  $\beta$ -mercaptoethanol, 0.5% *N*-lauroylsarkosine, 50 mM lithium citrate, pH 6.5). The lysate was layered on a cushion of 4 ml of 5.7 M CsCl in 0.1 M EDTA, pH 7.5. After centrifugation in a Spinco SW41 rotor at 28,000 rpm at 20° for 20–24 hr, the gelatinous pellet was dissolved in a small amount of H<sub>2</sub>O. Following ethanol precipitation and digestion with proteinase K, the RNA was extracted with phenol and chloroform-isoamyl alcohol and precipitated with ethanol. Polyadenylated [poly (A)<sup>+</sup>] RNA was prepared by passing total RNA over a column of oligodeoxythymidylic acid-cellulose (Bethesda Research Laboratories) (Aviv and Leder, 1972). Poly (A)<sup>+</sup> RNA was size fractionated on sucrose-formamide gradients (Siddell *et al.*, 1980). A 50- $\mu\text{g}$  sample of poly (A)<sup>+</sup> RNA was denatured in 60% deionized formamide, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA at 37° for 5 min before centrifugation. The RNA was layered on top of a to 5 to 20% sucrose formamide gradient containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM LiCl, 0.5% sodium dodecyl sulfate (SDS) and 50% deionized formamide. The gradients were centrifuged for 20 hr at 28,000 rpm and 20° in an SW41 rotor. Fractions were collected from the bottom of the gradient, and 30  $\mu\text{g}$  of calf liver tRNA per ml was added as a carrier. The RNA of each fraction was precipitated with ethanol and divided into two parts. One aliquot was analyzed by Northern blot hybridization, the other part was used for *in vitro* translation assays. Labeled ribosomal RNA from owl monkey (28 S, 5.2 kb; 18.5 S, 2.0 kb) and *Escherichia coli* (23 S, 3.1 kb; 16

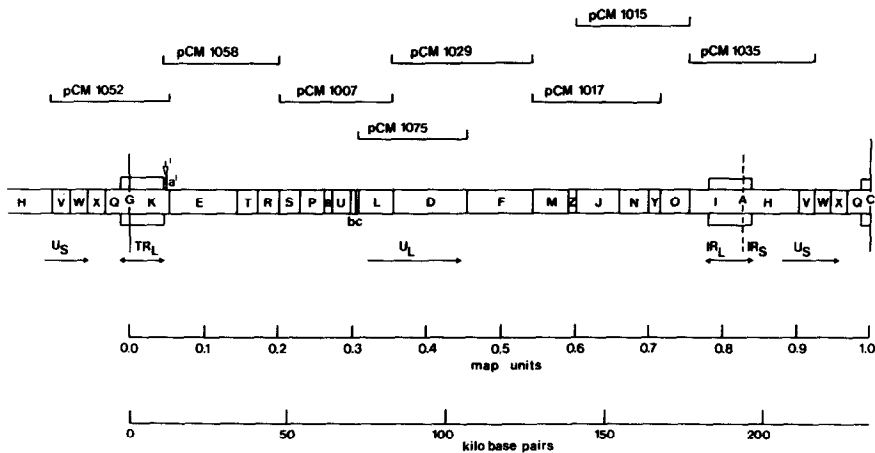


FIG. 1. Cleavage map of the HCMV (strain AD169) genome for restriction endonuclease *Hind*III. Virion DNA molecules consist of a long ( $U_L$ ) and a short ( $U_S$ ) unique sequence, each bracketed by a pair of inverted repeats ( $TR_L/IR_L$  and  $TS_S/IR_S$ , respectively). Eight cosmids clones representing the entire viral genome are indicated by horizontal brackets (Fleckenstein *et al.*, 1982).

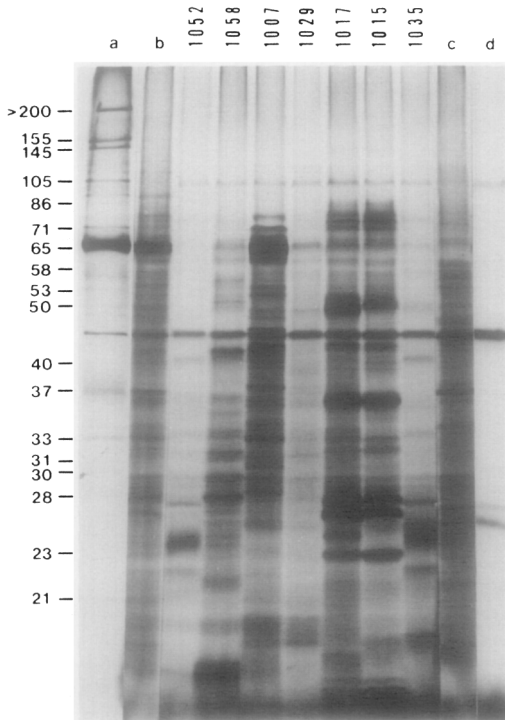


FIG. 2. Cell-free translation of late RNA selected by hybridization to cosmids-cloned viral DNA. Poly(A)<sup>+</sup> RNA was isolated from cells late after infection with HCMV and hybridized to cloned viral probes. The respective cosmids clones are marked by their numbers above the lanes. Selected mRNA was eluted and translated in a reticulocyte cell-free system containing [<sup>35</sup>S]methionine. The translation products were ana-

lyzed on a 15% SDS-PAGE, followed by fluorography. For Northern blot analysis, RNA was denatured with glyoxal (McMaster and Charnichael, 1977). The RNA was fractionated by electrophoresis on 1% agarose gels, transferred to nitrocellulose filters (Thomas, 1980), and hybridized with nick-repair, <sup>32</sup>P-labeled DNA probes (Knust *et al.*, 1983).

*Hybridization selection and cell-free translation.* The procedure for hybrid selection (Ricciardi *et al.*, 1979) was modified, following suggestions of H. Esche (personal communication). Twenty micrograms of cosmid-plasmid- or phage-cloned viral DNA was bound to a 0.4-cm-diameter nitrocellulose filter (Schleicher-Schull, BA 85). Cytoplasmic polyadenylated RNA (0.15–0.2 mg/ml) was incubated for 4 hr at 56° with several filter pieces in 20 mM PIPES (1,4-piperazinediethanesulfonic acid) (pH 6.4), 0.2% SDS, 50% formamide,

lyzed on a 15% SDS-PAGE, followed by fluorography. The sizes of polypeptides, indicated in kilodaltons, were determined by coelectrophoresis of marker proteins. Lane a, [<sup>35</sup>S] methionine-labeled proteins of purified AD169 virions; lane b, translated products of nonselected late poly(A)<sup>+</sup> RNA from AD169-infected cells; lane c, translated products of nonselected RNA from mock-infected cells; lane d, endogenous proteins synthesized in the cell-free system without addition of RNA.

TABLE 1  
SIZES OF POLYPEPTIDES TRANSLATED *IN VITRO* BY mRNA'S HYBRID SELECTED WITH THE pCM-SERIES OF COSMID CLONES OF THE HCMV GENOME

pCM1052	pCM1058	pCM1007	pCM1029	pCM1017	pCM1015	pCM1035
		145		155	155	
	90	90	110			
		86		77	77	
74		76				74
		71		73	73	
		65		70.5	70.5	
	55	62				
		54	53			
	51	52		51		
				50	50	
		49	49	49		49.5
		47				
		45				
		43				
41.5	42	42		42.5	42.5	
			41.5			41.5
		38				
		36		37	37	
				34		
33.5		33				33.5
	32			32.5	32.5	
	28	29				
27				28	28	
				26	26	27
				24		
23.5						23.5
				23	23	
22.5						22.5
	22					

Note. Sizes of polypeptides ( $\times 10^{-3}$ ) obtained by *in vitro* translation of late RNA selected with seven cosmid clones covering the entire HCMV genome. These polypeptides were detected in repeated experiments.

3  $\times$  SSC (1  $\times$  SCC; 0.15 M NaCl, 0.015 M sodium citrate) containing 0.1 mg/ml calf liver tRNA (Boehringer). Unbound RNA was removed by washing the filters five times at 56° in 0.1  $\times$  SSC, 0.5% SDS and two times in 2 mM EDTA, pH 7.0. For elution of CMV-specific RNA, filters were

heated to 95° for 3 min in 0.3 ml H<sub>2</sub>O, boiled for 1 min, and frozen in liquid nitrogen. Solubilized RNA was ethanol precipitated with 10  $\mu$ g of calf liver tRNA as carrier. The RNA was translated *in vitro* in a rabbit reticulocyte lysate system (Pelham and Jackson, 1976) with [<sup>35</sup>S]methionine ac-

TABLE 2

POLYPEPTIDES OBTAINED BY HYBRID-SELECTED *IN VITRO* TRANSLATION THAT COMIGRATE WITH STRUCTURAL VIRAL PROTEINS

<i>in Vitro</i> obtained polypeptides	Selecting <i>Hind</i> III DNA fragments
155 <sup>a,b</sup>	J-Y
145	a, U
86 <sup>b</sup>	a, U
71 <sup>a,b</sup>	b, c, L
65 <sup>b</sup>	b, c, L
53	D-F
50	Y-O
37	J-Y
33	U

<sup>a</sup> Identified as structural, phosphorylated viral proteins by monoclonal antibodies (Nowak *et al.*, 1984).

<sup>b</sup> Comigrating with phosphorylated virion proteins.

cording to the manufacturer's direction (Amersham-Buchler, Braunschweig).

*Immunoprecipitation and SDS-polyacrylamide gel electrophoresis.* Precipitations were carried out as described by Ross *et al.* (1980a). After preincubation with *Staphylococcus aureus* (Kessler, 1975), samples of *in vitro* translation assays were incubated on ice for 30 min with human immune sera or monoclonal antibodies. Antigen-antibody complexes were adsorbed to protein A bearing *S. aureus*. The washed immunoprecipitates were denatured and solubilized by the addition of SDS-sample buffer (Laemmli, 1970), followed by boiling for 3 min. *In vitro* translation products and immunoprecipitates were separated on 15% linear polyacrylamide gels (PAGE) (Bodemer *et al.*, 1980). Fixed gels were fluorographed by the method of Bonner and Laskey (1974).

## RESULTS

### *Gene Mapping by Hybrid-Selected Cell-Free Translation*

For hybridization selection of late viral RNA, eight cosmid clones were employed which cover the entire genome of HCMV strain AD169 (Fig. 1). The clones were derived from partial cleavage of purified virion DNA by *Hind*III (Fleckenstein *et al.*, 1982), and contain overlapping segments

of viral DNA. Purified, cosmid-cloned DNA, immobilized on nitrocellulose filters, was hybridized with polyadenylated RNA obtained at late times from infected cell cultures. Bound mRNA was eluted from the solid phase, and translated *in vitro* in a rabbit reticulocyte lysate system. Figure 2 gives an example showing the polypeptide patterns obtained with a set of cosmid clones. The *in vitro* translation products from hybrid-selected RNA gave a polypeptide pattern specific for each cosmid clone. Many identical bands were seen after selection with the cosmids pCM1017 and 1015, which contain largely overlapping segments of HCMV DNA. In four independent experiments, the most prominent polypeptides were synthesized after selection with the clones pCM1007 and pCM1017/pCM1015, respectively (Fig. 2). Table 1 summarizes the results of these experiments, listing the sizes of polypeptides that could reproducibly be hybrid selected and translated with individual cosmid clones. None of these polypeptides comigrated with the products of *in vitro*-translated RNA from mock-infected cells. Some of the polypeptides listed in Table 1 appeared to comigrate with virion proteins or polypeptides from extracts of infected cells; other *in vitro* translation products found after hybrid selection and translation could correspond to virus-specific proteins that have not been identified as yet. It remains possible that some peptides may be due to premature termination or aberrant initiation during *in vitro* translation.

The apparent comigration of some *in vitro*-translated polypeptides with native viral proteins allowed a tentative correlation with the DNA sequence encoding these products. Hybrid selection with overlapping cosmids and subcloned *Hind*III fragments allowed finer resolution of the putative genes for these proteins in individual restriction fragments. The results of these experiments are listed in Table 2.

### *Identification of Coding Sequences for the Phosphorylated Virion Protein of 71 kDa*

Correlation of *in vitro*-translation products with native virion proteins is possible

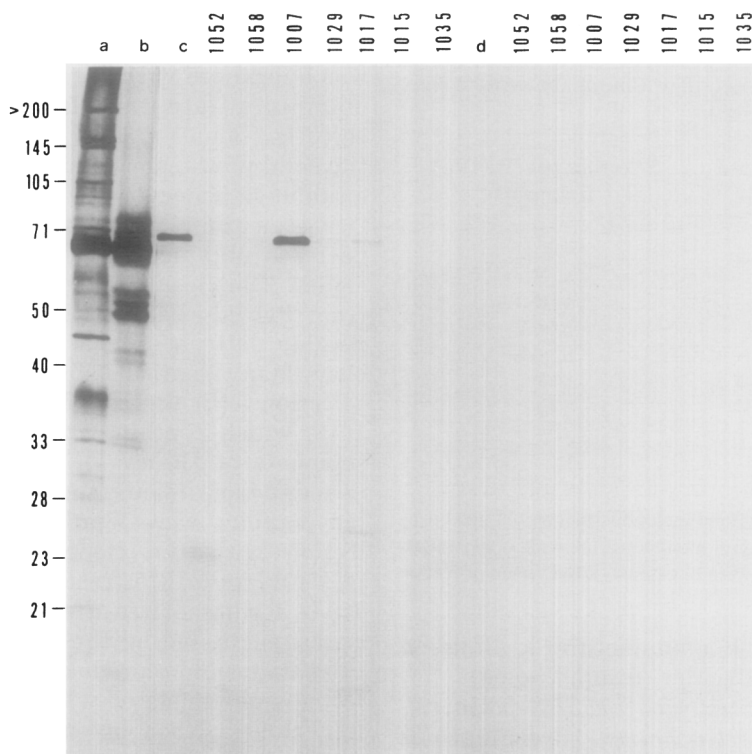


FIG. 3. Immunoprecipitation of cell-free translation products of RNA selected by hybridization to cloned viral DNA fragments. Late poly(A)<sup>+</sup> RNA from AD169-infected cells was selected by hybridization to viral DNA fragments (indicated by numbers above the lanes). The eluted RNA was translated in the reticulocyte cell-free system and the translation products were immunoprecipitated with monoclonal antibodies. Immunoprecipitation with the monoclonal antibody 355, directed against the phosphorylated 71-kDa HCMV protein, is shown in the left half; precipitation with the anti-adenovirus monoclonal antibody 2A6 (Sarnow *et al.*, 1982) is shown in the right half. The precipitated translation products were analyzed on a 15% SDS-PAGE, followed by fluorography. Lane a, [<sup>35</sup>S]methionine-labeled proteins of purified AD169 virions. Translated products of nonselected late poly(A)<sup>+</sup> RNA from AD169 infected cells were precipitated with a human anti-HCMV immune serum (lane b), with the monoclonal antibody 355 (lane c), or with monoclonal antibody 2A6 (lane d).

by precipitation with defined monoclonal antibodies. In a separate study a monoclonal antibody designated as PAb355 was obtained that was directed against a virion internal phosphorylated envelope protein of 71 kDa (Nowak *et al.*, 1984). Total RNA and poly(A)<sup>+</sup> mRNA were prepared from lytically infected cells during the late phase of viral replication and employed for *in vitro* translation. Monoclonal antibody 355 precipitated a single 71-kDa polypeptide from the mixture of *in vitro*-synthesized products (Fig. 3, lane c). Combining this method with hybrid selection, the sequence

coding for the phosphorprotein of 71 kDa (pp 71) could be located within the cosmid clone pCM1007 (Fig. 3). To determine more precisely the position of this structural gene, subcloned *Hind*III fragments of cosmid pCM1007 were employed for subsequent hybrid-selected *in vitro* translation combined with immunoprecipitation with monoclonal antibody PAb355. As shown in Fig. 4, the mRNA coding for pp71 hybridized with *Hind*III fragments b, c, and L (Fig. 1). Similarly, the mRNA encoding the *in vitro*-translation product of the 65-kDa protein was selected by the same subcloned

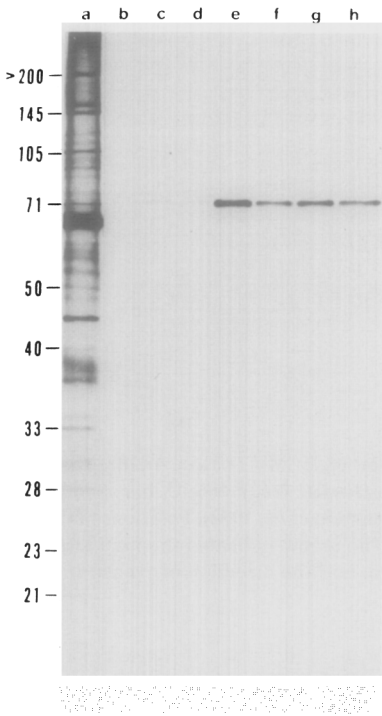


FIG. 4. Immunoprecipitation of cell-free translation products of RNA selected by hybridization to cloned viral *Hind*III DNA fragments. Late poly(A)<sup>+</sup> RNA from AD169-infected cells was selected by hybridization to the *Hind*III fragments S, P, a, b + c, b, c, and L + D (lanes b through h, respectively). The eluted RNA was translated and the products were immunoprecipitated with the monoclonal antibody 355. The immunoprecipitated translation products were analyzed on a 15% SDS-PAGE, followed by fluorography. Lane a shows [<sup>35</sup>S]methionine-labeled proteins of purified AD169 virions.

*Hind*III fragments (L, b, c) of cosmid clone pCM 1007. The 65-kDa *in vitro*-translated polypeptide comigrated with the major structural protein of HCMV strain AD169 (Fig. 2, Table 2). *In vitro* translation of RNA selected by *Hind*III fragments b and c resulted in about equivalent amounts of 65- and 71-kDa polypeptides; in contrast, *Hind*III L fragments selected the RNA molecules encoding the 71-kDa polypeptide more efficiently than the RNA for the 65-kDa protein (data not shown).

#### *Transcripts for Virion Polypeptides of 65 and 71 kDa*

The possibility that the coding sequences of the 65- and 71-kDa polypeptides over-

lapped led us to analyze the direction of transcription of these two mRNAs. *Hind*III fragment c was cloned in bacteriophage M13mp9 in both orientations. The orientation of the inserted *Hind*III c fragment was determined by taking advantage of an *Eco*RI recognition site which cleaves the insert of about 800 bp asymmetrically (Fig. 5). The message coding for pp71 was hybridized with single-stranded, cloned HCMV DNA in either of the two orientations (termed plus and minus, arbitrarily). The plus-strand DNA of extracellular bacteriophage contained the HCMV DNA which was transcribed as tested by hybrid selection and *in vitro* translation (Fig. 6A). This demonstrates that the mRNA coding for pp71 is transcribed from left to right in the prototype arrangement of the HCMV AD169 genome (Fig. 5). The message for the 65-kDa polypeptide was hybrid selected by the same M13 clone in the plus orientation as shown by *in vitro* translation of this mRNA and an analysis of the products directly (Fig. 6B).

Next, experiments were carried out to determine the sizes of the mRNAs encoding 65- and 71-kDa virion polypeptides. RNA was isolated from lytically infected cells during late times, fractionated to obtain poly(A)<sup>+</sup> RNA, and analyzed by Northern blot hybridizations. <sup>32</sup>P-Labeled *Hind*III fragments that were subcloned from cosmid pCM1007 were used as radioactive probes. A single type of an abundant poly(A)<sup>+</sup> RNA of about 4 kb was detected when *Hind*III fragments L and D, b, or c were used as probes (Fig. 7). *Hind*III fragment U, on the other hand, did not hybridize with this late 4-kb mRNA. This implies that the 4-kb mRNA hybridized with the same set of *Hind*III DNA fragments which hybrid selected the RNA encoding the two polypeptides of 65 and 71 kDa. Finally, late poly(A)<sup>+</sup> RNA was fractionated by sedimentation in sucrose gradients. RNA from each fraction was analyzed in parallel by Northern blot hybridization with the labeled *Hind*III c fragment and by *in vitro* translation. The products of *in vitro* protein synthesis were immunoprecipitated by monoclonal antibody 355 and by human immune sera. As shown in Fig.



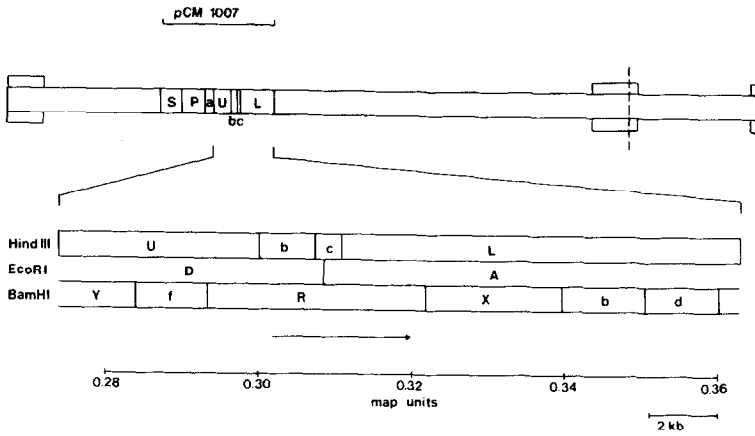


FIG. 5. Restriction endonuclease maps of the DNA region of HCMV (strain Ad169) that codes for the viral structural polypeptides of 65 and 71 kDa. The *Hind*III fragments U, b, c, and L of the cosmid clone pCM1007 are drawn on expanded scale (Fleckenstein *et al.*, 1982). Positions of cleavage sites for *Bam*HI are as given by Greenaway *et al.* (1982). The arrow indicates the map location of the coding sequences for the polypeptides of 65 and 71 kDa, and the direction of transcription for both polypeptides.

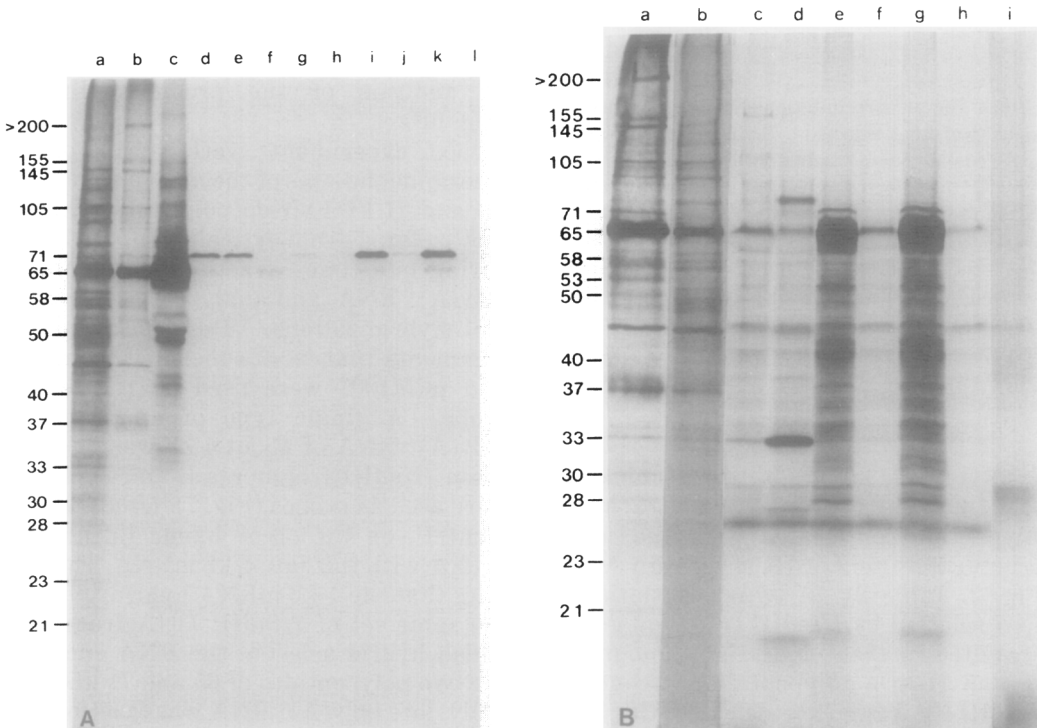


FIG. 6. (A) Immunoprecipitation of cell-free translation products of RNA selected by hybridization to cloned viral *Hind*III fragments. Late poly(A<sup>+</sup>) RNA from AD 169-infected cells was selected by hybridization to the plasmid-cloned *Hind*III fragments a, U, and c (lanes g through i) to the *Hind*III c fragment cloned in bacteriophage vector M13mp9 in both orientations (lanes j and k), and the replicative form of the M13 vector (lane l). The eluted RNA was translated, and the products were immunoprecipitated with the monoclonal antibody 355. The polypeptides were analyzed on a 15% SDS-PAGE, followed by fluorography. Lane a shows [<sup>35</sup>S]methionine-labeled proteins from AD169-

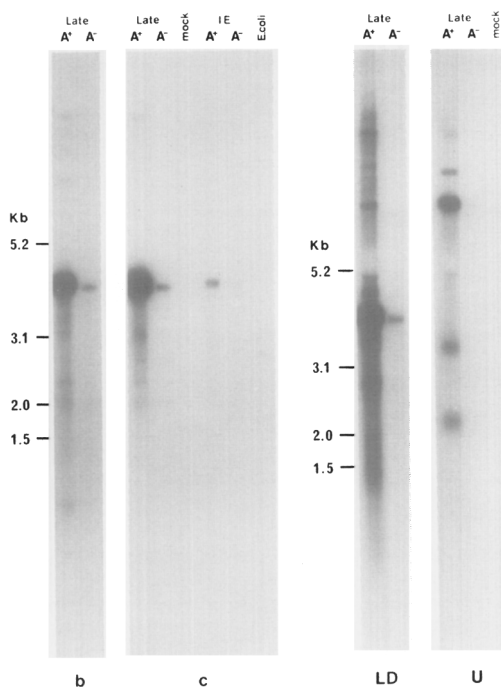


FIG. 7. Autoradiogram of Northern blot hybridizations of cloned *Hind*III DNA fragments to HCMV late and immediate-early (IE) RNAs. RNA was isolated from cells late after infection. IE RNA was extracted at 15 hr postinfection in the presence of 0.1 mg/ml cycloheximide. The RNAs were separated into polyadenylated (A<sup>+</sup>) and nonadenylated (A<sup>-</sup>) fractions, denatured, electrophoresed on agarose gels, and transferred to nitrocellulose filters. Cosmid clone pCM1075 (*Hind*III L and D) and cloned *Hind*III fragments b, c, or U were <sup>32</sup>P labeled *in vitro* and hybridized to 2.5 μg of blotted poly(A)<sup>+</sup> or poly(A)<sup>-</sup> RNA, to 7.5 μg of *Escherichia coli* RNA, or to 10 μg of RNA from uninfected HFF (Mock).

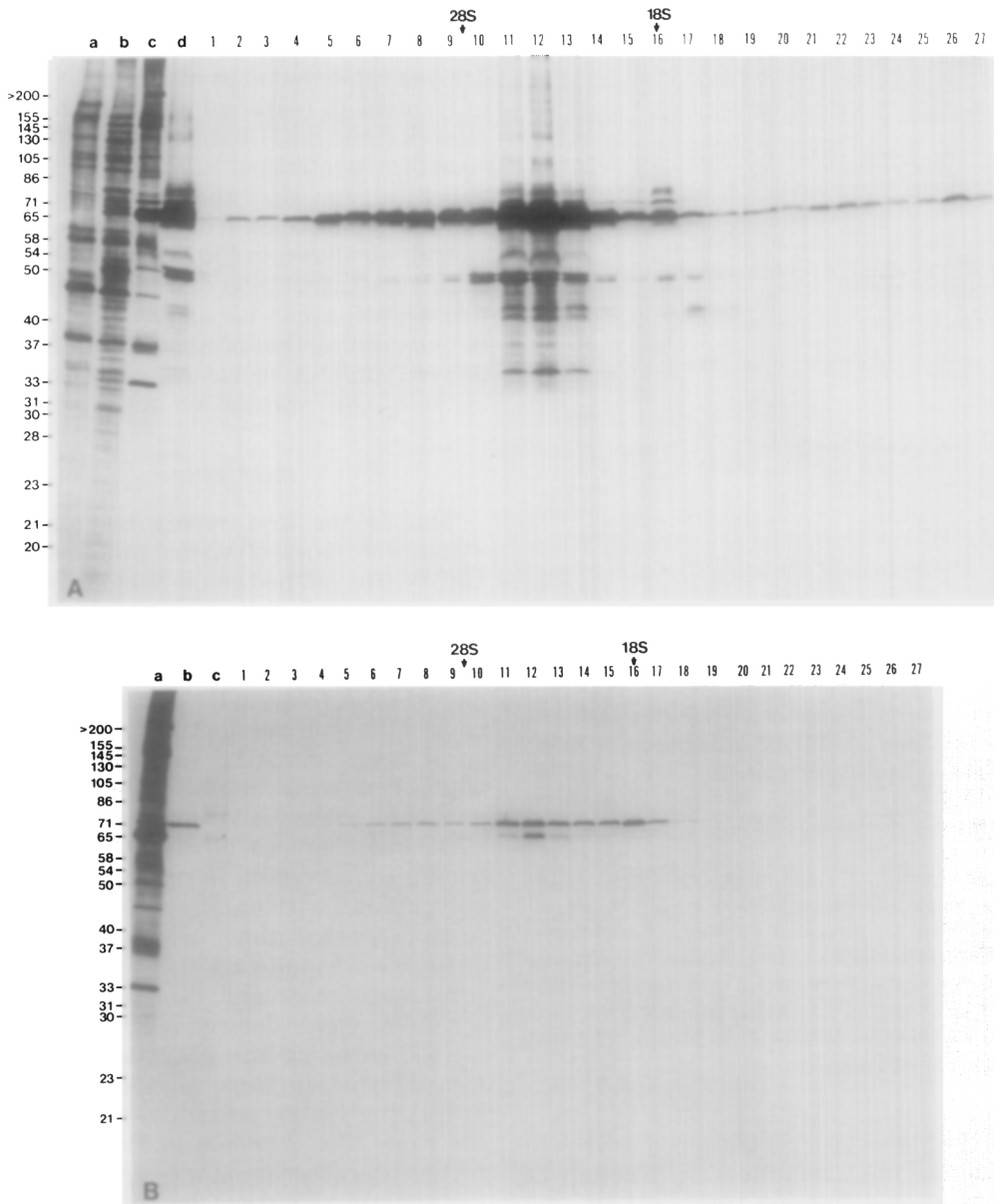
8B, the highest concentration of mRNA instructing pp71 was found in fraction no. 12; the same fraction contained the ma-

jority of mRNA for 65-kDa polypeptide (Fig. 8A). Northern blot hybridization demonstrated that most of 4-kb mRNA was in fraction 12 (data not shown). This implies that the mRNA molecules coding the structural proteins of 65 and 71 kDa have about the same sizes and map coordinates, and are transcribed from the same DNA strand. As shown in Fig. 8A, a small level of *in vitro* translation of 71-kDa polypeptide was also seen with smaller RNAs (>1.8 kb), suggesting that processed or degraded poly(A)<sup>+</sup> mRNA molecules can be active in the *in vitro* translation assay.

#### DISCUSSION

This is the first report mapping the structural genes of virion proteins from HCMV. The method employed to map DNA sequences coding for virion polypeptides was based on cell-free translation of viral RNA selected by hybridization to cosmid-cloned DNA fragments. This approach localized the map coordinates for nine structural genes encoding nine polypeptides which comigrate in SDS-polyacrylamide gels with authentic virion proteins. For a more unambiguous identification, it was useful to correlate *in vitro*-synthesized polypeptides with native viral proteins by immunoprecipitation using monoclonal antibodies. Employing this approach the map coordinates and direction of transcription were determined for a 4-kb mRNA encoding the phosphorylated 71-kDa internal envelope protein of HCMV. Surprisingly, viral polyadenylated RNA of the same size, genomic location, and direction of transcription was also translated *in vitro* into a 65-kDa polypeptide. This 65-

infected cell extracts; lane b, <sup>35</sup>S-labeled proteins of purified AD169 virions. Translated products of nonselected late poly(A)<sup>+</sup> RNA from AD169-infected cells after immunoprecipitation with a human positive anti-HCMV serum (lane c), with the monoclonal antibody 355 (lanes d and e), or with the anti-adenovirus monoclonal antibody 2A6 (lane f). (B) Cell-free translation products of RNA selected by hybridization to cloned viral *Hind*III fragments. Late poly(A)<sup>+</sup> RNA from HCMV-infected cells was selected by hybridization to the *Hind*III fragments a, U, and c (lanes c through e) to the *Hind*III c fragment cloned in bacteriophage vector M13mp9 in both orientations (lanes f and g), and to the replicative form of M13 vector (lane h). The eluted RNA was translated and the products were analyzed on a 15% SDS-PAGE, followed by fluorography. Lane a shows [<sup>35</sup>S]methionine-labeled proteins of purified AD169 virions; lane b, <sup>35</sup>S-labeled proteins from AD169-infected cell extracts; lane i, endogenous proteins synthesized in the cell-free system without added RNA.



**FIG. 8.** Immunoprecipitation of cell-free translation products of RNA size-fractionated in sucrose gradients. Fifty micrograms of late poly(A)<sup>+</sup> RNA from AD169-infected cells was size fractionated on a 5 to 20% sucrose-formamide gradient. The RNA in each fraction (1 through 27) was ethanol precipitated and half of the RNA was translated in the reticulocyte cell-free system. The suspension with the translated products was divided into two aliquots. One aliquot of each fraction was precipitated with a human anti-HCMV immune serum (A), the other aliquot was precipitated with the monoclonal antibody 355 (B). The immunoprecipitated products were analyzed on a 15% SDS-PAGE, followed by fluorography. (A) (lane a) shows [<sup>35</sup>S]methionine-labeled proteins of mock-infected cell extracts, (lane b) of AD169 infected cell extracts, (lane c) of purified AD169 virions, and (lane d) of translated products of late poly(A)<sup>+</sup> RNA from AD169-infected HFF after immunoprecipitation with human serum. (B) (lane a) shows [<sup>35</sup>S]methionine-labeled proteins of purified AD169 virions, (lane b) translated products of late poly(A)<sup>+</sup> RNA from AD169 infected cells after immunoprecipitation with the monoclonal antibody 355, or (lane c) with the anti-adenovirus monoclonal antibody 2A6.

kDa protein appeared to be equivalent to the major structural protein of virus particles and dense bodies. The coding sequences for the 65-kDa polypeptide might be localized more upstream (5') within the 4-kb mRNA than the sequence instructing the 71-kDa protein. This suggestion is based upon the efficiency of hybrid selection and *in vitro* translation in subcloned *Hind*III fragments and from translation of size-fractionated mRNA (Figs. 6, 8).

There are several possible interpretations of the experimental results presented here. First, the two polypeptides, 71 and 65 kDa, could be encoded in two different 4-kb m-RNAs that derive from the same region of the CMV genome. These mRNAs could arise from two distinct transcripts or from differential splicing patterns of a primary transcript, giving rise to two related but distinct 4-kb mRNAs. Alternatively, a single 4-kb mRNA species could give rise to two different proteins, 71 and 65 kDa, by using two different reading frames in the same mRNA. This is apparently the case with the Adenovirus E1b-22 S mRNA and E1b-19 kDa and E1b-58 kDa proteins read in two different reading frames (Bos *et al.*, 1981). Finally, it is formally possible that the 71-kDa protein is a precursor of the 65-kDa protein. The reason this appears to be an unlikely hypothesis is that two different monoclonal antibodies directed against the 71-kDa protein do not react with the 65-kDa protein (Nowak *et al.*, 1984). Furthermore, the cleavage of the 71-kDa protein into a 65-kDa protein would have to occur very efficiently in the rabbit reticulocyte *in vitro* translation extract, and that possibility appears to be unlikely as well. Peptide maps of the 71- and 65-kDa proteins should help to distinguish between some of these alternatives. What is clear from these data, however, is that identical or overlapping coding regions of the CMV genome are employed to produce two structural proteins of this virus.

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