Predominant Immediate-Early Transcripts of Human Cytomegalovirus AD 169

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Transcription of the human cytomegalovirus genome (strain AD 169) was investigated at the immediate-early (IE) time after infection, by using cycloheximide to suppress virus-specific protein synthesis. In total cell RNA, four predominant IE transcripts were found which were encoded by one contiguous region of the long unique segment between map units 0.06 and 0.16 in prototype arrangement of human cytomegalovirus AD 169 DNA. Analysis by Northern blot hybridizations demonstrated that the transcripts possessed a size of 1.9, 2.2, 2.3, and 5.0 kilobases, respectively. Coding sequences and directions of transcriptions were mapped by Northern blots and hybridizations with oligodeoxythymidylic acid-primed and randomly primed cDNA. The 1.9-, 2.2-, and 2.3-kilobase RNAs were found in the polyadenylated fraction of IE RNA exclusively; in contrast, a part of the 5.0-kilobase RNA appeared polyadenylated, although the majority of the same transcript was found in the nonpolyadenylated pool. Also, different from the other IE genes, the DNA coding for the 5.0-kilobase IE RNA was transcribed in high quantities during the late phase of virus replication, suggesting an exemption from the temporal regulation of herpesvirus transcription.

Transcription and translation of rapidly replicating herpesviruses such as herpes simplex and pseudorabies viruses are coordinately regulated. Initial expression of immediate-early (IE) genes is a prerequisite for initiation of the second early phase of replication. This is followed by the start of viral DNA replication and a third late phase of virion polypeptide synthesis (3, 8, 19). Likewise, human cytomegalovirus (HCMV), which has been placed into a separate taxonomic subgroup of herpesviruses (29), has a similar sequential order of transcription (41). During a short initial period of replication, virus-coded IE proteins are readily detected by immunofluorescence (16, 24) or gel electrophoresis after immunoprecipitation (23). In the IE phase, abundant transcription is restricted to a limited number of viral genes (9, 21, 40).

The genome of HCMV is a linear duplex DNA molecule of about 235 kilobase pairs (kbp), which is significantly larger than the DNA of all other herpesviruses characterized before. The molecules contain two pairs of inverted repeats which subdivide the DNA into a long (L) segment of about 195 kbp and a short (S) segment of about 40 kbp. Each pair of inverted repeats borders a unique stretch of DNA designated U_L and U_S, respectively. Because both the L and S segments can occur in either orientation, DNA molecules of CMV particles are found in four isomeric conformations that are present in about equal proportions (15, 20). Several workers have defined the abundantly transcribed IE regions by hybridizing IE mRNA with restriction fragments of virion DNA. Wathen and Stinski found that the *Xba*I E and N fragments of DNA from HCMV Towne (map units 0.66 to 0.77) code for two size classes of IE mRNA (4.8 and ~2.0 kilobases (kb), respectively) (40). Recently, the same work-

ers described the map locations for three IE transcripts of strain Towne (34). Also, De Marchi found the coding sequences of the major IE transcripts between map units 0.686 and 0.733 of HCMV Davis (9); this genomic area appeared to code for a highly abundant 2.2-kb transcript and a second, less-abundant RNA species of 4.8 kb (10). McDonough and Spector (21) placed the dominant IE region into the corresponding genomic area. This is the section between map units 0.061 and 0.142 on the DNA molecule of HCMV AD 169, as the prototype arrangement of the U_L region generally adopted for HCMV AD 169 (15, 26, 31, 43) is in the opposite orientation with respect to the maps drawn for the HCMV Davis (9) and HCMV Towne (20, 38). Thus, it is generally agreed upon that the dominant IE DNA sequences of HCMV are located in the part of the U_L region which is mostly represented by the *Hind*III E fragment of strain AD 169 or in the equivalent genomic section of other HCMV strains.

In a recent study, it was found that NIH 3T3 cells and primary rat fibroblasts can be transformed by transfection with a cloned *Hind*III-*Xba*I fragment of 2.9 kbp (pCM4000) which represents the rightmost portion of restriction fragment *Hind*III-E (25). Since the location of both the abundantly transcribed IE region and the transforming DNA is within the same restriction fragment (*Hind*III-E), we investigated the IE transcription patterns of this genomic section in more detail. This study describes the coding region, the direction of transcription, and the relative abundancies of the dominant IE transcripts that we found to be encoded by the respective genomic area of HCMV AD 169.

MATERIALS AND METHODS

Virus and cell culture. HCMV AD 169 was provided by U. Krech, St. Gallen, Switzerland. Propagation of virus on human foreskin fibroblasts (HFF) followed standard methods (11, 33, 35). HFF cells were infected at 80% confluence with 1 to 5 PFU per cell. For isolation of IE RNA, the cells were infected and maintained in the presence of 100 µg of cycloheximide per ml and were harvested 12 to 14 h postin-

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FIG. 1. Physical map of the *Hind*III E fragment of HCMV AD 169. Only the cleavage sites of the clones and subclones used are shown. The transforming sequence (pCM4000) is indicated by the slanting lines on the right of cosmid clone pJN201. Brackets indicate DNA fragments cloned in plasmid pACYC 184, pA 10, or pHC 79.

fection. For isolation of late RNA, HFF cells were harvested approximately 90 h postinfection at 60 to 80% cytopathic effect. RNA from uninfected cells (mock RNA) was extracted from confluent HFF cells.

Isolation of RNA. The procedures for the isolation and purification of RNA from whole cells infected with HCMV AD 169 were modified from published procedures (6, 41). All buffers and glassware were autoclaved before use, and the first steps in preparation were carried out on ice. Cells were washed free of media with phosphate-buffered saline, collected by low-speed centrifugation at 1,500 rpm for 5 min, and washed once more with ice-cold phosphate-buffered saline. Cell pellets (about 10^9 cells) were lysed in 6 ml of 5.8 M guanidine hydrochloride (Sigma Chemical Co., St. Louis, Mo.), containing 50 mM lithium citrate, 0.1 M β -mercaptoethanol, and 0.5% Sarkosyl (pH 6.5). The cell lysate was centrifuged through a 4-ml cushion of 5.7 M CsCl, containing 0.1 M EDTA (pH 7.0) in a SW 41 cellulose nitrate tube for approximately 20 h at 28,000 rpm and 20°C (SW 41 rotor; Spinco). The supernatant was carefully removed. The pellet was dried and suspended in 0.6 ml of 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), and 20 mM Tris-hydrochloride (pH 7.4), and was precipitated after adding 0.1 volume of 2 M NaCl with 2.5 volumes of ethanol at -20°C. The precipitated RNA was centrifuged in a SS 34 rotor (Ivan Sorvall, Inc., Norwalk, Conn.) at 10,000 rpm for 1 h at -10°C. The pellet was washed in 70% ethanol and digested with 500 μ l of proteinase K (Boehringer, Mannheim, Federal Republic of Germany) (100 μ g per ml), 10 mM Tris (pH 7.4), 10 mM NaCl, 1 mM EDTA, and 0.5% SDS. The digest was extracted twice with 100 μ l of phenol-chloroform-isoamyl alcohol (25:24:1) and once more with chloroform-isoamyl alcohol (24:1) and was then precipitated with ethanol.

Polyadenylated [poly(A)⁺] RNA was selected from total RNA by three cycles of oligodeoxythymidylic acid [oligo(dT)]-cellulose chromatography (Bethesda Research Laboratories) (2), according to the directions of the manufacturer. To test the integrity and biological activity of the IE RNAs, all fractions of RNA {whole-cell RNA, poly(A)⁺ RNA, and nonpolyadenylated [poly(A)⁻] RNA} were translated in vitro with a rabbit reticulocyte lysate system (Amersham Corp., Braunschweig, Federal Republic of Germany) by the method of Pelham and Jackson (27). Polypeptides obtained by in vitro translation were separated by electrophoresis in 15% SDS-polyacrylamide gels as previously described (13, 32).

Isolation, purification, and labeling of recombinant DNA. Subfragments of a cosmid-cloned HCMV gene library of AD 169 DNA (15, 25) were cloned in plasmid vectors pACYC 184 (5), pA 10 (kindly provided by P. Gruss), or pHC 79 (18). Procedures for extraction of plasmid DNA, construction of recombinant DNAs, and transformation of *Escherichia coli* HB101 with recombinant DNA were performed as previously described (17). Each viral DNA insert was identified after endonuclease digestion by electrophoretic comigration with digests of cosmid-cloned viral DNA and verified by Southern blot hybridizations (30) to different DNA fragments of virion DNA. Recombinant DNA, purified from amplified bacterial clones, was labeled by nick translation to high specific activity (1×10^8 cpm/ μ g of DNA) with [α -³²P]dATP (800 Ci/mmol; New England Nuclear Corp., Boston, Mass.) following the procedure of Rigby et al. (28).

Gel electrophoresis of denatured RNA and Northern blot hybridization. Purified RNA, denatured for 15 min at 56°C in the presence of 1 M glyoxal (22), was separated on horizontal gels of 1% agarose containing 2.2 M formaldehyde and 20

mM morpholinepropanesulfonic acid-electrophoresis buffer, 5 mM sodium acetate, and 1 mM EDTA at 40 V for 15 h. Molecular weight standards were 23S (3.3 kb) and 16S (1.7 kb) *E. coli* rRNA (Boehringer) and 28S (5.3 kb) and 18S (2.0 kb) human cell rRNA (1, 4). After electrophoresis, gels were treated with 50 mM NaOH for 1 h and neutralized in 0.5 M Tris-hydrochloride (pH 6.5) for 30 min. The RNA was transferred to nitrocellulose filters (SS 85, Schleicher & Schuell, Inc., Keene, N.H.) with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), fixed to the filters by heat (5 h at 80°C), and prehybridized for 5 h at 65°C (5× SSC, 5× Denhardt solution [12], and 100 µg of yeast RNA per ml) (37). The hybridization solution contained 50% formamide (purified with Amberlite before use), 5× SSC, 0.1% SDS, 1× Denhardt solution, 20 mM sodium phosphate (pH 7.0), and 100 µg of yeast RNA per ml. Nick-repair-labeled DNA was hybridized to RNA blots for 48 h at 44 to 46°C at about 4×10^5 cpm/cm² of filter in a volume of 200 µl/cm². The filters were washed several times after hybridization with buffer containing 20 mM sodium phosphate, 0.1% SDS, and decreasing concentrations of SSC (2× SSC, 1× SSC, and 0.1× SSC) for 3 h at 62 to 65°C. The dried filters were autoradiographed with Kodak X-OMAT AR and S films.

Synthesis of cDNA and Southern blot hybridization. DNA complementary to RNA (cDNA) was synthesized with avian

FIG. 2. Autoradiograms of Northern blot hybridizations of a cloned fragment of HCMV DNA (*Hind*III-E, pJN201) to poly(A)⁺ and poly(A)⁻ fractions of HCMV IE and late RNA. Total IE RNA was extracted at 14 h postinfection in the presence of 100 µg of cycloheximide per ml, and then it was denatured, separated by agarose gel electrophoresis, and transferred onto nitrocellulose filters. Cosmid clone pJN201 (*Hind*III E fragment) was labeled in vitro with [α -³²P]dATP and hybridized to Northern blots of RNA from 20 µg of mock RNA (lane 1), 2.5 µg of poly(A)⁺ IE RNA (lane 2), 2.5 µg of poly(A)⁻ IE RNA (lane 3), 20 µg of mock RNA (lane 4), 2.5 µg of poly(A)⁺ late RNA (lane 5), and 2.5 µg of poly(A)⁻ late RNA (lane 6).

FIG. 3. Autoradiograms of Northern blot hybridizations of ³²P-labeled cloned viral DNA fragments to HCMV IE RNA. Hybridization of ³²P-labeled clone pGJ7 with 20 µg of mock RNA (lane 1), 2.5 µg of poly(A)⁺ IE RNA (lane 2), and 2.5 µg of poly(A)⁻ IE RNA (lane 3). Hybridization of ³²P-labeled clone pGJ6 with 20 µg of mock RNA (lane 4), 2.5 µg of poly(A)⁺ IE RNA (lane 5), and 2.5 µg of poly(A)⁻ IE RNA (lane 6). Hybridization of ³²P-labeled clone pGJ5 with 20 µg of mock RNA (lane 7), 2.5 µg of poly(A)⁺ IE RNA (lane 8), and 2.5 µg of poly(A)⁻ IE RNA (lane 9).

myeloblastosis virus reverse transcriptase (J. W. Beard, Life Sciences, Inc.) as previously described (1, 7, 39). The reaction mixture (100 µl) contained 5 µg of RNA; 0.4 mM each of dCTP, dGTP, and dTTP; 0.04 mM dATP; 150 µCi of [α -³²P]dATP (800 Ci/mmol; New England Nuclear Corp.); 5 µM oligo(dT) (Boehringer); or 250 µg of random primer (36) per ml, 50 mM Tris-hydrochloride (pH 8.3), 140 mM KCl, 10 mM MgCl₂, 10 mM dithiothreitol, and 300 U of reverse transcriptase per ml. The mixture was incubated for 1 h at 37°C. The reaction was stopped by adding EDTA to a final concentration of 20 mM. The DNA was extracted with an equal volume of phenol and, after the addition of salmon sperm DNA (final concentration, 1 mg/ml), the nucleic acids were precipitated with 2.5 volumes of ethanol. After centrifugation, the pellet was suspended in 0.3 M NaOH and 1 mM EDTA and incubated for 20 min at 65°C to hydrolyze the RNA. The reaction was chilled on ice and diluted with hybridization buffer. Viral DNA fragments were separated on 0.6% horizontal agarose gels and transferred to nitrocellulose filters (30). The filters were prehybridized in solution containing 0.1% Ficoll and 0.1% polyvinylpyrrolidone (Sigma) for 5 h at 65°C. The hybridization solution contained 50% formamide, 5× SSC, 0.1% SDS, 20 mM sodium phosphate, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 50 µg of polyadenylic acid (Sigma) per ml, and the ³²P-labeled cDNA. Hybridization was carried out at 42°C for 16 h. Washing procedures and autoradiography were carried out as described for Northern blots.

FIG. 4. Autoradiograms of Northern blot hybridizations of ^{32}P -labeled cloned viral DNA fragments to HCMV IE RNA. Hybridization of ^{32}P -labeled clone pGJ8 with 20 μg of mock RNA (lane 1), 2.5 μg of poly(A)⁺ IE RNA (lane 2), and 2.5 μg of poly(A)⁻ IE RNA (lane 3). Hybridizations of ^{32}P -labeled clone pGJ3 with 20 μg of mock RNA (lane 4), 2.5 μg of poly(A)⁺ IE RNA (lane 5), and 2.5 μg of poly(A)⁻ IE RNA (lane 6). Hybridization of ^{32}P -labeled clone pCM4000 with 20 μg of mock RNA (lane 7), 2.5 μg of poly(A)⁺ IE RNA (lane 8), and 2.5 μg of poly(A)⁻ IE RNA (lane 9). Hybridization of ^{32}P -labeled clone pGJ10 with 20 μg of mock RNA (lane 10), 2.5 μg of poly(A)⁺ IE RNA (lane 11), and 2.5 μg of poly(A)⁻ IE RNA (lane 12).

RESULTS

Localization of DNA coding for the predominant IE transcripts. For detailed RNA mapping, subfragments of cosmid clone pCM 1058 (15) were cloned in plasmid vectors by conventional cloning procedures. Fragments generated by restriction endonucleases *Hind*III or *Eco*RI were cloned in cosmid vector pHC 79; fragments obtained by double cleavage with *Xba*I-*Eco*RI and *Sall*-*Eco*RI were inserted between the respective cleavage sites of plasmid pA 10, and restriction fragments by *Bam*HI, *Hind*III-*Xba*I, and *Bam*HI-*Xba*I were cloned in vector pACYC 184 (Fig. 1). The identity of each clone was confirmed by Southern blot hybridization with a number of appropriate cloned DNA fragments.

Northern blot hybridizations of the labeled clone pJN201 (*Hind*III E fragment) with total poly(A)⁺ IE RNA revealed two classes of abundant transcripts of approximately 2.0 and 5.0 kb, respectively. In addition, two minor transcripts of 7.0 and 4.0 kb were observed during this phase of transcription (Fig. 2, lane 2). To localize the exact coding region of these transcripts, further hybridizations were performed with subclones of pJN201. Clone pGJ7, representing the leftmost 6.8 kb of the *Hind*III E fragment, appeared to code for a single abundant poly(A)⁺ RNA of 1.9 kb (Fig. 3, lane 2). The adjacent clone, pGJ6 (5.2 kb), also hybridized weakly with a transcript of identical size, suggesting that the coding sequence for the 1.9-kb poly(A)⁺ RNA possibly extends a short distance into pGJ6 (Fig. 3, lane 5). A second abundant poly(A)⁺ transcript of 2.3 kb was also detected when pGJ6

was used as a hybridization probe. This RNA also hybridized very weakly with clone pGJ5 (Fig. 3, lane 8), suggesting that the position of the coding sequence for the 2.3-kb RNA exists at the left end of pGJ6. The two minor transcripts (7.0 and 4.0 kb) appeared in about equal proportions after hybridization with clones pGJ6 and pGJ5 (Fig. 3, lanes 5 and 8) and with clone pGHS4 (data not shown). A third strong poly(A)⁺ transcript of 2.2 kb appeared in Northern blots with radioactive probes from clones pGJ8 (Fig. 4, lane 2) and pGJ3 (Fig. 4, lane 5) and clones pGHS2 and pGHS6 (data not shown). The 2.2-kb IE RNA was also detected if the central *Pst*I-*Bgl*II fragment (1.0 kb) was excised from clone pGJ3 and hybridized to poly(A)⁺ IE RNA (data not shown).

The 5.0-kb IE RNA appeared as an abundant transcript when subclones pGJ3, pCM4000, and pGJ10 were used as probes with poly(A)⁺ RNA (Fig. 4, lanes 5, 8, and 11). The internal *Pst*I-*Bgl*II fragment isolated from pGJ3 did hybridize weakly with the 5.0-kb RNA, indicating that the left end of the coding region for the 5.0-kb IE RNA extends into the *Pst*I-*Bgl*II subfragment (data not shown). A transcript of about the same size appeared in Northern blots if the poly(A)⁻ fraction of IE RNA was hybridized with the same labeled cloned probes (Fig. 4, lanes 6, 9, and 12). A higher level of hybridization was usually seen with poly(A)⁻ RNA if equal amounts of poly(A)⁺ or poly(A)⁻ IE RNA were used. Since most of the total RNA appears in the poly(A)⁻ fraction, this may indicate that the majority of 5.0-kb RNA synthesized during the IE phase of virus replication is not poly(A)⁺. This is in clear contrast to the other IE transcripts described above which are strictly confined to the poly(A)⁺ fraction (Fig. 2, lane 2).

Direction of transcription. The orientations of the IE transcripts were determined by comparing the hybridization patterns of oligo(dT)-primed and randomly primed cDNA (39). With the conditions of these experiments (high salt), the average size of cDNA corresponds to approximately 500 base pairs, and hardly any transcript exceeds 1 kb, as was shown by agarose gel electrophoresis (19a). Since oligo(dT)-primed cDNA is synthesized mainly from the 3' end of poly(A)⁺ RNA molecules, short cDNA preferentially hybridizes with 3' coding DNA sequences, and hybridization with the 5' coding sequences would be weak or negative. Figure 5A shows the Southern blot hybridization of ^{32}P -labeled, oligo(dT)-primed cDNA synthesized from IE RNA [poly(A)⁺] to DNA fragments covering the coding sequence for the 5.0-kb RNA. The cDNA hybridized clearly with clones pGHS6, pGJ10, and pCM4000; however, subclones pGJ1 and pGJ3 hybridized weakly, if at all. The experiment suggests that the transcription termination site of the poly(A)⁺ 5.0-kb IE RNA is located in the insert of clone pGJ10. This was confirmed by hybridization of randomly primed cDNA from the same IE RNA; Fig 5B, lane 4, shows strong hybridization of the randomly primed cDNA to clone pGJ3. Oligo(dT)-primed cDNA synthesized from the 5.0-kb transcript was found to hybridize more strongly with pCM4000 than expected from the putative position of the 3' end. This phenomenon could be due to hybridization of primer to oligo(dT) clusters within the known DNA sequence of pCM4000 (25a). In analogy, the directions of the other dominant IE transcripts were determined by hybridization with oligo(dT)-primed and randomly primed cDNA. Figure 5A shows strong hybridization of oligo(dT)-primed cDNA to clone pGHS2 (lane 2) but no or weak hybridization with clone pGJ3 (lane 6). The randomly primed cDNA hybridized strongly with both clones (Fig. 5B, lanes 2 and 4). This indicates that the 3' end of the 2.2-kb transcript is in

FIG. 5. (A) Autoradiogram of Southern blot hybridization of ^{32}P -labeled oligo(dT)-primed cDNA, synthesized from poly(A)⁺ IE RNA, to cloned fragments of HCMV DNA. Fragments of *Eco*RI-digested HCMV DNA, clone pGHS4 (lane 1); fragments of *Eco*RI-digested HCMV DNA, clone pGHS2 (lane 2); fragments of *Xba*I- and *Hind*III-digested HCMV DNA, clone pGJ11 (lane 3); fragments of *Xba*I- and *Hind*III-digested HCMV DNA, clone pGJ10 (lane 4); fragments of *Xba*I- and *Hind*III-digested HCMV DNA, clone pCM4000 (lane 5); fragments of *Xba*I- and *Sal*I-digested (only partially cleaved) HCMV DNA, clone pGJ2 (lane 7); fragments of *Xba*I and *Eco*RI-digested HCMV DNA, clone pGJ1 (lane 8); fragments of *Bam*HI-digested HCMV DNA, clone pGHS6 (lane 9); fragments of *Kpn*I-digested *Herpesvirus saimiri* DNA, *Kpn* d/e fragments (lane 10). Arrows show the positions where the viral insert of PGJ1 and PGJ3 had to be expected. (B) Autoradiogram of Southern blot hybridization of ^{32}P -labeled, randomly primed cDNA, synthesized from poly(A)⁺ IE RNA, to cloned fragments of HCMV DNA. DNA fragments of clone pGHS4 (lane 1); DNA fragments of clone pGHS2 (lane 2); DNA fragments of clone pGJ8 (lane 3); DNA fragments of clone pGJ3 (lane 4); DNA fragments of *H. saimiri*, according to A above (lane 5); DNA fragments of clone pCM4000 (lane 6). Cleavage with restriction enzymes *Xba*I and *Hind*III. Fragments of *Hpa*II-digested HCMV DNA of clone pCM4000 (lane 7); fragments of *Hind*III- and *Xba*I-digested HCMV DNA of clone pGJ10 (lane 8); fragments of *Bam*HI- and *Xba*I-digested HCMV DNA of clone pGJ10 (lane 9); and fragments of *Bam*HI-, *Xba*I-, and *Hind*III-digested HCMV DNA of clone pGJ10. Hybridization with ^{32}P -labeled cDNA was carried out at 42°C with 50% formamide for 16 h. Sizes of DNA fragments (viral insert or vector) were determined from internal markers (fragments of *Hind*III-digested λ DNA).

clone pGHS2. The direction of the 2.3-kb RNA was determined by hybridization of ^{32}P -labeled cDNA with clones pGJ6 and pGJ5. Figure 6 shows that the oligo(dT)-primed cDNA hybridized strongly with clone pGJ7 and pGJ6, however, not with clone pGJ5 (lane 3); randomly primed cDNA hybridized with all three clones. Thus, the direction of the 2.3-kb RNA is from right to left. cDNA hybridization indicated that the coding region for the 1.9-kb transcript spans from the interior of a 2.1-kb *Pst*I fragment (pCM 5027) (data not shown) to the right border of pGJ7, probably extending into pGJ6 (see above). A more detailed analysis of promoter and terminator sequences will be reported elsewhere.

Expression of IE genes during late time after infection. The

mapping of IE genes by Northern blot hybridization has shown that the *Hind*III E fragment of HCMV AD 169 is coding for four predominant transcripts (1.9-, 2.2-, 2.3-, and 5.0-kb RNA) and two additional scarce RNA species (4.0 and 7.0 kb) (Fig. 7). If virus replication proceeded for 90 h, no transcripts from the *Hind*III E fragment could be identified except for one class of RNA corresponding to 5.0 kb (Fig. 2, lanes 5 and 6). If equal amounts (2.5 μg) of poly(A)⁺ and poly(A)⁻ late RNA were transferred to nitrocellulose filters in parallel, the poly(A)⁻ fraction would appear to contain more of the 5.0-kb RNA than did the poly(A)⁺ fraction. Hybridization with subclones pGJ3, pCM4000, and pGJ10 (Fig. 8) indicated that map positions of the coding sequence for the late 5.0-kb RNA are identical with the

FIG. 6. Autoradiograms of Southern blot hybridizations of cDNA, synthesized from poly(A)⁺ IE RNA, to cloned fragments of HCMV DNA. Hybridization of ³²P-labeled oligo(dT)-primed cDNA with fragments of *Bam*HI-digested HCMV DNA, clone pGJ7 (lane 1); fragments of *Bam*HI-digested HCMV DNA, clone pGJ6 (lane 2); and fragments of *Sal*I-digested HCMV DNA, clone pGJ5 (lane 3). Hybridization of ³²P-labeled, randomly primed cDNA with fragments of *Bam*HI-digested HCMV DNA, clone pGJ7 (lane 4); fragments of *Bam*HI-digested HCMV DNA, clone pGJ6 (lane 5); and fragments of *Sal*I-digested HCMV DNA, clone pGJ5 (lane 6).

corresponding IE gene, as far as can be determined by Northern blot hybridizations (Fig. 8).

DISCUSSION

This study has shown that four dominant viral transcripts (1.9-, 2.2-, 2.3-, and 5.0-kb RNA) are synthesized from one genomic area during the IE phase of virus replication in HCMV AD 169-infected cells. All of these RNA molecules are encoded by one segment of DNA which extends between map units 0.06 and 0.15 of the HCMV genome and is mostly comprised within the *Hind*III E fragment. In addition, two scarce classes of RNA (7.0 and 4.0 kb) are specified by DNA from the same region. If total IE RNA were fractionated by oligo(dT)-cellulose chromatography, all of these IE RNA species would elute with the poly(A)⁺ fraction. However, unlike the other IE RNAs of this region, a high proportion of the 5.0-kb IE transcript was found in the poly(A)⁻ fraction as well. The 5.0-kb transcripts are also distinct from the other IE RNAs by the expression during the late phase of viral replication. A separate study from our laboratories showed by hybridization of DNA cloned in bacteriophage M 13 that the direction of transcription for the poly(A)⁺ and poly(A)⁻ fractions of 5.0-kb RNA (IE and late) is identical, confirming the orientation by cDNA hybridizations (Nelson et al., submitted for publication). These observations suggest that the 5.0-kb RNAs are continuously formed during all phases of productive infection; it represents an exemption from the general rule of temporal transcription regulation in herpesvirus replication.

McDonough and Spector (21) have determined the genomic regions of HCMV AD 169 which are transcribed at IE times by dot blot hybridizations and by hybridizing ³H-labeled IE RNA with cloned *Eco*RI fragments of virion DNA. Our data are consistent with their results, confining

CMV AD 169

FIG. 7. Summary of the map positions of HCMV DNA coding for IE RNAs and direction of transcription. Brackets indicate some of the cloned HCMV DNA fragments used for Northern blot hybridizations (IE RNAs) and Southern blot hybridizations (cDNA synthesized from IE RNA).

FIG. 8. Autoradiograms of Northern blot hybridization of ^{32}P -labeled cloned viral DNA fragments to HCMV late RNA. Hybridization of ^{32}P -labeled clone pGJ3 with 2.5 μg of poly(A) $^{+}$ late RNA (lane 1), 2.5 μg of poly(A) $^{-}$ late RNA (lane 2), and 20 μg of mock RNA (lane 3). Hybridization of ^{32}P -labeled clone pCM4000 with 2.5 μg of poly(A) $^{+}$ late RNA (lane 4), 2.5 μg of poly(A) $^{-}$ late RNA (lane 5), and 20 μg of mock RNA (lane 6). Hybridization of ^{32}P -labeled clone pGJ10 with 20 μg of mock RNA (lane 7), 2.5 μg of poly(A) $^{+}$ late RNA (lane 8), and 2.5 μg of poly(A) $^{-}$ late RNA (lane 9).

the dominant IE genes to a single contiguous region of U_L between map units 0.06 and 0.015.

The cleavage maps of HCMV AD 169 and strain Towne show similarity in the IE regions, allowing a comparison of their transcription programs. Stinski and colleagues have identified the IE genes of HCMV Towne coding for two size classes of IE RNAs of about 2 and 4.8 kb (40, 41) and have recently determined the map locations of three different IE transcripts (34). The same workers have mapped the promoters and 3' ends of two transcripts (1.95 and 2.25 kb) which apparently are equivalent to our 1.9- and 2.3-kb RNAs. The direction of the 1.95- and 2.25-kb IE RNAs of HCMV Towne were found to be convergent (34), and there was evidence for RNA splicing in the formation of the 1.95-kb RNA. We mapped the positions of the four dominant IE transcripts of HCMV AD 169 by Northern blot and cDNA hybridizations to short labeled virion DNA clones and isolated restriction fragments. It appeared that the 5.0-kb IE transcript of AD 169 is equivalent to the 4.8-kb RNA of strain Towne; the 1.9- and 2.3-kb IE RNAs of strain AD 169 seemed to correspond to the 1.95- and 2.3-kb RNAs of Towne. The IE RNA of 2.3 kb in strain AD 169 appeared as one size class, whereas the Towne strain made five size classes ranging from 2.3 to 1.1 kb (34). This may be due to differences in splicing patterns between the two virus strains. With the exception of the 1.9-kb transcript, there appeared little, if any, space for sizeable splicing during the formation of the other dominant IE RNAs of strain AD 169. Transcription analysis of IE RNA by means of single-strand-specific nucleases will give the definite answer.

De Marchi (10) has analyzed IE transcription of HCMV

Davis by a series of Northern blots using cloned *EcoRI* and *HindIII* fragments. Based on her studies, the dominant IE genes of HCMV AD 169 and strain Davis are comparable. The organization of IE genes may be similar in strains AD 169 and Davis, in spite of possible profound differences in the sequence arrangement of other parts of the viral genomes (9). However, De Marchi described a single size class of 2.0-kb RNA (10), whereas we found three transcripts of 1.9, 2.2, and 2.3 kb, respectively. This discrepancy may be due to strain variability or, more likely, to the fact that cloned DNA probes which are significantly larger than the transcripts cannot discriminate small RNAs of similar sizes. De Marchi observed that a 4.8-kb RNA is confined to the nuclei of infected cells, and transcription of the respective DNA is carried on from IE to late times after infection (10). This is in good agreement with our data on the continuous synthesis of the 5.0-kb RNA [(poly(A) $^{+}$ and poly(A) $^{-}$] in HCMV AD 169.

HCMV and herpes simplex virus are similar in their overall genome structure, in spite of largely different genome sizes. Both viruses possess two stretches of unique (U_L and U_S) DNA, with each stretch framed by two pairs of inverted repeats and oriented in both directions. Thus, the striking difference in the map positions of IE genes between HCMV and herpes simplex virus is remarkable. By far, most IE transcription of HCMV occurs within one area of less than 20 kbp in the U_L region. In contrast, herpes simplex virus IE transcripts primarily originate from the inverted repeats of the S segments and from adjacent U_S sequences (1, 8, 42); also, IE transcription of pseudorabies virus appears to be restricted to inverted repeats (14). The DNA of herpesviruses may change in evolution with extension and contraction of certain structural domains, preserving the gross DNA sequence arrangement, whereas at the same time, the genomes are profoundly reorganized with respect to the detailed functional scheme of essential viral genes. This is another striking example of the pronounced molecular diversity within the herpesvirus group.

Cloned fragments of HCMV AD 169 are capable of transforming rodent cells in vitro (25). Recent experiments have shown that the transforming activity is confined to a small stretch of DNA (≈ 600 base pairs) which is unable to code for a sizeable polypeptide (Nelson et al., submitted for publication). Since this sequence is a subset of clone pCM4000, it is totally comprised within the DNA coding for the 5.0-kb RNA. This raises the question as to whether transformation by HCMV is in any way related to IE transcription or whether it is a function of an IE gene product.

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