Temporal Regulation of Murine Cytomegalovirus Transcription and Mapping of Viral RNA Synthesized at Immediate Early Times After Infection

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The replication of murine cytomegalovirus strain Smith in murine embryonic fibroblasts was investigated at immediate early, early, and late times after infection. Cloned subgenomic HindIII fragments of murine cytomegalovirus DNA served to define the regions of transcription. At immediate early times viral RNA classes ranging in size from 5.1 to 1.05 kilobases (kb) were transcribed mainly from the fragments HindIII-K and -L, whereas low levels of transcription were detected from the two termini HindIII-E and HindIII-N. A characteristic pattern of proteins could be translated from immediate early RNA in vitro. At early and late times after infection transcription from all HindIII fragments occurred, but different patterns of transcripts and proteins could be identified. Inhibitors of DNA synthesis induced differences in the late transcription pattern, located in the HindIII-F fragment. The coding region for abundant immediate early transcription could be located at between 0.769 and 0.817 map units. A plasmic clone containing the main part (0.769 to 0.815 map units) of this region was constructed. This region coded for six polyadenylated immediate early RNA species of 5.1, 2.75, 2.0, 1.75, 1.65, and 1.05 kb in size. Only the 1.75-kb RNA originated entirely from the HindIII-L fragment. The 5.1- and 2.75-kb RNA species were encoded by both the HindIII-L and HindIII-K fragments, and the 2.0-, 1.65-, and 1.05-kb RNA species were entirely transcribed within HindIII-K.

The species-specific cytomegaloviruses (CMV) of humans and animals are able to cause asymptomatic persistent and latent infection in the immunocompetent and illness and fatal disease in the immunocompromised host. In humans the question of latent CMV infection is difficult to assess experimentally. Therefore, appropriate animal models are required, and the murine cytomegalovirus (MCMV) system appears to be particularly useful because it resembles the human infection in the main biological properties (9).

We have initiated a series of in vivo and in vitro studies designed to analyze the principles that govern MCMV latency, persistency, and reactivation (7, 24, 24a). Knowledge of the individual steps of MCMV replication under permissive conditions in vitro is a prerequisite for the understanding of reactivation from latency in vivo. The genome of MCMV is a linear duplex DNA of 235 kilobases (kb) and thus of a size similar to that of human CMV (HCMV). In contrast to HCMV, it consists of one large unique segment that lacks isomeric forms and large terminal as well as internal repeats (7, 17). We have recently cloned the genome of MCMV and mapped the fragments for the enzymes HindIII, XbaI, and EcoRI (7). This library of cloned fragments enabled us to perform a more detailed study of MCMV transcription. As in other herpesviruses, the regulation of MCMV gene expression is divided into three phases (1, 10): (i) the immediate early phase observed in cells arrested for protein synthesis (3), (ii) the early phase which requires the synthesis of at least one immediate early gene product, and (iii) the late phase after the onset of viral DNA synthesis (3, 19). This report describes the time course of the sequential appearance of MCMV RNA. The genomic regions encoding immediate early, early, and late genes were determined. The region of abundant immediate early transcription was mapped in detail, and a recombinant plasmid

spanning the region of immediate early transcription was constructed for further studies of immediate early transcription.

MATERIALS AND METHODS

Virus and cell culture. MCMV (mouse salivary gland virus, strain Smith; ATCC VR-194) was obtained from the American Type Culture Collection, Rockville, Md., and propagated on BALB/c mouse embryo fibroblasts. Fibroblasts were prepared from 15- to 17-day-old BALB/c mouse embryos by trypsinization (0.25% trypsin–0.125% EDTA, sterilized by filtration through a 100-nm filter [Millipore Corp., Bedford, Mass.]) and subsequent selection during two 4- to 6-day culture passages in minimum essential medium with Earle salts, complemented with 5% fetal calf serum (Seromed, Munich, Germany), 1,000 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.35 mg of L-glutamine per ml in a humidified 5% CO_2 atmosphere at 37°C. For isolation of RNA, cells were seeded in 100-mm plastic tissue culture dishes (Greiner, Nürtingen, Germany) and infected 20 h later with 0.2 PFU per cell under the influence of a centrifugal field of 800 × g for 30 min. This method results in a 30- to 80-fold increase of infectivity (12). Cycloheximide (50 μg/ml) was added to the cultures at various times postinfection (p.i.) and cells were maintained in the presence of the inhibitor for 4 h.

Isolation of RNA. Total cellular RNA was isolated following published procedures (4, 28), with some modifications. All glassware and plastic tubes were siliconized and autoclaved. Cells were trypsinized in the presence of cycloheximide, and about 5 × 10^7 cells were washed twice in 50 ml of phosphate-buffered saline-A supplemented with cycloheximide. The cell pellets were lysed in 6 ml of 5.8 M guanidine hydrochloride (Sigma Chemical Co., Munich, Germany) containing 50 mM lithium citrate, 0.1 M β-mercaptoethanol, and 0.5% Sarkosyl, pH 6.5. The lysate was centrifuged through a

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4.5-ml cushion of 5.7 M CsCl in 0.1 M EDTA (pH 7.0) for 22 to 24 h at 32,000 rpm in a Beckman SW41 rotor. The supernatant was carefully decanted, the pellet was resuspended in distilled water, and 0.2 volume of 1 M potassium acetate was added. RNA was precipitated with 2.5 volumes of ethanol at $-20°C$. The precipitate was collected by centrifugation, washed twice with 70% ethanol, and dried under vacuum. The RNA was resuspended in a small volume of distilled water and stored at $-70°C$. Polyadenylated [poly(A)$^+$] RNA was selected from total RNA by oligodeoxythymidylic acid-cellulose chromatography (BRL, Neu Isenburg, Germany) following published procedures (15).

In vivo labeling of RNA. Infected cells were trypsinized and transferred to a small volume of phosphate-free minimum essential medium containing 50 μg of cycloheximide per ml and labeled with 2.5 mCi of $^{32}P_i$ (Amersham Corp., Braunschweig, Germany) per ml for 4 h.

Labeled RNA was purified as above and prepared for hybridization as described previously (27). In some experiments phosphonoacetic acid (PAA; 250 μg/ml) was added to infected cells at 6 h p.i. to prevent viral DNA replication. Hybridization of ^{32}P-labeled RNA to Southern blots was carried out as described before (27).

In vitro translation of RNA. The biological activity of the purified total cell RNA and poly(A)$^+$ RNA was tested in a cell-free rabbit reticulocyte lysate system prepared according to Pelham and Jackson (23). In vitro synthesized, [^{35}S]methionine-labeled proteins were separated by electrophoresis in 10% polyacrylamide gels and visualized by fluorography as previously described (14).

Gel electrophoresis of RNA and Northern blot hybridization. Purified RNA, denatured for 15 min at 56°C in the presence of 1 M glyoxal, was separated in 1% agarose gels containing 2.2 M formaldehyde and 20 mM morpholinopropanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA. Gels were stained with ethidium bromide, destained in water, and photographed with Polaroid type 665 or 667 film.

The RNA was transferred from gels to nitrocellulose filters (Schleicher & Schüll, Dassel, Germany) with 20× SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate) after treatment with 50 mM NaOH for 60 min and neutralization in 0.5 M Tris-hydrochloride–1.8 M NaCl, pH 7.5, for 50 min. The filters were baked at 80°C for 2 h under vacuum and prehybridized for 4 to 6 h at 68°C in 5× SSC with 5× Denhardt solution (6) and 190 μg of yeast RNA per ml. Hybridization and washing procedures were as described for Southern blot hybridization (7). The following served as molecular weight markers: 23S (3.3 kb) and 16S (1.7 kb) *Escherichia coli* rRNA and cellular 28S (5.3 kb) and 18S (2.0 kb) rRNA.

Purification, labeling, and gel electrophoresis of viral DNA fragments. Total MCMV DNA and cloned fragments were purified, electrophoresed, and labeled as described previously (7). The terminal *Hind*III-E fragment was isolated out of gels after cleavage of MCMV DNA with *Hind*III and separation of fragments in 0.4% agarose gels following described procedures (15).

Synthesis of cDNA and blot hybridization. DNA complementary to poly(A)$^+$ was synthesized with avian myeloblastosis virus reverse transcriptase (J. W. Beard, Life Sciences Inc., St. Petersburg, Fla.). The reaction mixture (100 μl) contained 10 μg of poly(A)$^+$ RNA, 0.04 mM dCTP, 0.04 mM dTTP, 0.4 mM dATP, 0.4 mM dGTP, 100 μCi each of [α-^{32}P]dTTP and [α-^{32}P]dCTP, 10 mM dithiothreitol, 6 mM MgCl$_2$, 40 mM KCl, 50 mM Tris (pH 8.3), 50 μg of random primer (26) or 10 μg of oligodeoxythymidylic acid (Boeh-

ringer, Mannheim, Germany), and 36 U of reverse transcriptase. After 1.5 to 2 h at 37°C the reaction was stopped by addition of EDTA, pH 8.5, to a final concentration of 20 mM. The mixture was extracted with an equal volume of phenol-ether, and after addition of salmon sperm DNA (final concentration, 0.5 mg/ml) it was precipitated with 2.5 volumes of ethanol. After centrifugation the pellet was resuspended in 0.3 M NaOH–1 mM EDTA and incubated for 30 min at 65°C. The mixture was chilled on ice, diluted with hybridization buffer (1.5× SSC, 0.03% sodium dodecyl sulfate, 6 mM sodium phosphate, 20 mg of polyadenylic acid per ml [Sigma, Munich, Germany]) and hybridized at 68°C to Northern or Southern blots, prehybridized with a solution containing 0.1% Ficoll and 0.1% polyvinylpyrrolidone (Sigma) for 4 to 6 h at 68°C. The washing procedure and autoradiography were carried out as previously published for Southern blots (7).

In vivo labeling of DNA. Cells were seeded in 35-mm plastic culture dishes (Greiner) and infected 20 h later. A 100-μCi/ml amount of [^3H]thymidine or 50 μCi of deoxy-[^3H]adenosine, deoxy-[^3H]cytosine or deoxy-[^3H]guanosine per ml was added for 4 h at the times indicated. Cells were lysed with 2% Sarkosyl in 20 mM Tris (pH 7.5)–10 mM EDTA, treated with RNase (100 μg/ml) and proteinase K (100 μg/ml) for 60 min at 56°C, and centrifuged to equilibrium in CsCl at 35,000 rpm in a Beckman 50Ti rotor. Fractions (50 μl each) were collected from the bottom and the density of each fraction was determined. Trichloroacetic acid (10%)-precipitable radioactivity was determined from 5-μl aliquots of each fraction. For restriction enzyme analysis fractions with a density of 1.718 (viral; 21) or 1.69 to 1.7 (cellular) g/ml were pooled, dialyzed against 20 mM Tris (pH 8.5), and precipitated with 2.5 volumes of ethanol. Restriction enzyme digests were analyzed on 0.6% agarose gels or 7.5 to 20% polyacrylamide gradient gels, prepared for fluorography, and exposed to Kodak X-Omat S films as described previously (8).

Molecular cloning of *Bam*HI fragments of MCMV DNA. MCMV DNA was cloned with *Bam*HI (Boehringer), and the fragments were inserted into the *Bam*HI site of the plasmid vector pACYC184 as described previously (7).

RESULTS

Temporal regulation of MCMV replication. Transcription in the absence of de novo protein synthesis characterizes the immediate early phase of herpesvirus replication, and the transition to the early phase requires protein synthesis. The onset of viral DNA replication defines the initiation of late viral gene expression. To determine the beginning of the late phase, the incorporation of radioactivity into DNA of infected cells was analyzed sequentially after infection in the absence of cycloheximide (Fig. 1). Viral and cellular DNAs were separated by CsCl gradient centrifugation. Incorporation of label into viral DNA was detectable about 16 h p.i. and reached a maximum at 20 to 24 h. Incorporation of [^3H]thymidine and deoxy-[^3H]adenosine, deoxy-[^3H]cytosine, or deoxy-[^3H]guanosine into cellular DNA declined after infection and stopped during the early phase between 4 and 8 h p.i. Presence of cycloheximide from 0 to 4 h p.i. did not shift the shutoff of the cellular DNA synthesis but delayed the onset of viral DNA replication, the production of viral structural proteins, and the release of infectious virus by 4 h (data not shown).

MCMV-specified RNA generated in productively infected murine embryonic fibroblasts was analyzed for the appearance of different size classes of RNA after infection. To
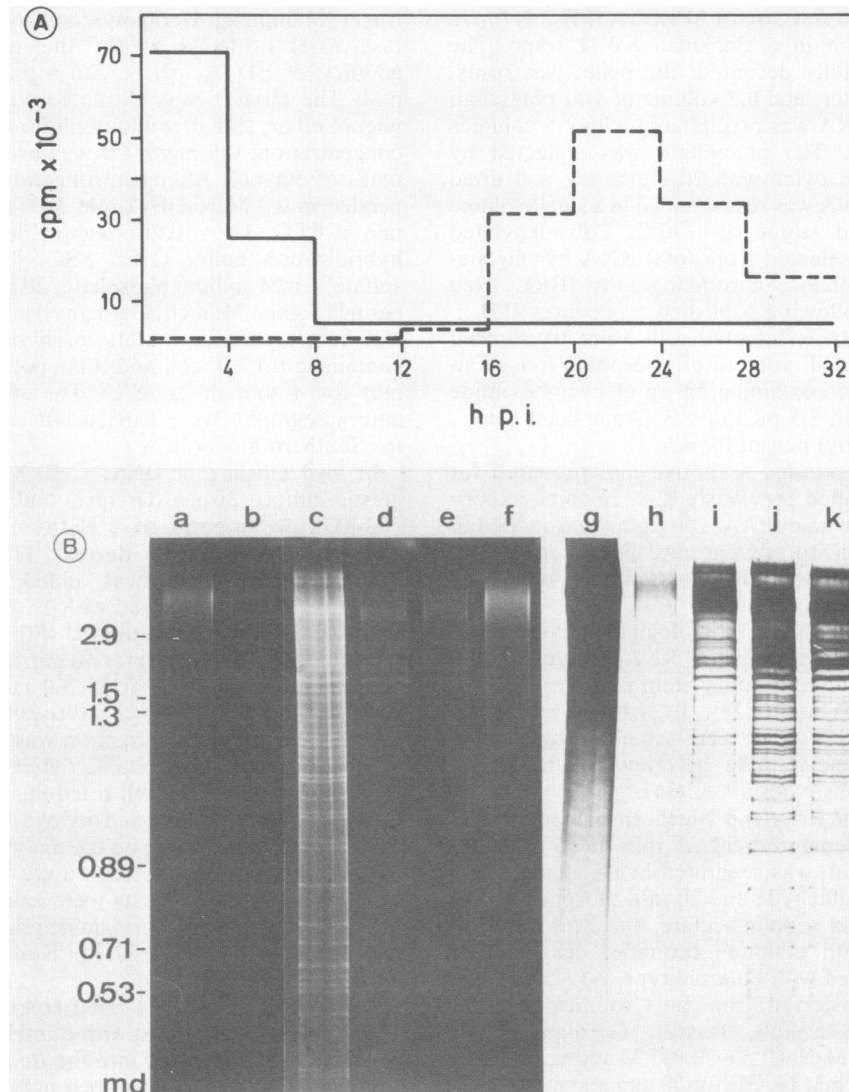
FIG. 1. Kinetics of [³H]thymidine incorporation into cellular and viral DNA during productive infection of mouse embryo fibroblasts. (A) [³H]thymidine-labeled DNA from infected cells was fractionated after CsCl centrifugation. A 5-µl portion of each 50-µl fraction was precipitated with 10% trichloroacetic acid and collected on glass-fiber filters, and the precipitable radioactivity was determined by liquid scintillation counting. The label in DNA with densities of 1.69 to 1.7 (cellular, ———) and 1.718 (viral, - - -) g/ml is shown for various periods p.i. (B) The amount of incorporated radioactivity per microgram of DNA was determined in pooled fractions of labeled cellular and viral DNA. After cleavage with BamHI, the restriction fragments were separated on 5 to 20% polyacrylamide gradient gels. Bands were visualized after staining with ethidium bromide (lanes a to f) or by fluorography (lanes g to k). Lanes: Cellular DNA labeled from 0 to 4 h p.i. (204,000 cpm/µg) (a, g) and 4 to 8 h p.i. (43,000 cpm/µg) (b, h); unlabeled virion DNA (c); viral DNA labeled from 16 to 20 h p.i. (55,000 cpm/µg) (d, i) and 20 to 24 h p.i. (119,000 cpm/µg) (e, j); whole-cell DNA labeled from 16 to 24 h p.i. (46,000 cpm/µg) (f, k). (The fainter than expected band in lane b [see also lane a] is due to DNA loss after precipitation which followed the determination of incorporated radioactivity.)

analyze immediate early RNA, it was advantageous to accumulate transcripts by the addition of the protein synthesis inhibitor cycloheximide. For comparable conditions this treatment was also applied for the RNA preparations from later stages of infection. Cycloheximide was added either at the beginning of the infection or at various times later. After a 4-h period in the presence of the inhibitor the whole-cell RNA was extracted. Therefore, the time points given in the results represent, if not otherwise indicated, the period of permissive infection preceding a 4-h period nonpermissive for protein synthesis. The RNA was transferred to nitrocellulose filters, and viral transcripts were detected by autoradiography after hybridization to a ³²P-labeled MCMV DNA probe (Fig. 2).

Immediate early RNA, which accumulated in the absence of protein synthesis during the first 4 h p.i., comprised several classes of 2.75, 2, and 1.75 kb in size. The RNA species of 2 and 1.75 kb are difficult to distinguish in Fig. 2. The existence of these two RNA classes is demonstrated in experiments discussed later. After 4 h of infection under permissive conditions, the transcription pattern had changed and new transcripts of 4.2 and 1.9 kb in size became visible, indicating the appearance of early transcripts. The 1.9-kb RNA is a true early transcript because it hybridizes to the cloned HindIII-F fragment of MCMV DNA (unpublished data) which is not transcribed at immediate early times, as shown later. When the permissive period was extended to 8 and 12 h, no further RNA classes became detectable. During
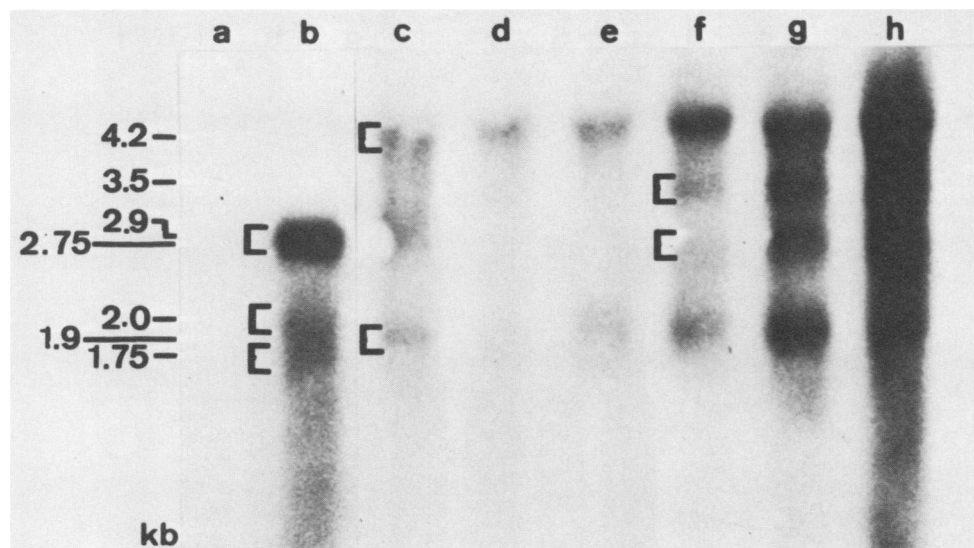
FIG. 2. Autoradiograph of a Northern bot hybridization of whole-cell RNA from infected cells. RNA was extracted from noninfected cells (a) or cells treated for 4 h with 50 μg of cycloheximide per ml at 0 (b), 4 (c), 8 (d), 12 (e), 16 (f), 20 (g), and 24 (h) h p.i. A 5-μg portion of each RNA was fractionated in a 1% agarose gel, transferred to nitrocellulose paper, and hybridized to ^{32}P-labeled MCMV DNA followed by autoradiography. Abundant size classes are designated to indicate the temporal changes in the transcription pattern.

the first 4 to 8 h of the early phase, the immediate early transcripts gradually disappeared; this could be traced by the declining concentration of the 2.75-kb RNA, the most abundant size class of immediate early RNA. In addition, the weak hyridization of RNA extracted after 8 and 12 h of permissive infection demonstrated that the transcriptional activity of the viral genome is rather low during the later stages of the early phase.

Increased transcriptional activity and the appearance of new RNA size classes became detectable after 16 h of permissive infection. The abundant transcripts were 4.2, 3.5, 2.9, and about 2 kb in size (Fig. 2). RNAs of 4.2 and about 2 kb were also transcribed at earlier phases of infection. Whether these represent accumulating identical transcripts or RNAs with similar size needs to be investigated in more detail. This augmented transcriptional activity could be clearly assigned to the late phase since viral DNA synthesis started at 16 h p.i.

The temporal regulation of MCMV gene expression was further investigated by in vitro translation of the RNAs obtained as described above. Amounts of 1 μg of total RNA from infected and noninfected cells were incubated in a cell-free rabbit reticulocyte translation system, and the proteins were separated in 10% acrylamide gels and visualized by fluorography (Fig. 3). Three major proteins were translated from immediate early RNA but not from RNA from noninfected cells. After 2 h of infection permissive for protein synthesis, additional proteins were already detectable, indicating the transition from immediate early to early transcription at this time. The decreasing activity of transcription seen in later stages of the early phase is in accordance with the reduced translation resulting from respective RNA preparations. During the late phase after the onset of DNA synthesis at 16 h, a different pattern of polypeptides was obtained after in vitro translation. Altogether, the different patterns of translation products indicated the switch from immediate early to early transcription after 2 h and confirmed the sequence from early to late transcription after 16 h of permissive infection.

Mapping the origin of immediate early, early, and late RNAs. The library of HindIII-cleaved fragments cloned in plasmids was used for the genomic mapping of the transcripts. HindIII-cleaved MCMV DNA, a representative set of HindIII clones, and the gel-purified HindIII-E fragment were arranged following the physical map (7) and separated on 0.8% agarose gels (Fig. 4a). DNA was transferred to nitrocellulose paper and hybridized to whole-cell RNA labeled in vivo with ^{32}P$_i$ for 4 h in the presence of 50 μg of cycloheximide per ml. Prominent immediate early transcription was found only from one region (Fig. 4b). This region of abundant transcriptional activity corresponds to the HindIII-K fragment (0.753 to 0.787 map units) and to the adjoining HindIII-L fragment (0.787 to 0.818 map units). There was only weak hybridization to the terminal HindIII-E fragment. To investigate the transcription from the small HindIII fragments M, N, O, and P, 5 μg of unlabeled RNA from different phases of infection was spotted on nitrocellulose paper and hybridized to ^{32}P-labeled cloned fragments. The HindIII-N fragment showed a weak but specific hybridization to immediate early RNA (Fig. 5). Only the HindIII-D fragment repeatedly hybridized to both immediate early RNA and RNA from noninfected cells to the same extent. This result raises the possibility of cross-hybridization of the HindIII-D DNA with cellular sequences, although labeled RNA from noninfected cells did not hybridize to any MCMV fragment. RNA from infected cells treated with cycloheximide 1 and 1.5 h p.i. also hybridized only to the HindIII-K, -L, and -E fragments (data not shown). cDNA prepared with oligodeoxythymidylic acid primer from the poly(A)$^+$ fraction of immediate early RNA hybridized to the HindIII-K, -L, and -E fragments. In contrast to whole-cell ^{32}P-labeled immediate early RNA, the cDNA hybridization to the HindIII-K fragment was prominent, whereas the HindIII-L fragment showed weak hybridization (Fig. 4c) and hybridization to the HindIII-E fragment was detectable only after very long exposure. Similar results were obtained when random primed cDNA from the poly(A)$^+$ fraction was used (Fig. 4d). This suggests that most of the poly(A)$^+$ immediate early
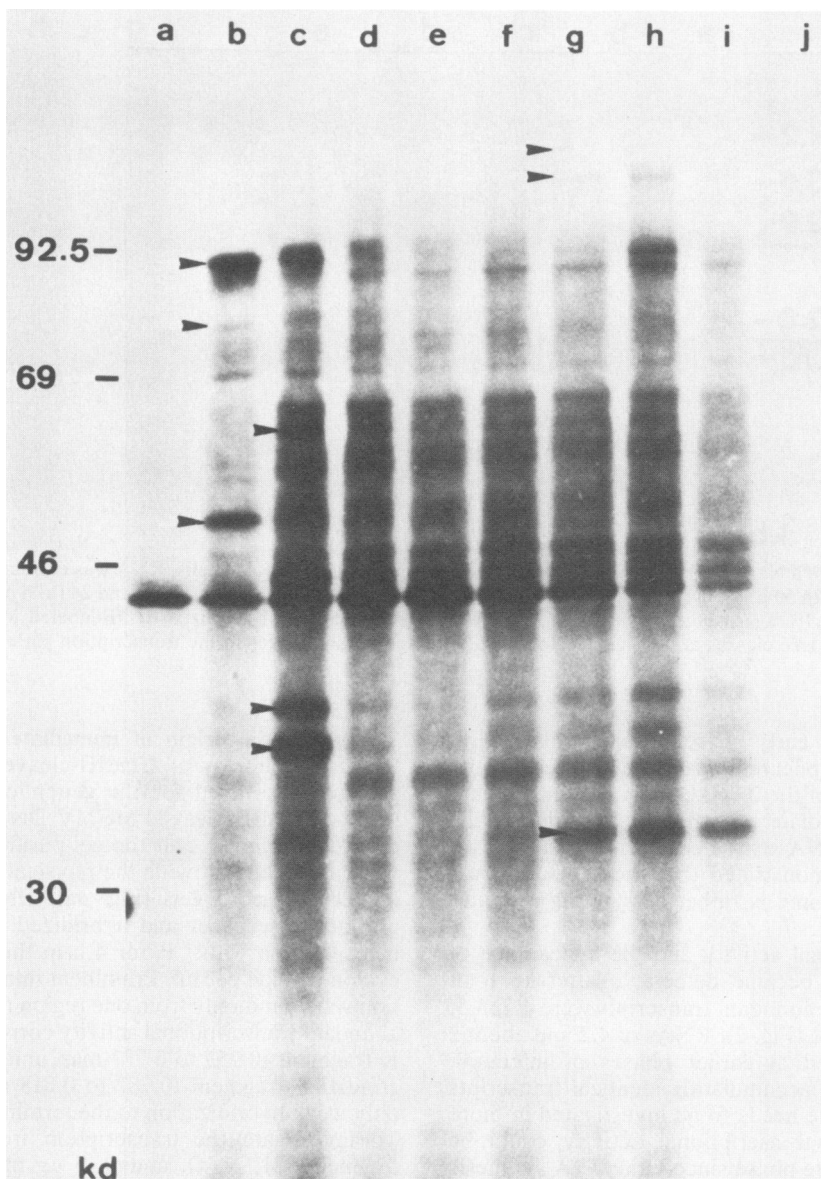
FIG. 3. Temporal pattern of in vitro translation products of RNA from productively infected cells. Fluorogram of in vitro translation products of the RNAs analyzed by Northern blotting in Fig. 1. Lanes: RNA from noninfected cells (a), immediate early RNA (b), RNA from cells blocked with cycloheximide at 2 (c), 4 (d), 8 (e), 12 (f), 16 (g), 20 (h), and 24 (i) h p.i.; no RNA added (j). The infection phase-specific appearance of in vitro translation products is indicated by arrowheads.

RNA molecules terminate within the HindIII-K fragment and that the majority of the transcripts from the HindIII-L fragment are not poly(A)$^+$.

Two hours of permissive infection resulted in a change from limited to extensive transcription of the MCMV genome (Fig. 6a). RNA from these cells hybridized to all HindIII fragments of MCMV DNA. These early RNAs hybridized strongly to HindIII fragments B, F, K, and J and relatively weakly to fragments A, H, D, C, and G. Hybridization to small fragments was seen after long exposure and also in the dot spot experiments (Fig. 5). Hybridization to fragments HindIII-O and HindIII-P was detectable only after very long exposure. RNAs labeled from 6 to 10 h or 12 to 16 h revealed the same transcription patterns (data not shown).

At late times after infection RNA transcription from all subgenomic fragments contained (Fig. 6b). Substantial tran-

scription originated from HindIII fragments B, M, H, D, C, G, and F. There was still transcription of RNA from the regions that code for immediate early RNA (HindIII-K and -L fragments). Whether the late transcription from some regions of the genome was dependent upon MCMV DNA synthesis was further investigated. The addition of PAA at 6 h p.i. in a concentration of 250 μg/ml blocks MCMV DNA synthesis completely (unpublished data), but did not cause major changes of the hybridization pattern of late RNA (data not shown). To detect minor changes of transcription due to the action of PAA, double and triple digests of the cloned fragments HindIII-A, -B, -C, -D, -F, -G, and -K were hybridized after transfer to nitrocellulose paper with late RNA labeled with ^{32}P$_i$ in the presence or absence of PAA. Late RNA synthesized in the presence of PAA lacked detectable transcription from some subfragments within

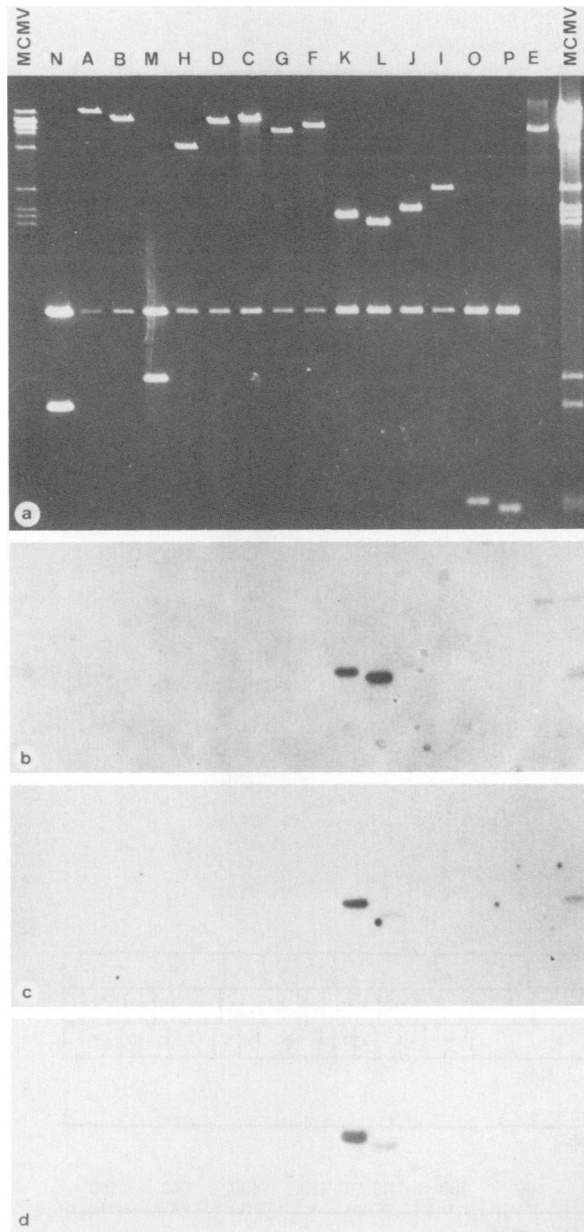FIG. 4. Southern blot of whole-cell immediate early RNA and immediate early cDNA hybridized to MCMV DNA fragments. MCMV HindIII fragments are arranged according to their map positions. MCMV DNA and recombinant plasmids were cleaved with HindIII and electrophoresed together with the gel-purified HindIII-E fragment on 0.8% agarose gels. Bands were visualized with ethidium bromide (a) and transferred to nitrocellulose filters. The gels were run under identical conditions. Amounts of DNA varied from 0.25 μg for large fragments to 1.5 μg for small fragments. Filters were exposed for autoradiography after hybridization to whole-cell ^{32}P-labeled immediate early RNA (b), to oligodeoxythymidylic acid-primed ^{32}P-labeled cDNA transcribed from poly(A)$^+$ immediate early RNA (c), and to random primed ^{32}P-labeled cDNA transcribed from poly(A)$^+$ immediate early RNA (d). Exposure times were 72 (b) and 16 (c, d) h.

HindIII-F (Fig. 7). Three of four fragments to which RNA from PAA-treated cells did not hybridize could be identified as the internal XbaI fragments T, U, and W of 2.0, 1.4, and 1.1 megadaltons (Md) in size (lanes b, f, and i) (7). Hybridiz-

ing fragments obtained after HindIII/XbaI/BamHI and HindIII/BamHI cleavage of about 1.4 and 1.1 Md in size were HindIII/BamHI digestion products derived from the right-hand HindIII/XbaI subfragment (Fig. 7, lanes c, g, and j and d, h, and k). In addition, a 1.6-Md BamHI cleavage product of the left-hand HindIII/XbaI subfragment, which is not precisely mapped so far, also showed a reduced hybridization (Fig. 7, lanes c, g, and j). These results prove the existence of PAA-sensitive transcripts. Their identification requires further analysis.

Size classes of immediate early RNA synthesized from the HindIII-K and -L fragments. Analysis of the RNA size classes encoded by the HindIII-K and -L fragments and the identification of RNA classes that contain poly(A)$^+$ RNA species was carried out by Northern blotting. Amounts, 5 μg each, of mock and immediate early whole-cell RNA, 5 μg of poly(A)$^-$ immediate early RNA, and 1.25 μg of poly(A)$^+$ immediate early RNA were separated on 1% agarose gels and transferred to nitrocellulose filters (Fig. 8). The filters were hybridized to ^{32}P-labeled cloned HindIII-K or -L fragments. The size classes detected by these clones were the same as those detected by total viral DNA. Thus, the transcription from the termini of the genome did not seem to add abundant RNA species different in size. The hybridization to cloned fragments was at least as sensitive as hybridization to MCMV DNA (Fig. 1), and additional size classes of immediate early RNA could be detected (Fig. 8). The K fragment hybridized to five classes within the poly(A)$^+$ fraction of the immediate early RNA of 5.1, 2.75, 2.0, 1.65, and 1.05 kb in size. The poly(A)$^-$ RNAs that hybridized to the HindIII-K fragment probably represent contaminations with poly(A)$^+$ immediate early RNA because the same size classes also hybridized to ^{32}P-labeled cDNA synthesized from poly(A)$^+$ immediate early RNA (data not shown). The HindIII-L fragment also hybridized to the 5.1- and 2.75-kb RNA and to an additional size class of 1.75 kb (Fig. 8). In addition, a set of poly(A)$^-$ RNA in the HindIII-L fragment
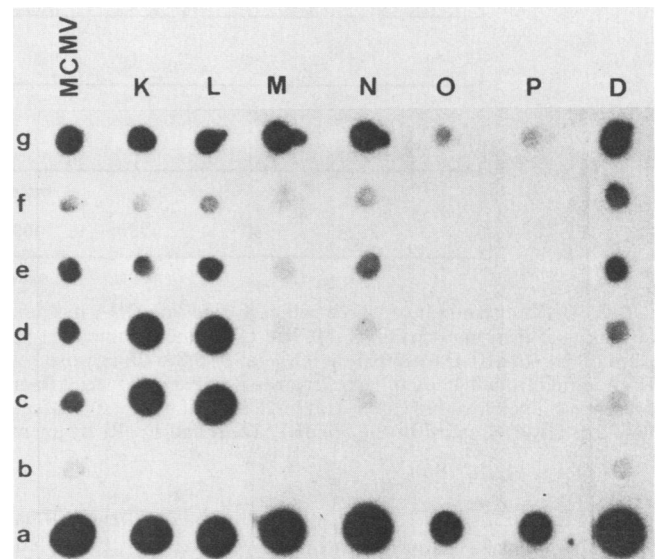

FIG. 5. Dot spot hybridization of ^{32}P-labeled MCMV DNA and cloned HindIII fragments to whole-cell RNA. Cells were incubated with cycloheximide for 4 h at 0 (c), 2 (d), 6 (e), 12 (f), and 20 (g) h p.i. A 5-μg portion of RNA was spotted on nitrocellulose filters. Controls were RNA (5 μg) from noninfected cells (b) and 100 ng of MCMV DNA (a).
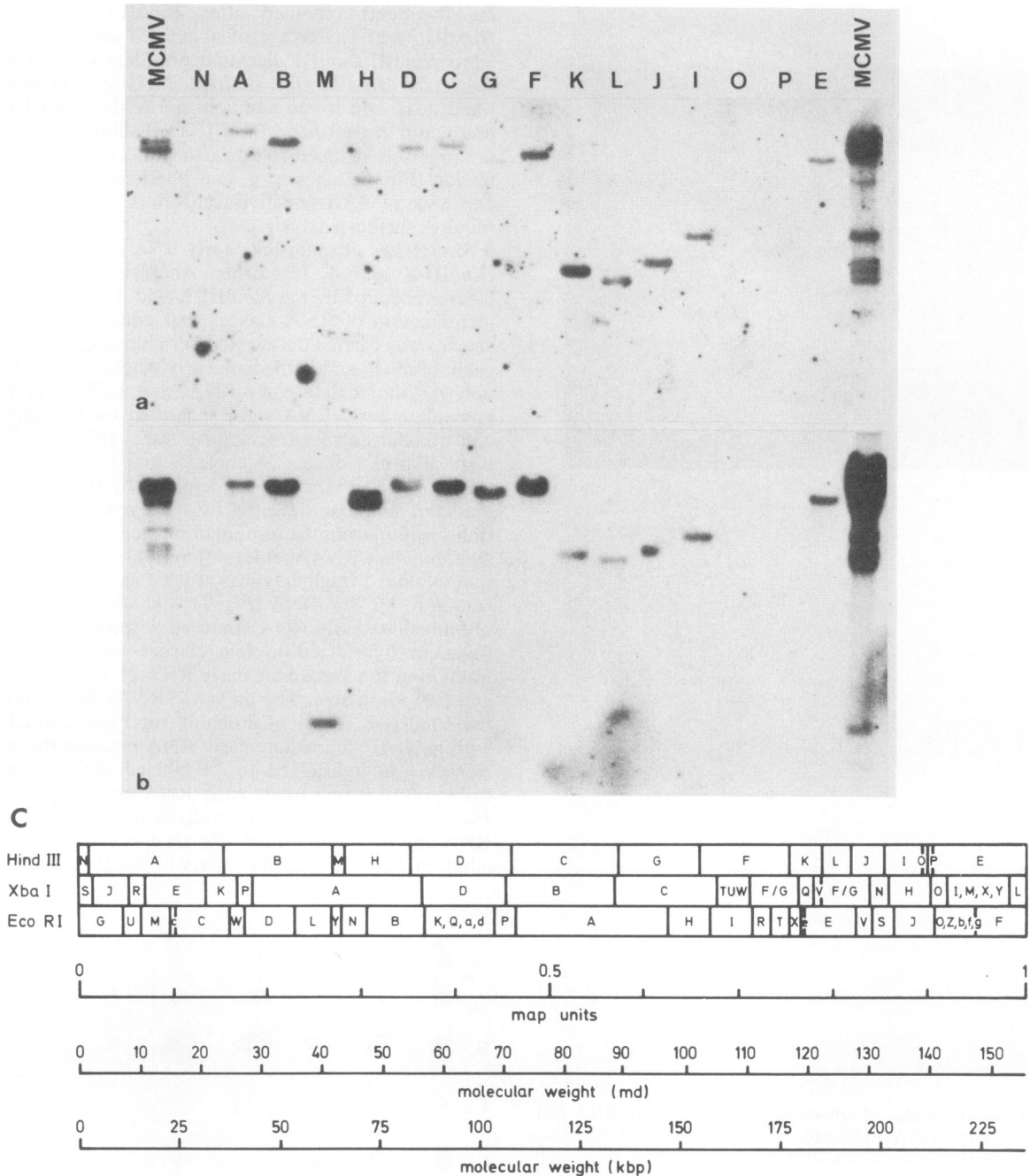
FIG. 6. Southern blot of whole-cell early and late RNA hybridized to MCMV DNA fragments. MCMV HindIII fragments are arranged according to their map positions. MCMV DNA and recombinant plasmids were cleaved with HindIII and electrophoresed together with the gel-purified HindIII-E fragment on 0.6% (a) or 0.8% (b) agarose gels. DNA fragments were transferred to nitrocellulose filters. Amounts of DNA varied from 0.25 μg of large fragments to 1.5 μg of small fragments. Whole-cell RNA from infected cells labeled with ^{32}P for 4 h in the presence of cycloheximide after 2 (a) or 20 (b) h of permissive infection was used for hybridization. Filters were exposed for autoradiography for 72 h. Physical maps for the HindIII, XbaI, and EcoRI fragments of MCMV DNA are given in (c).

ranging from 2.25 to <1.0 kb could be detected. These RNAs from the poly(A)⁻ fraction did not hybridize to ^{32}P-labeled cDNA synthesized from poly(A)⁺ immediate early RNA (data not shown) and therefore represent true transcripts.

Analysis of the region of abundant immediate early transcription. The cloned fragments HindIII-K and HindIII-L were digested with various combinations of enzymes, and hybridization to whole-cell immediate early RNA was car-

ried out. Within the cloned HindIII-K fragment (Fig. 9A; also Fig. 10) immediate early RNA hybridized to the K fragment after cleavage with HindIII (lane a) and to the internal XbaI fragments Q and V (7), as well as to the small right-hand HindIII/XbaI subfragment (not visible in lane b). Hybridization to this subfragment is shown in lane c, where the residual viral sequences that remained linked to the vector DNA after XbaI cleavage faintly hybridized. After
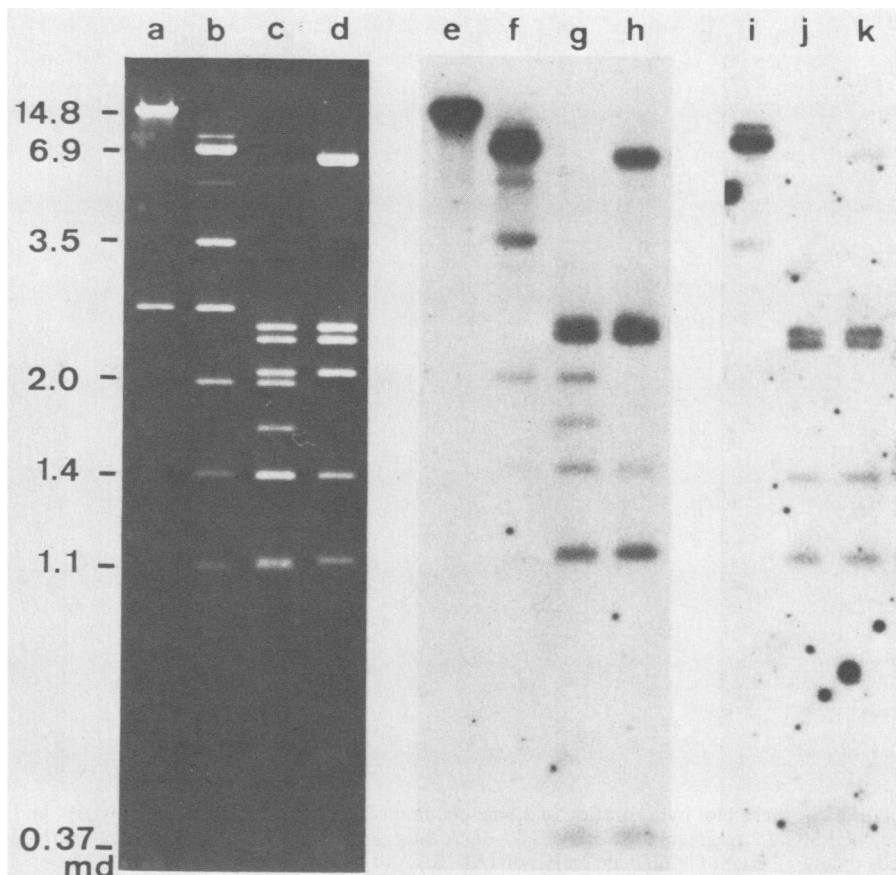
FIG. 7. Southern blot of whole-cell late RNA synthesized in the presence or absence of PAA hybridized to the MCMV HindIII-F fragment. The recombinant plasmid containing HindIII-F was fractionated in a 0.8% agarose gel after cleavage with HindIII, BamHI, and XbaI. After staining with ethidium bromide (a to d), fragments were transferred to nitrocellulose filters and hybridized with ^{32}P-labeled RNA from late (20 h) MCMV-infected cells (e to h) or with ^{32}P-labeled RNA from late (20 h) MCMV-infected cells in the presence of 250 μg of PAA per ml (i to k). The recombinant plasmid was digested with HindIII (a and e), HindIII/XbaI (b f, i), HindIII/XbaI/BamHI (c, g, j), and HindIII/BamHI (d, h, k). Exposure time was 72 h.

cleavage with HindIII/PstI (lane d) and PstI (lane e), hybridization was found to all three fragments, although the hybridization to the large left-hand fragment of 3.5 Md in size was only weak. Cleavage with HindIII/BamHI (lane f) and BamHI (lane g) revealed a singular hybridizing fragment of 2.85 Md in size which corresponds to map units (0.769 to 0.787. Map position 0.769 therefore marks the left-hand limit of the region that codes for immediate early RNA.

Within the recombinant plasmid containing HindIII-L (Fig. 9B; also Fig. 10) hybridization of immediate early RNA to the total L fragment (lane a) was found, and after HindIII/BamHI (lane b) and BamHI cleavage (lane c) hybridization to the large left-hand HindIII/BamHI subfragment and faintly to the small internal BamHI fragment of 0.33 Md in size was seen. The hybridization of the latter fragment indicated the right-hand end of immediate early RNA transcription at map position 0.817. However, the possile hybridization to the right-hand 0.15-Md HindIII/BamHI subfragment was not determined. Therefore, further digestions of the HindIII-L clone with HindIII/PstI (lane d), PstI (lane e), HindIII/PstI/BamHI (lane f), and PstI/BamHI (lane g) were carried out. It is shown in lane d that all MCMV fragments generated after HindIII/PstI cleavage hybridized whereas the two vector fragments did not. PstI cleavage gave six fragments which all showed hybridization. After triple cleavage (lane f) hybrid-

ization of the 0.33-Md internal BamHI fragment was only weak in comparison with that of the 0.44-Md left-hand HindIII/PstI subfragment. In lane g the larger of the two vector fragments shown in lane d is increased in size by the 0.15-Md HindIII/BamHI right-hand subfragment but still does not hybridize with immediate early RNA, confirming the limit of immediate early transcription at map position 0.817 as indicated above. In conclusion, the region of abundant immediate early RNA transcription spans from map unit 0.769 to 0.817. Almost the complete region is included with a BamHI fragment of 7.17 Md in size. This fragment was cloned in vector pACYC184 and the resulting plasmid clone pAMB25 was obtained. The results are given in detail in the map (Fig. 10).

DISCUSSION

The data presented in this paper are part of an attempt to elucidate the mechanisms of MCMV latency. After molecular cloning and mapping of the MCMV genome (7), we have used the plasmid clones to analyze the transcriptional activity during the different phases of viral replication and to locate the transcripts of the immediate early phase.

The analysis of the temporal pattern of transcription, translation, and viral DNA synthesis provides clear evidence
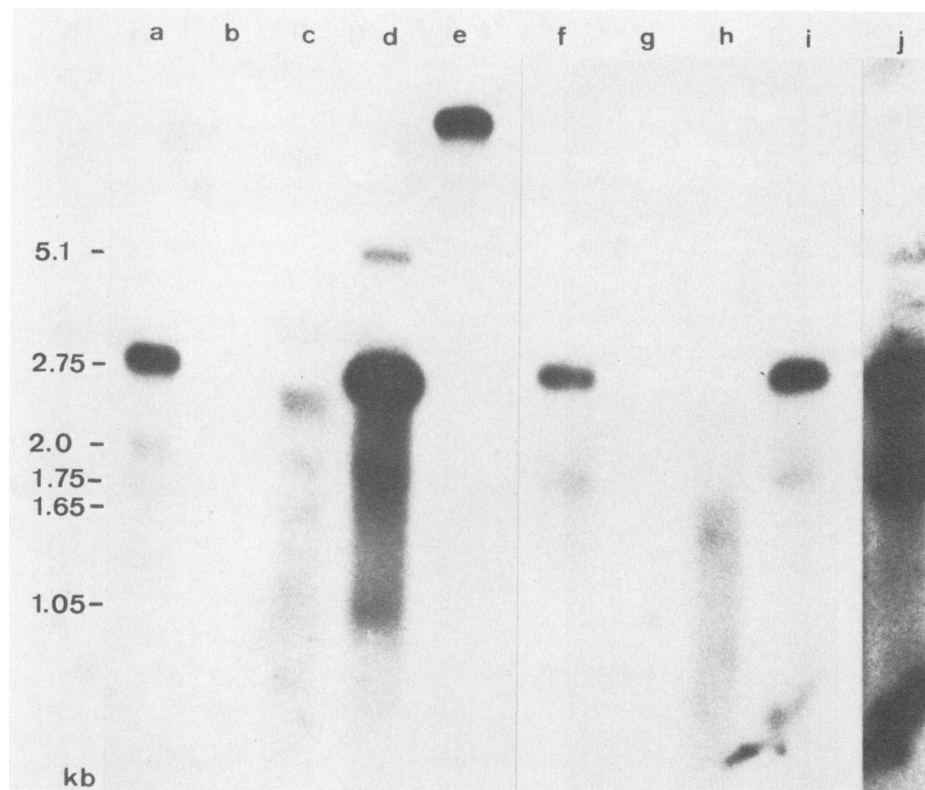
FIG. 8. Autoradiograph of a Northern blot hybridization of whole-cell immediate early RNA and the poly(A)$^+$ and poly(A)$^-$ fractions of immediate early RNA to cloned MCMV fragments HindIII-K and -L. A 5-μg portion of whole-cell immediate early RNA (a and f), 5 μg of RNA from noninfected cells (b and g), 5 μg of immediate early poly(A)$^-$ RNA (c and h), 1.25 μg of immediate early poly(A)$^+$ RNA (d and i), and 100 ng of MCMV DNA (e) were fractionated in 1% agarose gels. After transfer to nitrocellulose paper the filters were hybridized to ^{32}P-labeled HindIII-K (a to e) and -L (f to j). Exposure times were 3 days for lanes a to i. In lane j the hybridization shown in lane i was exposed for 10 days. The sizes of viral RNAs are indicated in kilobases.

that gene expression in MCMV is regulated and that the transcription program consists of three phases.

In the first phase viral transcription occurs in the absence of de novo protein synthesis, whereas the switch from the immediate early to the early phase requires viral protein synthesis. Under permissive conditions the immediate early phase is rather short and early proteins can be translated from RNA after 2 h of permissive infection. Chantler and Hudson (3) have described a 4-h period of immediate early translation. This discrepancy may be explained by the fact that in their report no early proteins could be detected at all and that elevated salt concentrations were required to visualize early proteins in subsequent studies (2). Our results are consistent with their observations with regard to the decline of immediate early protein translation after 4 h p.i. and the low translaional activity of viral RNA during the early phase. We could show this effect also on the level of transcription. However, the early phase should not be defined as a lag phase (3) since it is more appropriately characterized by a continuous decline of transcriptional and translational activity.

The switch from early to late transcription and translation at about 16 h p.i. is correlated with the beginning of viral DNA synthesis. The starting point of DNA synthesis is in agreement with some (20) but not all (19) other estimations. These differences may be explained by different cell culture conditions which can considerably influence the kinetics of MCMV propagation (22).

There has been no unequivocal answer to the question of whether MCMV influences cellular DNA synthesis (11, 20). Our data provide evidence that in growing cells MCMV blocks the incorporation of radiolabeled nucleotides into cellular DNA. Treatment of infected cells with cycloheximide did not delay the inhibition of cellular DNA synthesis, which could indicate that this inhibition is induced by a structural component of the infecting virus as has been suggested before (20). The same treatment, however, delayed both the start of viral DNA synthesis and release of infectious virus, which reveals that the shutoff of cellular DNA synthesis and the initiation of viral DNA synthesis are regulated independently.

The mapping studies of viral RNA accumulating in infected cells revealed the following: in the immediate early phase there is transcription from only two distinct regions of the genome. These are represented by the neighboring HindIII fragments K and L and the terminal HindIII fragments N and E which are joined after circularization of the genome (17). The transcription from HindIII-K and -L is abundant whereas that from fragments E and N is small. The switch from immediate early to early transcription is accompanied by a change from limited to extensive transcription. Early transcripts arise from all HindIII fragments of the MCMV genome, and the most abundant transcription can be located to HindIII fragments B, F, K, I, and J. In the late phase the transcription from all fragments continues. In addition to the accumulation of stable transcripts from the early phase, new
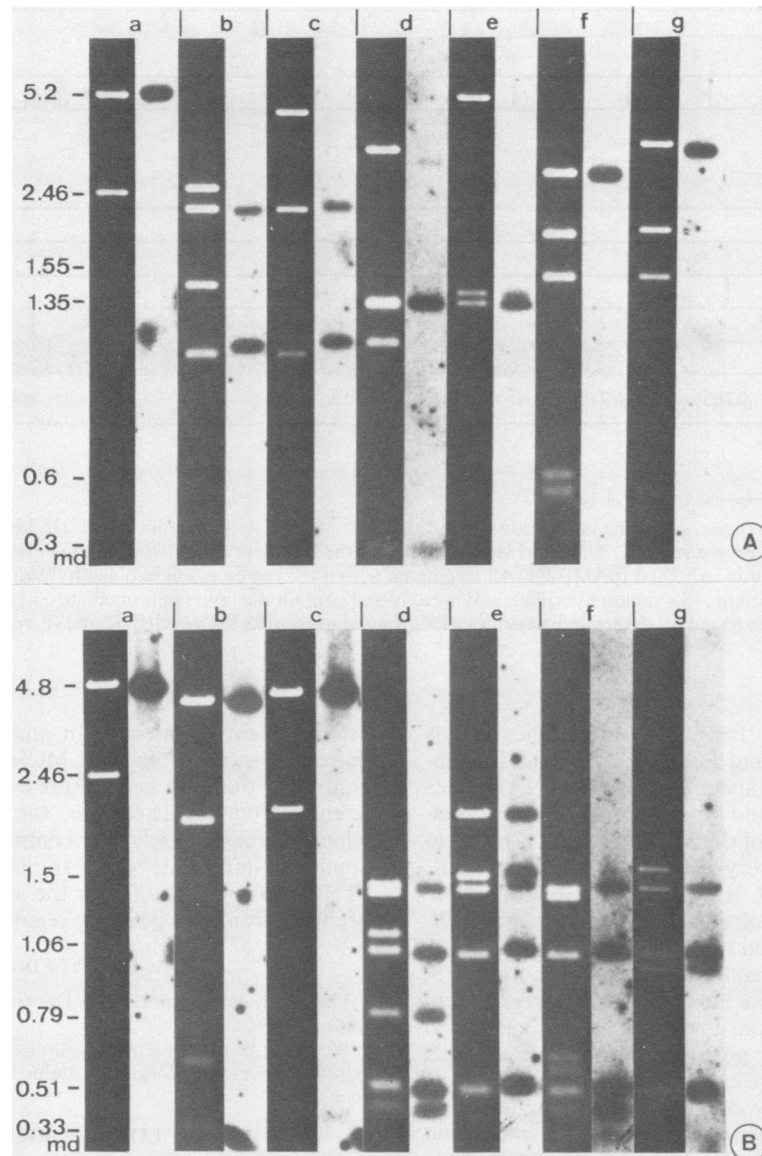
FIG. 9. Southern blot of ^{32}P-labeled whole-cell immediate early RNA hybridized to cloned fragments HindIII-K and HindIII-L of MCMV. The recombinant plasmids were digested with different combinations of the enzymes HindIII/XbaI, PstI, and BamHI and fractionated by gel electrophoresis. DNA bands were visualized by ethidium bromide staining and transferred to nitrocellulose filters. After hybridization with ^{32}P-labeled whole-cell immediate early RNA, autoradiographs were exposed for 3 days. DNA bands stained by ethidium bromide and autoradiograms of individual digests were mounted in parallel for comparison. Due to slight size alteration of the nitrocellulose filters, the DNA bands visualized by ethidium bromide and by autoradiography are not always at identical positions. (A) Hybridization to digests of the HindIII-K clone: cleavage with HindIII (a), HindIII/XbaI (b), XbaI (c), HindIII/Pst-I double cleavage (d), PstI (e), HindIII/BamHI (f), and BamHI (g). (B) Hybridization to digests of the HindIII-L clone: cleavage with HindIII (a), HindIII/BamHI (b), BamHI (c), HindIII/PstI (d), PstI (e), HindIII/PstI/BamHI (f), PstI/BamHI (g).

late transcripts arise. HindIII fragments B, C, D, F, G, H, and M are the regions of most abundant transcription at the peak of transcription during the late phase at 20 to 24 h p.i. There was no major difference in transcription from individual HindIII fragments in the presence or absence of PAA. Only by a more detailed restriction enzyme mapping could PAA-sensitive RNAs be detected that are transcribed within the HindIII-F fragment.

Whether the abundant immediate early transcripts arise from a discontinuous stretch of DNA or whether a cluster of RNAs is transcribed from a limited region within the two fragments HindIII-K and -L was investigated in detail. The

size of the tested individual fragments from this region ranged from 0.33 to 1.495 Md. It was found that immediate early RNA hybridized to all fragments between map units 0.769 and 0.817. Thus, a continuous stretch of DNA gives rise to the bulk of immediate early RNAs which cluster between map units 0.769 and 0.817. Preliminary data show that several immediate early transcripts originate from this region. Within the poly(A)$^+$ RNA fraction only one RNA of 1.75 kb in size is entirely transcribed within HindIII-L, and it terminates between map units 0.815 and 0.817.

Cleavage experiments revealed that the less abundant species of RNA hybridizing to fragment HindIII-E is proba-
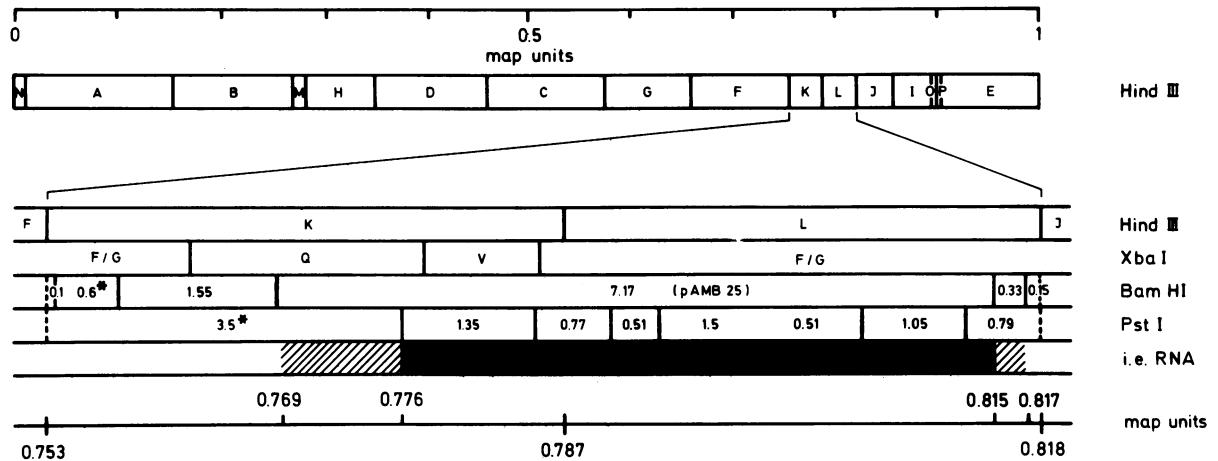
* = sizes of Bam HI and Pst I fragments are given in md

FIG. 10. Physical map of the region encoding immediate early RNA. The MCMV genome consists of 235-kb unique sequences of double-stranded DNA without terminal repeat regions. The XbaI letters represent the fragment designation of the entire XbaI physical map (7). The cloned 7.17-Md BamHI fragment is indicated (pAMB25). All fragments within the region given in black hybridize with whole-cell immediate early RNA. The shaded areas flanking this region hybridize only weakly and contain the limits of immediate early transcription. The map units of the immediate early region are based on determining various DNA fragments within the HindIII-K and -L region, using several DNA size standards.

bly encoded mainly by the far end, namely, the regions represented by the XbaI-L and EcoRI-F fragments (unpublished data). It is tempting to speculate that the RNA species encoded by the HindIII-N and -E fragments are only transcribed after circularization of the MCMV DNA (17). Circularization seems to occur very early after infection (J. R. Marks, J. A. Mercer, and D. H. Spector, personal communication). The sum of the molecular weights of the immediate early RNA species derived from map units 0.769 to 0.817 is higher than the coding capacity for asymmetric transcription of this region. This indicates, that symmetrical transcription or transcription from the same DNA region with different reading frames or posttranscriptional processing may be involved in immediate early transcription of MCMV.

That MCMV DNA simply consists of one unique region without isomeric forms and lacks terminal or internal repeat regions or both (7, 17) complicates direct comparisons between the transcription programs of MCMV and HCMV. However, in HCMV strains Towne and Davies (5, 28) the abundant immediate early transcription is initiated in the long unique segment between map positions 0.660 and 0.770. An analogous region was found for HCMV strain AD169, although the published map represents the orientation of the U_L segment in the other isomeric form and therefore other map positions of abundant immediate early transcription (13, 16). These map positions of abundant immediate early transcription are similar to those of MCMV. In addition, in HCMV there is also low immediate early transcription from both termini (16, 27). At early and late times after infection transcription occurs from all segments of the MCMV and HCMV genome (5, 16, 28), and in both viruses some distinct regions are extensively transcribed. All of these similarities raise the questions of whether they represent functional homologies between the two viruses and whether the functions encoded by the repeat regions in HCMV are present in MCMV.

It has been hypothesized by Stinski et al. (25) that the predominant immediate early protein(s) of HCMV regulates later events in viral transcription and may play a role in the establishment of latency. In analogy, one or more of the immediate early proteins of MCMV may be involved in the control of the viral gene expression during productive and latent infections. Therefore, the pAMB25 plasmid clone, which contains nearly the complete sequences coding for abundant immediate early transcription was constructed. This clone will be useful for the analysis of the function and products from this genomic region during latent infection.

ACKNOWLEDGMENTS

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