Cell-Related Sequences in the DNA Genome of Human Cytomegalovirus Strain AD169

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Human cytomegalovirus (HCMV) cloned *Eco*RI fragments R and b hybridized strongly, under standard high-stringency conditions, to uninfected cellular DNA of human, murine, or sea urchin origin. Less hybridization was detected with fragments A, C, E, WL(F), WN(H), I, M, O, P, Q, V, c, d, and e. Southern blot analysis of the HCMV-related human DNA localized the major sites of hybridization of HCMV *Eco*RI fragments R, b, and d to defined regions of the 28S rRNA gene.

Human cytomegalovirus (HCMV) is of considerable importance in medicine as the leading viral cause of birth defects and as an opportunistic pathogen in immunosuppressed individuals (for a review see reference 9). Like other members of the herpesvirus group, HCMV is able to establish latent and persistent infections and may have oncogenic potential (for reviews see references 15 and 21).

The recognition of HCMV as an important human pathogen has made it imperative to develop a means of rapid diagnosis and to study the mechanisms of viral replication at a molecular level. Nucleic acid hybridization provides a sensitive and useful technique for these studies. To provide the reagents for such studies, Spector and co-workers have cloned *Eco*RI fragments representative of the entire genome of HCMV strain AD169 and determined their order on the linear genome (20, 23). Before these cloned HCMV DNA fragments could be used as sensitive hybridization reagents, it was necessary to identify any regions of homology between the HCMV genome and uninfected cell DNA. In this paper, we have identified the HCMV strain AD169 *Eco*RI fragments that hybridize to uninfected cell DNA and have characterized the human DNA to which HCMV hybridizes under standard conditions of high-stringency hybridization.

To determine rapidly whether the recombinant HCMV DNA clones share homology with uninfected cell DNA, we spotted samples of uninfected human placenta, mouse, sea urchin, and phage lambda DNA onto nitrocellulose filters by the procedure of Thomas (24), with several modifications. The DNA samples were denatured by boiling for 10 min and adjusted to a salt concentration of 1 M NaCl before being spotted. The filters were prehybridized at 37°C for 12 to 16 h in buffer containing 50% (vol/vol) formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50 mM NaH₂PO₄ (pH 6.5), 5× Denhardt solution, 4 mM EDTA, 0.2% sodium dodecyl sulfate, and 250 µg of salmon sperm DNA per ml. The filters were hybridized in buffer containing 50% (vol/vol) formamide, 5× SSC, 50 mM NaH₂PO₄ (pH 6.5), 1× Denhardt solution, 1 mM EDTA, 0.05% sodium dodecyl sulfate, 100 µg of salmon sperm DNA per ml, 10% (wt/vol) dextran sulfate, and 8 × 10⁶ cpm of individual, ³²P-labeled cloned HCMV DNA fragments per ml purified as described previously (23). The cloned DNA fragments were

labeled with [α-³²P]dCTP by nick translation to a specific activity of 2 × 10⁸ to 5 × 10⁸ cpm/µg (17). To standardize the experiments for variation in complexity and specific activity of the labeled fragments, we also spotted various quantities of purified HCMV virion DNA (1, 0.1, and 0.01 ng) onto each filter. After hybridization the filters were washed under the high-stringency conditions described previously (22) and subjected to autoradiography. Hybridization to uninfected cell DNA was assessed as positive if the intensity of the cellular DNA spot on the autoradiogram was equal to or greater than that for the 0.01-ng virion DNA spot. Representative autoradiograms are shown in Fig. 1A, and the data are summarized in Fig. 1B. The cloned HCMV *Eco*RI fragments R and b showed the most intense hybridization to DNA from uninfected cells, whereas less hybridization was detected with HCMV *Eco*RI fragments I and V. Hybridization to uninfected cell DNA was also detected with HCMV *Eco*RI fragments A, C, E, WL(F), WN(H), M, O, P, Q, c, d, and e, but the hybridization approached the limits of the sensitivity of our assay and was not detected in every experiment.

Modification of the hybridization conditions altered the extent of hybridization of some of the HCMV *Eco*RI fragments to cellular DNA. For example, when the formamide concentration was lowered from 50 to 40% (low stringency) the extent of hybridization of many of the fragments increased. A representative example of this is shown for HCMV fragments d and P (Fig. 1A).

The sequences to which HCMV DNA hybridized were characterized by Southern blot analysis. Human placenta DNA was cleaved with *Eco*RI or *Eco*RI plus *Sst*I and subjected to electrophoresis through 0.8% agarose gels (23). In all cases, the extent of digestion was monitored by adding a small portion of the digestion reaction to a tube containing a suitable test DNA. The separated fragments were transferred to nitrocellulose filters by the method of Southern (19) and incubated at 37°C for 14 to 18 h in prehybridization buffer containing 50% (vol/vol) formamide, 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.0), 5× Denhardt solution, 3× SSC, 5 mM EDTA, 100 µg of denatured salmon sperm DNA per ml, and 0.1% sodium dodecyl sulfate. The filters were then hybridized with individual ³²P-labeled cloned DNA fragments for 18 to 24 h in prehybridization buffer containing 10% (wt/vol) dextran sulfate and 1 × 10⁶ to 2 × 10⁶ cpm/ml of the radiolabeled probe. Only HCMV *Eco*RI fragments R, b, and d reproducibly hybridized to specific fragments of human

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FIG. 1. Identification of HCMV *EcoRI* fragments that hybridize to uninfected cell DNA. (A) Dot blots were prepared that contained purified HCMV virion DNA (0.01, 0.1, and 1.0 ng), 5 μ g of lambda DNA, and 5 μ g each of uninfected mouse, human, and sea urchin DNA. Each filter was hybridized with the 32 P-labeled HCMV *EcoRI* fragment indicated at the left of the filter in high-stringency or low-stringency buffer (indicated by an asterisk) as described in the text. The filters were dried and subjected to autoradiography in the presence of Dupont Cronex Hi-Plus intensifying screens at -70°C for 19 h. (B) Diagram of *EcoRI* restriction endonuclease sites of HCMV strain AD169 in the prototype orientation (20). Fragments which show hybridization to uninfected cell DNA are indicated by a bar. The *EcoRI* L-S junction fragment WN has also been designated fragment H. In the inverted orientation of the genome, the *EcoRI* L-S junction fragment would be WL (also designated fragment F). The slanting lines indicate the position of the inverted repeats bounding the long and short segments of the genome.

DNA (Fig. 2). The predominant *EcoRI* band detected by each of these HCMV fragments was 7.3 kilobase pairs (kbp) in length. In addition, HCMV *EcoRI* fragment b hybridized to multiple additional *EcoRI* fragments ranging in size from 2.5 to 15 kbp, to which fragment R also hybridized, albeit

less strongly. Very faint hybridization to a 7.3-kbp *EcoRI* fragment of human DNA was also detected with HCMV *EcoRI* fragment V, and minor bands with long exposures of the autoradiograms were seen in some experiments after hybridization with HCMV *EcoRI* fragment I (data not shown). No significant hybridization to specific bands of *EcoRI*-cleaved human DNA was detected when the other cloned *EcoRI* fragments of HCMV DNA were used as hybridization probes. The same results were obtained with human DNA isolated from other organs and other individuals (data not shown).

Regions of fragments R and b that hybridized to human DNA were localized by hybridization of the indicated subfragments to the filters (Fig. 2). *Bgl*II divides HCMV *EcoRI* fragment R into two subfragments, of 3.65 and 2.25 kbp. The 3.65-kbp subfragment contains the oncogenic transforming region defined by Nelson et al. (11). Only the 2.25-kbp subfragment, however, hybridized to the uninfected cell DNA. Hybridization of a 0.43-kbp *Pst*I-*Bam*HI subfragment within this 2.25-kbp fragment of R to a Southern blot revealed the same pattern of hybridization as that seen with the entire R fragment. In similar experiments, a 375-base-pair *Hae*III subfragment within HCMV *EcoRI* fragment b appeared to hybridize to the same cellular fragments that were detected by the entire fragment b.

Spector and Vacquier previously reported that seven *EcoRI* fragments of HCMV strain AD169 (C, WL, WN, I, P, R, and b) shared limited homology with the 5' coding exon of *v-myc*, the oncogene of the retrovirus MC-29 (22). In subsequent experiments the region of *v-myc*-related sequences in R and b has been localized to the same subfragments which hybridize to uninfected human DNA (16). To determine whether the HCMV-related human sequences were also related to *v-myc* or human *c-myc*, Southern blots containing human placenta DNA cleaved with *EcoRI*, *Bam*HI, *Sst*I, *Hind*II, and *Pvu*II were hybridized with HCMV *EcoRI* fragments R and b, with the 0.5-kbp *Pst*I-*Sal*I subfragment of *v-myc* (1, 25) that shared homology with HCMV, and with the corresponding 1.5-kbp *Sst*I subfragment of the human *c-myc* gene (4). The results of these experiments (Fig. 3) showed that HCMV fragments R and b and *v-myc* hybridized to many of the same human bands, including a 7.3-kbp *EcoRI* fragment, a 5.4-kbp *Bam*HI fragment, a 5.7-kbp *EcoRI* plus *Sst*I fragment, an 11-kbp *Sst*I fragment, a 3.4-kbp *Hind*II plus *EcoRI* fragment, 12-kbp and 5.4-to-5.8-kbp *Pvu*II fragments, and a 6.1-kbp *EcoRI* plus *Pvu*II fragment. HCMV fragments R and b also detected a 0.5-kbp fragment in the *Bam*HI digest, a 2.7-kbp fragment in the *Sst*I and *Sst*I plus *EcoRI* digests, and a 2.7-kbp fragment in the *Pvu*II and *Pvu*II plus *EcoRI* digests. None of the major human bands detected by HCMV fragments R and b corresponded to bands detected by the human *c-myc* probe. The *v-myc* probe hybridized faintly to the fragments detected by the human *c-myc* subfragment. This low level of hybridization of *v-myc* to the human *c-myc* gene under the given conditions was not surprising in view of the significant divergence of the nucleotide sequence of this region of *v-myc* from that of the corresponding region in the human *c-myc* gene (2).

The results of the above experiments indicated that although the pattern of hybridization to human DNA of *v-myc* was similar to that of HCMV fragments R and b, the HCMV fragments were not detecting bands detected by the human *c-myc* gene. In a separate study, Rasmussen et al. determined that the regions in HCMV fragments R and b that cross-hybridize with *v-myc* contain local regions which are G+C rich and share limited sequence homology with regions

FIG. 2. Hybridization of ^{32}P -labeled cloned HCMV DNA fragments to Southern blots of restriction endonuclease-cleaved human DNA. Southern blots containing 0.5 ng of *Eco*RI-cleaved HCMV virion DNA (lane 1) and 5 μg of human placenta DNA cleaved with *Eco*RI alone (lane 2) or with *Eco*RI plus *Sst*I (lane 3) were prepared, hybridized with ^{32}P -labeled cloned HCMV *Eco*RI fragments d, R, or b or with the subfragments of R and b indicated below each panel, washed, and subjected to autoradiography as described in the text. The restriction endonuclease maps of the fragments used for the hybridization are shown below the autoradiograms. Restriction sites, indicated by letters, are as follows: E, *Eco*RI; Bg, *Bgl*II; P, *Pst*I; Ba, *Bam*HI; H, *Hae*III. Not all of the *Hae*III sites in *Eco*RI fragment b are shown. The numbers indicate the lengths of fragments in kilobase pairs.

in *v-myc* which are also G+C rich (16). These observations suggested that at least some of the HCMV-related human DNA might also correspond to G+C-rich sequences. One possibility, easily tested, was that *v-myc* and HCMV fragments R and b were hybridizing to the human ribosomal locus, a G+C-rich sequence present at high copy number. This possibility was further strengthened by the observation that some of the major human restriction endonuclease fragments detected by HCMV fragments R and b were similar in length to those predicted from the restriction endonuclease maps of the human 28S rRNA gene (10, 26). To test this possibility directly, a Southern blot of restriction endonuclease-cleaved human DNA identical to those described above was hybridized with a 7.3-kbp *Eco*RI fragment (designated rDNA A) that contains approximately 0.2 kbp of the 3' coding sequences of the 18S rRNA, the sequences encoding the 5.8S rRNA, most of the 28S rRNA, and the internal transcribed spacer region (26). The results indicated that one of the major sites of hybridization of HCMV fragments R and b and *v-myc* to human DNA was located within the 28S rRNA gene. From the pattern of hybridization, the region of cross-hybridizing sequences was localized to a 1.2-kbp region bounded by a *Hind*II site and a *Bam*HI site near the 5' end of the coding segment of the 28S rRNA gene (shown schematically at the bottom of Fig. 3).

The sizes of the major *Eco*RI and *Hind*II fragments of human DNA detected by fragment d (Fig. 3) were similar to those of the fragments detected by fragments R and b. However, in the other digests HCMV fragment d hybridized to fragments different from the major ones to which R and b

hybridized, including an 8-kbp *Bam*HI fragment, a 1.6-kbp *Sst*I and *Sst*I plus *Eco*RI fragment, a 2.7-kbp *Pvu*II fragment, and a 1.2-kbp *Eco*RI plus *Pvu*II fragment. A comparison of the hybridization patterns of HCMV fragment d and the rDNA probe indicated that the bands detected by fragment d were a subset of those detected by the rDNA probe. These sequences were localized to a 200-base-pair region of the 28S rRNA gene bounded by *Hind*II and *Pvu*II sites.

By hybridization of the 0.5-kbp *Pst*I-*Sal*I subfragment of *v-myc* and HCMV *Eco*RI fragments R, b, and d individually to Southern blots of restriction enzyme digests of the cloned 18S and 28S rRNA genes (data not shown), we confirmed that these four fragments were detecting portions of the 28S rRNA gene. Additional confirmation was provided by hybridization of the above four fragments to Northern blots (8) of nonpolyadenylated RNA prepared as described previously (7) from uninfected human fibroblasts (Fig. 4). The *v-myc* subfragment and HCMV fragments R, b, and d each detected the 28S rRNA species but not the 18S rRNA. *Eco*RI fragment S served as a negative control and failed to hybridize to the rRNA.

In summary, we have used both dot blot and Southern blot analysis to identify and characterize cross-hybridizing sequences between the HCMV DNA genome and uninfected cell DNA. By dot blot analysis, we found that HCMV *Eco*RI fragments R and b showed the most extensive hybridization to DNA from uninfected human, mouse, and sea urchin cells. Less hybridization was detected with the cloned HCMV fragments I and V, and limited hybridization was observed with HCMV *Eco*RI fragments A, C, E, WL, WN,

FIG. 3. Comparison of HCMV-related human DNA to *myc*-related human DNA and ribosomal human DNA. Southern blots containing 5 μ g of human placenta DNA cleaved with the restriction endonucleases indicated above each lane were prepared and hybridized with 32 P-labeled HCMV *Eco*RI fragments R, b, or d, with the 0.5-kbp *Pst*I-*Sal*I subfragment of *v-myc* (*v-myc*_{0.5}), with the 1.5-kbp *Sst*I subfragment of the human *c-myc* gene (*c-myc*_{1.5}) or with the 7.3-kbp *Eco*RI fragment of the human ribosomal locus (rDNA A). The filters were washed and subjected to autoradiography as described in the text. The filter which was initially hybridized with HCMV *Eco*RI fragment R was dehybridized before hybridization with *v-myc*_{0.5}. The filter which was hybridized to *c-myc*_{1.5} had been previously hybridized with HCMV *Eco*RI fragment d and dehybridized. The filter hybridized with the rDNA A probe contained 0.1 ng of *Eco*RI-cleaved rDNA A recombinant plasmid DNA in the left-hand lane. All other filters contained 0.5 ng of *Eco*RI-cleaved HCMV virion DNA in the left-hand lane. The regions of the human 28S rRNA gene to which HCMV *Eco*RI fragments R, b, and d hybridized are illustrated at the bottom. Restriction sites indicated by letters are as follows: E, *Eco*RI (Δ); B, *Bam*HI ($\blacktriangle$); S, *Sst*I ($\circ$); H, *Hind*II ($\bullet$); P, *Pvu*II ($\square$).

M, O, P, Q, c, d, and e in some experiments. The remaining cloned HCMV *Eco*RI fragments, which constitute approximately 50% of the genome, were consistently negative in our assays. Rger et al. (18) have recently reported similar

results when cloned HCMV strain AD169 fragments from a cosmid library were used to detect cellular sequences. The results of these studies suggest the use of caution in the interpretation of hybridization experiments in which labeled

FIG. 4. Northern analysis of cytoplasmic nonpolyadenylated RNA from uninfected human foreskin fibroblast cells. Cytoplasmic nonpolyadenylated RNA (0.16 μ g per lane) was run on a denaturing formaldehyde gel and transferred to diazotized paper (7, 8). Each lane on the filter was hybridized individually to 32 P-labeled *v-myc* (0.5-kbp *Pst*I-*Sal*I subfragment), or HCMV *Eco*RI fragments R (2.25-kbp *Eco*RI-*Bgl*II subfragment), b, d, or S. The filter strips were washed and subjected to autoradiography. The positions of the 28S and 18S rRNAs are indicated.

virion DNA or cloned HCMV subfragments that were positive by dot blot analysis for hybridization to human DNA are used to detect the presence of HCMV nucleic acid in human biological specimens.

A critical question concerns the function, if any, of the cell-related sequences in the HCMV genome. The observed hybridization of HCMV sequences to human DNA is probably due in part to G+C-rich sequences, particularly in the 28S ribosomal RNA gene. G+C-rich sequences also have been found to account for other cases of observed hybridization between DNA viral genomes and normal cell DNA. For example, sequences around the origin of replication of the DNA of simian virus 40 share homology with G+C-rich sequences in simian and human genomes (3, 6, 14). It has also been observed that human repetitive sequences hybridize to G+C-rich regions in the genomes of herpes simplex virus types 1 and 2, Epstein-Barr virus, and the Towne strain of HCMV (5, 12, 13). In the case of the Towne strain of HCMV, the homology to human repetitive DNA was localized to the L-S junction (12). This result is consistent with our observation that HCMV strain AD169 L-S junction fragments WL and WN also hybridized to dot blots of uninfected cell DNA. Further experiments addressing the role of these cell-related sequences in viral replication and gene expression are required to elucidate their functional or evolutionary significance.

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