Cleavage Maps for Human Cytomegalovirus DNA Strain AD169 for Restriction Endonucleases *EcoRI*, *BglII*, and *HindIII*

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We have used cloned *EcoRI* fragments of the human CMV (HCMV) genome, strain AD169, to prepare restriction endonuclease maps of the DNA. Individual ^{32}P -labeled cloned fragments were hybridized to Southern blots of HCMV DNA cleaved to completion with the restriction endonucleases *BglII* and *HindIII* and cleaved partially with *EcoRI*. By determining which *EcoRI* fragments hybridized to the same band on a Southern blot, we were able to establish linkage groups. This information coupled with the data derived from digestion of the cloned fragments with the enzymes *BglII* and *HindIII* (Tamashiro et al., J. Virol. 42:547-557, 1982) provided the basis for the construction of detailed maps for the enzymes *EcoRI*, *BglII*, and *HindIII*. We also identified the *EcoRI* fragments derived from the termini of this genome and mapped them with respect to the *BglII* and *HindIII* terminal fragments. From our mapping data, we conclude that the genome of HCMV is approximately 240 kilobases in length and is divided into long (198 kilobases) and short (42 kilobases) regions. Both regions consist of a unique sequence bounded by inverted repeats (11 to 12 kilobases for the long region and 2 to 3 kilobases for the short region). Furthermore, the long and short regions can invert relative to each other.

Human cytomegalovirus (HCMV), a member of the herpesvirus group, has been associated with a large variety of diseases, including cytomegalic inclusion disease of newborns, mononucleosis, postperfusion syndrome in patients receiving multiple blood transfusions, interstitial pneumonia in immunocompromised individuals, and possible oncogenesis. The medical significance of this virus coupled with its ability to establish permissive, persistent, and latent infections makes it essential to understand its molecular biology.

The structures of the herpesvirus genomes in general are complex. The results of numerous studies involving electron microscopy, partial denaturation mapping, and restriction endonuclease mapping have indicated that herpesvirus genomes are characterized by tandem repeats, inverted repeats, and unique sequences which may be arranged in a variety of ways. The mapping data presented in this paper for HCMV strain AD169 support electron microscopic observations and mapping experiments of others which suggest that HCMV has a structure similar to that of herpes simplex virus (8, 17). The HCMV DNA appears to consist of two components designated L (long) and S (short). The L component consists of a unique segment, U_L (~174 kilobases [kb]), bounded by inverted re-

peat sequences of 11 to 12 kb each. The S component contains the unique sequence U_S (~38 kb), also flanked by inverted repeat sequences approximately 2 kb in length. Furthermore, the unique sequences U_S and U_L may be relatively inverted in different DNA molecules within the population such that four genome arrangements are possible.

Because the HCMV DNA genome is large (150×10^6 to 160×10^6 M; 2, 3, 8, 9), viral gene expression is likely to be complex. Studies on the molecular biology of this virus can be greatly simplified by (i) having access to large quantities of pure subgenomic DNA fragments for use as hybridization reagents and (ii) having some understanding of the structural organization and arrangement of these subgenomic fragments on the viral DNA genome. In the preceding paper, we described the cloning and characterization of all of the *EcoRI* restriction fragments of the AD169 strain of HCMV (15).

The goal of the experiments presented in this paper was to study the structural organization of the HCMV genome and to determine the order of the cloned *EcoRI* DNA fragments on the HCMV genome. While this work was in progress, Westrate and his co-workers, before the publication of their work, graciously made available to us the results of their mapping studies

with the restriction endonucleases *Hind*III and *Bgl*II (17). In their studies, they used the technique of cross-blot hybridization to detect homologies between different *Bgl*II and *Hind*III fragments. This information coupled with the results of the digestion of separated *Hind*III and *Bgl*II fragments with either *Bgl*II or *Hind*III, respectively, formed the basis of their maps. In several cases, however, we found that their arrangement of the *Bgl*II and *Hind*III fragments was not consistent with our data obtained from hybridization experiments with cloned *Eco*RI fragments. These discrepancies were most notable for the left half of the U_L segment and for the terminal fragments. We attribute most of the differences in the maps to alternative interpretations of the cross-blot hybridization data and to the increased sensitivity afforded by the hybridization and double digestion of cloned subgenomic fragments. In this paper, we present the order of the *Eco*RI, *Bgl*II, and *Hind*III fragments.

MATERIALS AND METHODS

Cells, viruses, and purification of HCMV and cellular DNA. The procedures for growing human embryonic lung cells, infecting the cells with HCMV strain AD169, and purifying the viral DNA have been described by Tamashiro et al. (15). To prepare ³²P-labeled HCMV DNA, infected cells were exposed to 50 μ Ci of ³²PO₄ per ml in low-phosphate (0.01 mM sodium phosphate) Earle minimal essential medium supplemented with 3% dialyzed calf serum for 4 days before harvesting the extracellular virus. High-molecular-weight uninfected and infected cell DNA was extracted according to procedures described by Hughes et al. (5). The high-molecular-weight intracellular HCMV DNA was purified from infected human embryonic lung cells 4 to 5 days after the cells showed 80% cytopathic effect and was enriched for viral sequences by isopycnic cesium chloride centrifugation. The equilibrium density of HCMV DNA (1.716 g/ml) differs sufficiently from that of human cell DNA (1.699 g/ml) to permit separation of the viral DNA from the majority of the cell sequences.

Growth, purification, and labeling of HCMV DNA from recombinant DNA clones. Colonies of *Escherichia coli* strain HB101 containing recombinant HCMV plasmids were picked from a freshly streaked agar plate into 100 ml of nutrient broth containing tetracycline (20 μ g/ml) and grown overnight at 37°C. Bacterial pellets were prepared by centrifugation of the culture at 6,000 rpm for 5 min in an RC-5 low-speed centrifuge at 4°C. Each pellet was suspended in 7.5 ml of 25% sucrose, 50 mM Tris-hydrochloride, pH 8.0, and 2.5 ml of lysozyme (10 mg/ml) in 25% sucrose–50 mM Tris-hydrochloride, pH 8.0, was added. The mixture was swirled gently and placed on ice for 15 to 30 min, and 3.5 ml of 0.25 M EDTA, pH 8.0, was then added slowly. After the lysate was placed on ice for an additional 10 to 12 min, 3.75 ml of 2% Sarkosyl in TES (50 mM Tris-hydrochloride, pH 8.0, 50 mM NaCl, and 5 mM EDTA) was added, and the lysate was kept on

ice for 15 to 45 min. The volume was then adjusted to 22.5 ml with TES, and CsCl (23.1 g) was added. After the addition of 2.5 ml of ethidium bromide (5 mg/ml) in TES, the mixture was centrifuged to equilibrium, using a Ti60 rotor at 40,000 rpm for 60 h at 20°C. The superhelical recombinant plasmid DNA was collected, the ethidium bromide was removed by successive extractions with isoamyl alcohol, and the solution was dialyzed against 5 mM Tris-hydrochloride–0.1 mM EDTA, pH 7.2. The isolated recombinant plasmids were then cleaved with *Eco*RI restriction endonuclease, and the fragments were separated by electrophoresis through 0.8% Seaplaque agarose (gels buffered with TEA [40 mM Tris-hydrochloride, 20 mM sodium acetate, 18 mM NaCl, and 2 mM EDTA, pH 8.1]) to separate the plasmid from the HCMV insert. The band containing the *Eco*RI HCMV DNA fragment was excised from the gel and purified away from the Seaplaque agarose according to the procedure described in the accompanying paper (15). To label the DNA to a high specific activity ($\sim 10^8$ cpm/ μ g) with [³²P]dCTP, we used the nick translation procedure of Rigby et al. (12).

Digestion of HCMV DNA with restriction endonucleases and electrophoresis of the DNA samples. Purified cytomegalovirus DNA was digested to completion with the restriction endonuclease *Eco*RI, *Bgl*II, or *Hind*III as described by Tamashiro et al. (15). High-molecular-weight infected cell DNA enriched for HCMV sequences was digested partially with *Eco*RI endonuclease. The extent of the digestion was monitored by removing a small portion of the reaction, which was then added to a tube containing 1 μ g of lambda DNA. The test samples were incubated in parallel with the digests containing only HCMV DNA for 1 h at 37°C. To terminate the reactions, all samples were heated to 65°C for 10 min. The test samples were analyzed by electrophoresis in 0.8% agarose gel, and the DNA was visualized by staining with ethidium bromide followed by transillumination with a UV light source. The reactions were judged to be complete if only the appropriate set of restriction fragments for lambda DNA were observed.

The HCMV DNA samples were analyzed by electrophoresis in 0.8% Seakem agarose preparative slab gels (5 mm thick) buffered with TEA. Gels were formed in a horizontal gel box (12.5 by 28 cm), and the samples were subjected to electrophoresis for 16 h at 50 to 75 mA. The unlabeled HCMV DNA samples were placed in a slot 9.5 cm wide. A parallel lane contained HCMV DNA uniformly labeled with ³²P and cleaved to completion with *Eco*RI, *Hind*III, or *Bgl*II.

Hybridization procedures. After electrophoresis through agarose, the HCMV DNA was denatured, neutralized in situ, and transferred to nitrocellulose sheets (0.45 μ m by 12.5 cm by 20 cm) according to the procedure of Southern (13). The filters were then baked (80°C in vacuo) and cut into parallel strips 3.4 mm wide. Each strip was preannealed overnight at 37°C in 50% formamide, 3 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl plus 0.015 M sodium citrate), 50 μ g of denatured salmon sperm DNA per ml, 10 mM EDTA, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.0), and 0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone. The filters were then annealed in the same buffer to individual nick-translated ³²P-labeled cloned HCMV DNA frag-

ments at 37°C for 96 h. After hybridization, unannealed DNA was removed by washing the filters once with $2\times$ SSC at room temperature for 1 h, twice with $0.1\times$ SSC–0.1% sodium dodecyl sulfate for a total of 1 h at 55°C, and five to eight times in rapid succession with $0.1\times$ SSC at room temperature. The filter strips were dried, placed in exact parallel with other strips cut from the original filter, and subjected to autoradiography in the presence of a Lightning Plus intensifying screen at -70°C . The filter strip containing the HCMV DNA uniformly labeled with ^{32}P and cleaved with the same restriction endonuclease allowed identification of each of the hybridized bands. In some cases, we also used HCMV restriction endonuclease fragments that were endlabeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and kinase as described in the accompanying paper (15).

Detection of terminal *EcoRI* fragments. The genome of HCMV contains numerous nicks. Therefore, it is difficult to use methods which either label 5' ends or processively digest from 3' ends to identify terminal fragments. To circumvent this problem, we used the following methods. First, we end labeled synthetic DNA linkers (octomers from Collaborative Research Products, bearing an *EcoRI* restriction endonuclease site) with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, using T4 polynucleotide kinase in a reaction mix (24 μl) containing 50 mM glycine, pH 9.5, 10 mM MgCl_2 , 5 mM dithiothreitol, 25% glycerol, 0.2 mM spermidine, 25 μg of linker, 1 mCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (ICN Radiochemicals), and 12 U of kinase (Bethesda Research Laboratories). After incubation at 37°C for 60 min, the reaction was terminated by incubation at 65°C for 10 min. A portion (5 μl) of the labeled linkers was then blunt-end ligated to 9 μg of HCMV DNA in a reaction mix (23 μl) containing 20 mM Tris-hydrochloride, pH 7.6, 10 mM MgCl_2 , 10 mM dithiothreitol, 30 mM NaCl, 1.7 mM ATP, and 10 U of ligase (Bethesda Research Laboratories). After incubation at 4°C for 48 h, the reaction was passed through a 20-ml column of Sephadex G-75 in buffer containing 0.1 M NaCl, 10 mM Tris-hydrochloride, pH 8.0, and 10 mM EDTA to remove unincorporated label and the majority of the low-molecular-weight linkers. The high-molecular-weight HCMV DNA with attached linkers was then precipitated with ethanol three times and electrophoresed through 0.8% Seaplaque agarose in TEA buffer. The band of high-molecular-weight DNA was excised and purified from the agarose by passage through a column of benzoyl-naphthol-DEAE-cellulose. After ethanol precipitation, the DNA was cleaved with the restriction endonuclease *EcoRI* and analyzed by electrophoresis in a 0.8% agarose slab gel. A sample of uniformly ^{32}P -labeled HCMV DNA digested to completion with *EcoRI* endonuclease was electrophoresed in a parallel lane. After electrophoresis, the gel was soaked in 95% ethanol for 3 h, vacuum dried, and autoradiographed in the presence of a Lightning Plus intensifying screen at -70°C .

RESULTS

Fragments generated by cleavage of the genome with restriction endonucleases *EcoRI*, *BglII*, and *HindIII*. Table 1 lists the fragments generated by cleavage of the HCMV genome with the restriction endonucleases *EcoRI*, *HindIII*, and *BglII*. For *EcoRI*, there were at least 35 fragments

generated; for *HindIII*, 29 fragments; and for *BglII*, 33 fragments. These restriction endonuclease patterns are identical to those published previously for HCMV strain AD169 (7, 17). We have assigned unique letters to each of these fragments, including all possible termini and L-S joint fragments. It should be noted that the letters given in Table 1 for the *BglII* and *HindIII* fragments differ from those published by Westrate et al. (17), who did not assign unique letters to the joint fragments. Furthermore, several differences in the number and sizes of fragments detected are reflected in the new lettering scheme. This nomenclature and the prototype arrangement were agreed upon at the International Herpesvirus Meeting held in Bologna, Italy, 27 to 31 July 1981. Table 1 also gives estimates of the molecular weights of the different fragments, calculated from their electrophoretic mobilities and from double-digestion data. Although the numbers for the smaller fragments are probably accurate, it should be noted that the given molecular weights for the larger fragments are only estimates. From the sum of the molecular weights of the unique fragments, we calculate a genome size of $158 \times 10^6 M_r$, a value which agrees well with other size determinants of the HCMV genome (2, 3, 6, 8, 9).

Identification of the *EcoRI* fragments at the termini of the genome. For the identification of the terminal fragments of the HCMV DNA genome, we found it necessary to develop a method which would circumvent the problem of a nicked genome. If the genome contains numerous nicks, methods which involve either the labeling of 5' ends or processive digestion from the 3' ends of the DNA will produce ambiguous results. We therefore used a method which utilized synthetic DNA linkers (octomers) bearing an *EcoRI* restriction site. These synthetic linkers were phosphorylated to high specific activity with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and kinase and covalently attached to the ends of the HCMV DNA by blunt-end ligation, using T4 ligase. In some cases we treated the HCMV DNA with exonuclease VII before ligation to ensure blunt ends. Treatment with exonuclease VII, however, had no effect on the results. After ligation, the HCMV DNA attached to linkers was cleaved with the endonuclease *EcoRI*, and the fragments were separated by electrophoresis through agarose. The gels were vacuum dried and autoradiographed in the presence of intensifying screens. Only the *EcoRI* fragments at the end of the genome became labeled (Fig. 1). On the basis of these experiments, we could identify three major terminal fragments with molecular weights of approximately 5.8×10^6 (8.8 kb), 5.0×10^6 (7.6 kb), and 2.8×10^6 (4.2 kb). Only the 8.8-kb fragment (fragment L) could be distinguished as

TABLE 1. Molecular weights of *Eco*RI, *Bgl*II, and *Hind*III fragments of HCMV DNA^a

<i>Eco</i> RI	M_r ($\times 10^6$)	<i>Bgl</i> II	M_r ($\times 10^6$)	<i>Hind</i> III	M_r ($\times 10^6$)
A	11.4	A (EF)	32 ^b	A (IH)	24 ^b
B	11.2	B (EI)	29 ^b	B (KH)	21 ^b
C	11.2	C (GF)	25 ^b	C (IQ)	16.3 ^b
D	11.2	D (GI)	21.5 ^b	D	15.9
E	10.3	E	20 ^c	E	14.3
F (WL)	8.1–9.2 ^d	F	12.5 ^c	F	14.2
G	8.3	G	12.5 ^c	G (HQ)	13.4 ^b
H (WN)	7.0–8.2 ^d	H	11	H	12.2 ^c
I	7.4	I	9 ^c	I	11.5 ^c
J	6.8	J	8.1	J	9.2
K	6.3	K	8.1	K	8.6 ^c
L	5.8 ^c	L	7.6	L	7.8
M	5.0	M	7.5	M	7.6
N	5.0 ^c	N	7.2	N	6.0
O	4.55	O	6.5	O	6.0
P	4.45	P	6.2	P	5.2
Q	4.3	Q	5.9	Q	5.0 ^c
R	3.9	R	5.9	R	4.6
S	3.45	S	5.2	S	4.2
T	3.45	T	4.2	T	4.2
U	2.85	U	3.7	U	3.9
V	2.8	V	3.7	V	3.8
W	2.8 ^c	W	2.8	W	3.8
X	2.6	X	1.9	X	3.5
Y	2.5	Y	1.35	Y	2.7
Z	2.4	Z	1.3	Z	1.5
a	2.3	a	1.05	a	1.4
b	1.9	b	0.9	b	0.88
c	1.65	c	0.88	c	0.50
d	1.1	d	0.78		
e	1.0	e	0.64		
f	0.95	f	0.52		
g	0.6	g	0.4		
h	0.56				
i	0.54				

^a The molecular weights were determined from the electrophoretic migration of the fragments through 0.8% agarose and from double-digestion data presented in Table 2 of the accompanying paper (15).

^b The *Bgl*II and *Hind*III 0.25 M joint fragments were not readily distinguishable on our gels. Therefore, the molecular weights were determined from summation of the *Eco*RI fragments contained within these joints. The letters in parentheses indicate the two terminal fragments contained within the joint.

^c The values given represent the molecular weights of the major terminal fragments. As discussed in text, the termini and joints are heterogeneous in size.

^d The values given for the sizes of the two *Eco*RI joint fragments represent the range of sizes observed for the cloned fragments.

a submolar fragment. The 7.6-kb fragment (labeled N) comigrated with the molar fragment M, and the 4.2-kb fragment (labeled W) comigrated with the molar fragment V. Since in the HCMV genome the U_L and U_S segments can invert relative to one another, there are a maximum of four termini if the restriction endonuclease cleaves outside of the repeat sequence. The presence of three *Eco*RI terminal fragments indicates that *Eco*RI cleaves within one set of repeats. The electron microscopic observations of P. Sheldrick (personal communication cited by Westrate et al. [17]) suggest that the S segment of AD169 HCMV DNA is bounded by inverted

repeats of approximately $1.6 \times 10^6 M_r$ and that the L segment is bounded by inverted repeats of approximately $5 \times 10^6 M_r$. Our mapping studies described here suggest that the repeats bounding the L segment are slightly larger ($\sim 8 \times 10^6 M_r$). We will present evidence that the three *Eco*RI terminal fragments are arranged such that terminal fragment W results from cleavage within the larger repeat region bounding the U_L segment and that terminal fragments L and N represent the termini of the S region in the two possible orientations.

In our library of cloned *Eco*RI fragments, we do not have the termini. However, the se-

FIG. 1. Identification of the *Eco*RI terminal fragments of HCMV DNA. Synthetic DNA linkers (ocotomers) bearing an *Eco*RI restriction site were phosphorylated to high specific activity with [γ - 32 P]ATP and kinase and attached to the ends of the HCMV DNA by blunt-end ligation, using T4 ligase. The HCMV DNA attached to linkers was then cleaved with the endonuclease *Eco*RI, and the fragments were separated by electrophoresis through 0.8% agarose. Only the three terminal *Eco*RI fragments L, N, and W became labeled and can be seen on the autoradiograph. The HCMV DNA in the far right lane was treated with exonuclease VII before attachment of the linker. 32 P-labeled HCMV DNA cleaved with *Eco*RI served as a marker (left lane).

quences present in the termini are represented in the *Eco*RI joint pieces F and H, where the molecular weight of joint piece F equals L plus W and joint piece H equals N plus W. As described below, these cloned joint pieces were used as hybridization probes to identify and align the *Bgl*III and *Hind*III terminal fragments of the genome.

Characterization of the cloned *Eco*RI joint pieces with the restriction endonucleases *Pvu*II, *Bam*HI, and *Hind*III. The joint pieces F and H include both common regions from the repeats bounding the L and S regions as well as unique sequences from the short region of genome. Therefore, both joint pieces will hybridize to all restriction endonuclease fragments from the termini and L-S joint of the genome. To use the *Eco*RI joint pieces to align the *Eco*RI, *Bgl*III, and *Hind*III terminal fragments, it was first necessary to subdivide the *Eco*RI joint pieces with additional restriction endonucleases, to identify the fragments both common and unique to each joint piece, and to map these fragments with respect to each other.

In Fig. 2A we show the pattern of fragments generated after *Bam*HI digestion of *Eco*RI joint pieces H (lane 1) and F (lane 2). Figure 2B shows the digestion of two different *Eco*RI-H joint pieces with *Pvu*II, and Fig. 2C and 2D show the restriction endonuclease patterns of two different *Eco*RI-F joint pieces after digestion with *Pvu*II (lane 1), *Pvu*II plus *Hind*III (lane 2), and *Hind*III (lane 3). Table 2 lists the molecular weights of the fragments generated. Cleavage of all of the joint pieces with the restriction endonuclease *Bam*HI (Fig. 2A) resulted in a small fragment of molecular weight 1.05×10^6 M_r (B2) and a second larger fragment whose molecular weight differed from clone to clone. We could distinguish the joint pieces F and H on the basis of their digestion with the enzymes *Hind*III and *Pvu*II. The joint piece H was not cleaved by *Hind*III (data not shown). Cleavage of the joint piece F with *Hind*III (lane 3 of Fig. 2C and 2D) yielded two fragments: a small fragment (labeled H2) and a larger fragment (H1) whose molecular weight was variable. Digestion of all *Eco*RI joint fragments with *Pvu*II (Fig. 2B for *Eco*RI-H and lane 1 of Fig. 2C and 2D for *Eco*RI-F) yielded three fragments with different but constant molecular weights (P2, P3, and P4 from *Eco*RI-H and P12, P13, and P14 from *Eco*RI-F) and one fragment with variable molecular weight (P1 from *Eco*RI-H and P11 from *Eco*RI-F). The fragments P1 and P11 appeared to be identical since they cross-hybridized, and when cleaved with *Bam*HI, both generated fragment B2 and a second fragment whose molecular weight was variable (data not shown). We know from analysis of the recombinant plasmids containing the

FIG. 2. Analysis of the *Eco*RI joint pieces F and H with restriction endonucleases. Recombinant plasmid DNA containing the *Eco*RI joint pieces F and H was cleaved with restriction endonucleases, fractionated by electrophoresis through 0.8% agarose, and stained with ethidium bromide. The lanes marked λ contain fragments of *Hind*III-cleaved λ DNA (M_r , 15.6×10^6 , 6.2×10^6 , 4.4×10^6 , 2.9×10^6 , 1.5×10^6 , and 1.3×10^6). The position of fragments representing plasmid alone is indicated by "O." The letters designating the viral fragments have been assigned arbitrarily and are used solely for identification purposes. We have summarized the data from these figures in Table 2. (A) Cleavage of joint H clone no. 310 (lane 1) and joint F clone no. 359 (lane 2) with *Eco*RI plus *Bam*HI. (B) Cleavage of joint H clone no. 293 (lane 1) and joint H clone no. 310 (lane 2) with *Eco*RI plus *Pvu*II. (C and D) Cleavage of joint F clone no. 359 (C) and joint F clone no. 360 (D) with *Eco*RI plus *Pvu*II (lane 1), *Eco*RI plus *Hind*III plus *Pvu*II (lane 2), and *Eco*RI plus *Hind*III (lane 3).

TABLE 2. Double-digestion products of cloned *EcoRI* joint pieces

Clone no.	Joint	Enzymes used for cleavage	Size of viral fragments generated ($M_r \times 10^6$) ^a	Letter designating fragments ^b
310	H	<i>Bam</i> HI + <i>Eco</i> RI	5.9	B1
			1.05	B2*
293	H	<i>Pvu</i> II + <i>Eco</i> RI	4.0	P1b*
			1.65	P2
			1.35	P3
			0.96	P4
310	H	<i>Pvu</i> II + <i>Eco</i> RI	2.8	P1a*
			1.65	P2*
			1.35	P3*
			0.96	P4*
359	F	<i>Bam</i> HI + <i>Eco</i> RI	7.7	B11
			1.05	B2
359	F	<i>Hind</i> III + <i>Eco</i> RI	7.7	H1
			1.1	H2*
359	F	<i>Pvu</i> II + <i>Eco</i> RI	3.5	P11b*
			3.1	P12
			1.1	P13*
			0.74	P14* ^c
360	F	<i>Pvu</i> II + <i>Eco</i> RI	2.8	P11a*
			3.1	P12*
			1.1	P13
			0.74	P14 ^c

^a The molecular weights of the fragments generated were determined from their electrophoretic mobility through 0.8% agarose relative to the *Hind*III λ DNA marker fragments (Fig. 2).

^b The fragments indicated by an asterisk were eluted from Seaplaque agarose (see Fig. 2), nick translated with [α -³²P]dCTP, and hybridized to Southern blots of HCMV DNA cleaved with *Eco*RI, *Hind*III, and *Bgl*III (Fig. 4, 5, and 6).

^c The *Pvu*II fragment P14 contains a *Hind*III cleavage site and is cleaved to yield a fragment of $0.66 \times 10^6 M_r$.

joint pieces that the fragments P1, P11, and B2 are at the end of the joint pieces and are directly linked to the plasmid before cleavage by *Eco*RI (data not shown). Therefore, P1, P11, and B2 are from a region common to both *Eco*RI joint pieces F and H and contain part of the terminal repeat bounding the U_L sequence. In addition, P1 and P11 include the heterogeneous L-S junction. *Pvu*II fragments P12 and P2 also cross-hybridized (data not shown). Since the molecular weight of P2 fragments from all *Eco*RI-H joint pieces was constant but differed from the molecular weight of P12 fragments, the simplest interpretation of the data is that P2 and P12 include segments from the junction of the U_S and S repeat segments of the genome and that the repeats bounding the S region of the genome are inverted. *Pvu*II fragments P3, P4, P13, and P14 arose from the U_S region of the genome. Since *Pvu*II fragment P14 was cleaved by *Hind*III (lane 2 of Fig. 2C and 2D), we placed this fragment at one end of the joint piece. The order of *Pvu*II fragments P3 and P4, however, is uncertain. In Fig. 3 we show the most likely arrangement of the *Bam*HI, *Hind*III, and *Pvu*II cleavage sites within the *Eco*RI joint pieces F and H.

Alignment of the *Eco*RI, *Bgl*III, and *Hind*III terminal fragments. To identify the *Bgl*III and *Hind*III terminal fragments, we first labeled the cloned *Eco*RI joint pieces by nick translation with [³²P]dCTP and hybridized them to Southern blots of HCMV DNA cleaved with the enzymes *Eco*RI (Fig. 4), *Bgl*III (Fig. 5), and *Hind*III (Fig. 6). In Fig. 4 we show that each joint piece could be independently used to identify all of the *Eco*RI terminal and joint fragments. Both joint fragments hybridized to multiple *Eco*RI fragments, including the two joints F and H and the terminal fragments L, N, and W (Fig. 4). There was also hybridization to multiple minor fragments resulting from heterogeneity at the termini and joints of different HCMV DNA molecules. As discussed in the preceding paper (15), this heterogeneity in the joint fragments was also revealed in the set of recombinant clones obtained for the joint pieces. The exact nature of the heterogeneity is currently under investigation in our laboratory. It should be noted that in Fig. 1 we saw only the major termini blunt-end ligated to the labeled *Eco*RI linkers. The minor fragments were below the limits of detection in that experiment.

When the joint pieces were hybridized to

FIG. 3. Restriction endonuclease cleavage maps of the DNAs of the cloned *EcoRI* joint pieces F and H. The major fragments generated by cleavage of the *EcoRI* joint pieces F (clone no. 360) and H (clone no. 310) with *PvuII*, *BamHI*, and *HindIII* are shown in relation to the *EcoRI*, *BglII*, and *HindIII* terminal fragments which in their inverted orientation comprise the joint. The letters in parentheses in the long repeat indicate the *BglII* and *HindIII* terminal fragments from the opposite orientation of the long segment of the genome. The letters in quotation marks have been arbitrarily assigned to the fragments in the joint piece for identification purposes only (see Table 2). All other letters are those assigned to the fragments in relation to the map of the entire genome. The portion of the *EcoRI* joint piece containing sequences from the long and short repeat are indicated by the slanting lines.

Southern blots of HCMV DNA cleaved with *BglII*, the terminal fragments E, F/G, and I could be detected (Fig. 5 and 7). The joint pieces also hybridized to *HindIII* terminal fragments H/I, K, and Q (Fig. 6 and 8). In addition, there was hybridization of the *EcoRI* joint pieces to some higher-molecular-weight *HindIII* fragments which probably represented the *HindIII* joint pieces. It should be noted that we could detect stepwise heterogeneity in the *HindIII*-Q fragments. The other *HindIII* and *BglII* terminal fragments were too large to be resolved on our gels into their multiple components and appeared instead as diffuse bands.

To align the *EcoRI*, *BglII*, and *HindIII* terminal fragments, representative restriction endonuclease fragments generated by cleavage of *EcoRI* joint pieces F and H with *BamHI*, *HindIII*, and *PvuII* (indicated by an asterisk in Table 2) were eluted from gels, nick translated to a high specific activity, and hybridized to Southern blots of HCMV DNA cleaved with *EcoRI* (Fig. 4 and Table 3), *BglII* (Fig. 5 and Table 4), and *HindIII* (Fig. 6 and Table 5). We will discuss below only the hybridization of the representative fragments to the *BglII* and *HindIII*

TABLE 3. Hybridization of ³²P-labeled subfragments of the L-S junction to Southern blots of HCMV DNA cleaved with the restriction endonuclease *EcoRI*^a

<i>EcoRI</i> subfragment	L-S junction subfragment												
	f	a	h	O	F	H	H	B2	P1a	P1b	P2	P3	P4
F					+	+	+	+	+	+	+	+	+
H					+	+	+	+	+	+	+	+	+
L					+	+	+	+	+	+	+	+	+
N					+	+	+	+	+	+	+	+	+
W					+	+	+	+	+	+	+	+	+
f	+												
a		+											
h			+										
O				+									

^a Data compiled from Fig. 4.

FIG. 4. Hybridization of ^{32}P -labeled subfragments of the L-S junction to Southern blots of HCMV DNA cleaved with *Eco*RI. Identical strips from a preparative Southern blot containing the *Eco*RI fragments of HCMV DNA were hybridized with cloned HCMV *Eco*RI fragments f, a, h, O, F, and H and with the restriction endonuclease subfragments of *Eco*RI joint pieces F and H described in Table 2. These fragments include sequences from the long and short inverted repeats as well as the unique sequences adjacent to the repeats. The ^{32}P -labeled probe used for the hybridization is indicated at the top of each strip. The filter strip labeled "CMV" represents *Eco*RI-cleaved HCMV DNA fragments which were end-labeled with ^{32}P and subjected to electrophoresis in a parallel lane of the gel. The letters along the right sides of the autoradiogram mark the positions of the HCMV *Eco*RI joint pieces F and H and terminal fragments L, N, and W.

terminal fragments and not to the higher-molecular-weight joint pieces, since the transfer of these higher-molecular-weight *Bgl*II and *Hind*III joint fragments to nitrocellulose filters was inefficient and thus could not be distinguished as separate bands on our filters.

The *Pvu*II fragments P3 and P4, which are unique to *Eco*RI joint piece H, hybridized to a heterogeneous set of *Eco*RI fragments comprising joint piece H and terminal fragment N. They also hybridized mainly to the terminal fragments *Bgl*II-I and *Hind*III-H/I (which we will designate as *Hind*III-H). Thus, the terminal fragments *Bgl*II-I, *Hind*III-H, and *Eco*RI-N were colinear at one end of the S segment. In the inverted orientation of the S segment, *Eco*RI fragment L also mapped at the terminus. As expected, the

*Pvu*II unique fragments P13 and P14 and the *Hind*III unique fragment H2 of *Eco*RI joint piece F hybridized to the heterogeneous set of *Eco*RI fragments comprising joint piece F and the terminal fragment L. The *Pvu*II unique fragment P13 also hybridized to the terminal fragments *Hind*III-Q and *Bgl*II-F/G (we will designate it *Bgl*II-F). Thus, *Eco*RI-L, *Hind*III-Q, and *Bgl*II-F all mapped at the same terminus. *Pvu*II fragment P14 and *Hind*III fragment H2 hybridized to *Bgl*II fragment F and to the internal *Hind*III fragment X. Thus, *Hind*III-X was adjacent to *Hind*III-Q and shared sequences with *Eco*RI-L and *Bgl*II-F. We have presented evidence above suggesting that the *Pvu*II fragments P2 (from joint H) and P12 (from joint F) include sequences from the junction of the U_S and S repeat

FIG. 5. Hybridization of ^{32}P -labeled subfragments of L-S junction to Southern blots of HCMV DNA cleaved with *Bgl*III. Identical strips from a preparative Southern blot containing the *Bgl*III fragments of HCMV DNA were hybridized with the labeled HCMV DNA probes as described in Fig. 4. The filter strip labeled "CMV" represents ^{32}P -labeled HCMV DNA cleaved with *Bgl*III and subjected to electrophoresis in a parallel lane of the gel. The letters along the right side of the autoradiogram mark the positions of the *Bgl*III terminal fragments E, F, G, and I.

segments of the genome. The finding that both P2 and P12 hybridized to the terminal fragments *Eco*RI-L and -N, *Bgl*III-I and -F, and *Hind*III-H and -Q further supports these map positions.

We mapped the small *Bam*HI fragment B2 common to both joint pieces in the repeat bounding the U_L segment of the genome. The hybridization data added further support to this map position. Fragment B2 hybridized to both sets of *Eco*RI joint pieces F and H and to terminal fragment *Eco*RI-W, but not to the terminal *Eco*RI fragments L and N. This fragment also hybridized to the terminal fragments *Bgl*III-E and -G and *Hind*III-K and -I. To determine the orientation of the *Hind*III terminus with respect to the *Bgl*III fragments, we used in our hybridization experiments an *Eco*RI fragment (*Eco*RI-f) which we mapped close to one terminus in the U_L region of the genome (data to be presented below). *Eco*RI-f hybridized only to *Bgl*III-E and

*Hind*III-I. Therefore, the terminal fragments *Hind*III-I and *Bgl*III-E mapped at one end of the L segment, and *Hind*III-K and *Bgl*III-G mapped at the other end. On the basis of the size and double digestion with *Bam*HI and cross-hybridization data, we had mapped the set of P1 and P11 fragments in the region including part of the repeat bounding the U_L sequences as well as the heterogeneous L-S junction. Additional evidence for this map position was provided by the hybridization of these fragments to Southern blots of *Bgl*III-, *Hind*III-, or *Eco*RI-cleaved HCMV DNA. When the *Pvu*II fragments (P1a, P1b, P11a, and P11b) were hybridized to *Eco*RI-cleaved HCMV DNA, there was hybridization to the two joint fragments F and H and terminal fragment W; there was also a small amount of hybridization to terminal fragments L and N. These fragments also hybridized extensively to *Bgl*III fragments E and F/G and *Hind*III frag-

FIG. 6. Hybridization of ^{32}P -labeled subfragments of the L-S junction to Southern blots of HCMV DNA cleaved with *Hind*III. Identical strips from a preparative Southern blot containing the *Hind*III fragments of HCMV DNA were hybridized with the labeled HCMV DNA probes as described in Fig. 4. The filter strip labeled "CMV" represents *Hind*III-cleaved intracellular HCMV DNA fragments which were end-labeled with ^{32}P and subjected to electrophoresis in a parallel lane of the gel; in this preparation fragments A,B,C,D, and G,H,I did not label well, and there is a labeled cellular DNA fragment appearing on the autoradiogram between HCMV *Hind*III fragments L,M and N,O. The letters along the right side of the autoradiogram mark the positions of the *Hind*III terminal fragments H, I, K, and Q and the *Hind*III internal fragment X. *Eco*RI fragment H was hybridized to a strip from a preparative Southern blot from a different gel.

ments H/I and K, but only to a limited extent to *Bgl*II fragment I and *Hind*III fragment Q. The simplest interpretation of these data is that the heterogeneous fragments from both joints flanked the junction of the L-S repeats, with only a small portion of the S repeat represented in the heterogeneous fragments. In summary, the results of our experiments suggested that *Eco*RI fragment W was located within the terminal repeats flanking the U_L region and was colinear with *Hind*III fragment I and *Bgl*II fragment E at one end and with *Hind*III fragment K and *Bgl*II fragment G at the other end. *Eco*RI fragments L and N bound the U_S region such that *Eco*RI fragment L was colinear with *Hind*III fragments Q and X and *Bgl*II fragment F, and *Eco*RI fragment N was colinear with *Hind*III fragment H and *Bgl*II fragment I (see Fig. 12).

Strategy for mapping the HCMV genome. The primary source of data for the construction of the restriction endonuclease maps (summarized

in Fig. 12) came from the hybridization of each of the ^{32}P -labeled cloned *Eco*RI fragments to identical nitrocellulose filter strips of HCMV DNA cleaved to completion with *Bgl*II (Fig. 7 and Table 6) and *Hind*III (Fig. 8 and Table 7) and cleaved partially with *Eco*RI (Fig. 9). By determining which cloned *Eco*RI fragments hybridized to the same *Bgl*II, *Hind*III, or partial *Eco*RI fragments, we could construct linkage maps. The nitrocellulose filter strips in Fig. 7 through 9 have been arranged from left to right in the prototype linkage order of the labeled cloned fragments used for the hybridization (beginning with fragments representing the S segment of the genome). We also localized more precisely the *Bgl*II and *Hind*III restriction sites within the *Eco*RI fragments by cleaving each cloned *Eco*RI fragment with either *Bgl*II or *Hind*III and determining the molecular weights of the given fragments after separation by agarose gel electrophoresis. The results of these double-digestion experiments are summarized in Table 2 of the

TABLE 4. Hybridization of ³²P-labeled subfragments of the L-S junction to Southern blots of HCMV DNA cleaved with the restriction endonuclease *Bgl*II^a

<i>Bgl</i> II subfragment	L-S junction subfragment													
	f	a	h	O	F	H	B2	P1a	P1b	P2	P3	P4	H1	H2
E	+	+	+	+	+	+	+	+	+	+	± ^b	± ^b	±	± ^b
F/G ^c		+	+	+	+	+	+	+	+	+	+	+	+	+
I					+	+		±	±	+	+	+	±	+

^a Data compiled from Fig. 5. Only the hybridization to the *Bgl*II terminal fragments E, F, G, and I is summarized.
^b The hybridization seen is probably to the high-molecular-weight *Bgl*II joint fragments A, B, C, and D which comigrate with terminal fragment *Bgl*II-E in our gels.
^c Fragments F and G cannot be electrophoretically separated on our gels.

TABLE 5. Hybridization of ³²P-labeled subfragments of the L-S junction to Southern blots of HCMV DNA cleaved with the restriction endonuclease *Hind*III^a

<i>Hind</i> III subfragment	L-S junction subfragment													
	f	a	h	O	F	H	B2	P1a	P1b	P2	P3	P4	H1	H2
F		+												
H/I ^b	+	±	+	+	+	+	+	+	+	+	+	+	+	+
K		+	+	+	+	+	+	+	+	+				
Q					+	+		±	±	+			+	+
X					+									+

^a Data compiled from Fig. 6. Only the hybridization to the *Hind*III terminal fragments H, I, K, and Q and internal fragments F and X is summarized.
^b Fragments H and I cannot be electrophoretically separated on our gels.

FIG. 7. Hybridization of ^{32}P -labeled cloned HCMV DNA fragments to Southern blots of *Bgl*/II-cleaved HCMV DNA. Identical strips from preparative Southern blots containing the *Bgl*/II fragments of HCMV DNA were hybridized with the labeled cloned HCMV DNA *Eco*RI fragments indicated at the top of each strip. The filter strip labeled "CMV" represents ^{32}P -labeled HCMV DNA cleaved with *Bgl*/II and subjected to electrophoresis in a parallel lane of the gel. Panels A and B represent the autoradiograms of filter strips from two separate gels.

preceding paper (15). Finally, we compared our results with the double-digestion and cross-blot hybridization data reported by Westrate et al. (17). In their paper, they separated *Bgl*/II and *Hind*III fragments and further cleaved them with *Hind*III and *Bgl*/II, respectively (see Tables 1

and 2 in reference 17 for summary). They also identified homologies between different *Bgl*/II and *Hind*III fragments, using the technique of cross-blot hybridization (see Table 4 in reference 17 for summary). Although some of their results for larger or comigrating fragments were

FIG. 8. Hybridization of ^{32}P -labeled cloned HCMV DNA fragments to Southern blots of *Hind*III-cleaved HCMV DNA. Identical strips from preparative Southern blots containing the *Hind*III fragments of HCMV DNA were hybridized with the labeled cloned HCMV *Eco*RI fragment indicated at the top of each strip. The filter strip labeled "CMV" represents ^{32}P -labeled HCMV DNA cleaved with *Hind*III and subjected to electrophoresis in a parallel lane of the gel. Panels A, B, and C represent the autoradiograms of filter strips from three separate gels. In panel C the electrophoresis time was shorter so that the hybridization of *Eco*RI fragments A and D to the smaller *Hind*III fragments could be seen.

ambiguous, their data, in general, do not conflict with ours. We attribute the differences in our *Bgl*II and *Hind*III maps mainly to alternative interpretations of the cross-blot hybridization data and to the increased sensitivity afforded by

the hybridization and double digestion of cloned DNA fragments. The detailed analysis which allowed the construction of the linkage groups will be presented below.

U_S region (*Eco*RI fragments B, T, and X) and

TABLE 6. Hybridization of cloned *Eco*RI fragments to Southern transfers of HCMV DNA cleaved with the restriction endonuclease *Bgl*II^a

<i>Bgl</i> II fragment ^b	<i>Eco</i> RI fragment																			
	F	B	T	X	H	O	H	f	G	i	g	K	c	Y	S	Q	b	Z	E	M
E	+				+	+	+	+	+	+	+	±								
[F]	+	+			+															
[G]	+				+															
H					+															
I	+		+	+	+							+	+	+	+					
[J]																			+	
[K]																				
[L]																				
[M]																				
N																				
O																				
P	+		+																	
[Q]																				
[R]																				
S																				
T																				
[U]																				
[V]																				
W																				
X																				
Y																				
Z																				
a																				
[b]																				
[c]																				
d																				
e	+																			
f																				
g																				

^a Data compiled from Fig. 7. The hybridization to *Bgl*II joint fragments A, B, C, and D could not be distinguished unambiguously and therefore is not presented.
^b The *Bgl*II fragments in brackets cannot be electrophoretically separated in our gels.

FIG. 9. Hybridization of ^{32}P -labeled cloned HCMV DNA fragments to Southern blots of intracellular HCMV DNA partially cleaved with *EcoRI*. The intracellular HCMV DNA was prepared and enriched for viral sequences as described in Materials and Methods. Identical strips from preparative Southern blots containing the *EcoRI* partial cleavage products of the intracellular HCMV DNA were hybridized with the labeled cloned HCMV *EcoRI* fragments indicated at the top of the strip. The filter strip labeled "CMV" represents ^{32}P -labeled HCMV virion DNA cleaved with *EcoRI* and subjected to electrophoresis in a parallel lane of the gel. Panels A and B represent the autoradiograms of filter strips from two separate gels. The positions of the partial *EcoRI* cleavage products containing two linked *EcoRI* fragments are indicated (—).

TABLE 7. Hybridization of cloned *Eco*RI fragments to Southern transfers of HCMV DNA cleaved with the restriction endonuclease *Hind*III^a

<i>Hind</i> III fragment ^b	<i>Eco</i> RI fragment																			
	F	B	T	X	H	O	h	f	G	i	g	K	c	Y	S	Q	b	Z	E	M
D																				
[E]																				
[F]																				
[H]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
[I]	+																			
[J]																				
[K]	+				+	±														
L																				
M																				
[N]																				
[O]																				
P									+	+										
Q																				
R	+			+																
[S]																				
[T]																				
U																				
[V]	+																			
[w]	+																			
X																				
Y	+																			
Z																				
a																				
b																				
c																				

^a Data compiled from Fig. 8. The hybridization to *Hind*III joint fragments A, B, C, and G could not be distinguished unambiguously and therefore is not presented.

^b The *Hind*III fragments in brackets cannot be electrophoretically separated on our gels.

the L-S joint (*Eco*RI fragments H, O, and h). Located within the U_S region are *Eco*RI fragments B ($M_r = 11.2 \times 10^6$), T ($M_r = 3.45 \times 10^6$), and X ($M_r = 2.6 \times 10^6$). All three of these cloned *Eco*RI fragments hybridized to one of the fragments in the *Hind*III-H/I group. The hybridization of *Eco*RI fragment B to *Hind*III fragment H/I is faint in Fig. 8, but can be seen clearly when the autoradiograms are overexposed. *Eco*RI fragment B also hybridized to *Hind*III fragments V, W, and X. Since the *Eco*RI terminal fragment L hybridized to *Hind*III-X and to terminal fragment *Hind*III-Q, we mapped *Eco*RI fragment B to the left of terminal *Eco*RI fragment L. Digestion of *Eco*RI fragment B with *Hind*III yielded 2 mol of a fragment with molecular weight of $3.8 \times 10^6 M_r$ (*Hind*III fragments V and W) and two fragments with molecular weights of $2.4 \times 10^6 M_r$ (part of *Hind*III-X) and $1.1 \times 10^6 M_r$ (part of *Hind*III-H/I). As described above, our mapping data for the terminal fragments seem to indicate that the terminal fragments *Hind*III-H and *Hind*III-Q flank the U_S sequence. The finding that *Eco*RI-B, a fragment with molecular weight of $11.2 \times 10^6 M_r$, includes sequences in both *Hind*III-X (which is adjacent to terminal fragment *Hind*III-Q) and terminal fragment *Hind*III-H supplies additional proof for our proposed map. On the basis of the above data, we ordered the *Hind*III fragments from right to left as Q, X, (V, W), H. *Eco*RI-B hybridized to *Bgl*II fragments F/G, P, and e (Fig. 7; the hybridization to fragment e can be seen when the autoradiogram is overexposed); since *Eco*RI-B also hybridized to *Hind*III-X and we mapped *Hind*III-X within *Bgl*II-F, *Eco*RI-B must contain sequences in common with *Bgl*II-F. Digestion of *Eco*RI-B with *Bgl*II yielded three fragments with molecular weights of 6.6×10^6 , 4×10^6 , and $0.63 \times 10^6 M_r$. The fragment with a molecular weight of $0.63 \times 10^6 M_r$ corresponds to *Bgl*II fragment e. Therefore, *Bgl*II-e must be located between *Bgl*II-P and the terminal *Bgl*II-F. Since *Eco*RI-T also hybridized to *Bgl*II-P, we placed *Eco*RI-T to the left of *Eco*RI-B. *Eco*RI-T also hybridized to *Bgl*II-I, the other terminal *Bgl*II fragment which shared sequences with *Bgl*II-F. Since *Eco*RI-X also hybridized to *Bgl*II-I, we placed *Eco*RI-X to the left of *Eco*RI-T. As discussed above, the *Eco*RI terminal fragment N also hybridized to *Bgl*II-I and *Hind*III-H. Thus, *Eco*RI-N mapped to the left of *Eco*RI-X (see Fig. 12). Consistent with the order of the *Eco*RI fragments being B, T, X, N (from right to left) is the observation that *Eco*RI fragments B and T hybridized to the same partial *Eco*RI fragment, and *Eco*RI fragments T and X hybridized to the same partial *Eco*RI fragment (Fig. 9). We also noted that when the *Hind*III autoradiograms were overexposed, *Eco*RI fragments B, T, and

X hybridized slightly to higher-molecular-weight *Hind*III fragments which probably correspond to the *Hind*III joint pieces resulting from the relative inversion of the U_L and U_S regions of the genome. We have already determined that *Eco*RI terminal fragments N and W (in the inverted orientation) form the L-S joint piece *Eco*RI-H, which we have placed to the left of *Eco*RI fragment X. Our mapping data indicated that three additional *Eco*RI fragments (O, h, and a) also contained sequences from the inverted repeat surrounding the U_L region. For ease of comparison, we have presented the hybridization data for these fragments in the figures and tables showing the hybridization of subfragments of the cloned joint pieces (Fig. 4, 5, and 6; Tables 3, 4, and 5). The results of the hybridization of *Eco*RI fragments O and a are also shown in the composite figures representing the entire genome (Fig. 7 and 8). We mapped *Eco*RI-O ($M_r = 4.55 \times 10^6$) to the left of *Eco*RI-H. Several lines of evidence indicate that this fragment is contained entirely within the inverted repeats bounding the U_L segment. First, digestion of the HCMV genome with *Eco*RI yielded 2 mol of fragment O. Second, fragment O hybridized to the same *Bgl*II and *Hind*III fragments as the small *Bam*HI fragment (B2 from the *Eco*RI joint fragments discussed above), which we have also mapped entirely within the repeats bounding the U_L sequence. *Eco*RI fragment O hybridized to terminal *Hind*III fragments H/I and K (Fig. 6 and 8; Tables 5 and 7) as well as to some higher-molecular-weight bands, most likely representing the *Hind*III joint pieces. It also hybridized to terminal *Bgl*II fragments F/G and E (Fig. 5 and 7; Tables 4 and 6).

*Eco*RI fragment h ($M_r = 0.56 \times 10^6$) is located to the left of *Eco*RI fragment O. This fragment has an *Eco*RI site in both the repeat and the U_L region. As can be seen in Fig. 4, *Eco*RI fragment h cross-hybridized with *Eco*RI fragment a ($M_r = 2.3 \times 10^6$), which we mapped at the other end of the U_L region and which also included part of the repeat bounding the U_L segment. We assume that the cross-hybridization was due to the fact that the repeats bounding the U_L region were inversions of each other, but we have not rigorously proven this. *Eco*RI fragment h hybridized to the terminal *Hind*III fragments I and K and *Bgl*II fragments E and G (Fig. 5 and 6; Tables 4 and 5). Due to the small size of this fragment, we cannot detect in our autoradiograms hybridization to the higher-molecular-weight bands which would comprise the joint pieces.

U_L region: *Eco*RI fragments h, f, G, i, g, K, and c. *Eco*RI fragments h, f ($M_r = 0.95 \times 10^6$), G ($M_r = 8.3 \times 10^6$), i ($M_r = 0.54 \times 10^6$), g ($M_r = 0.6 \times 10^6$), and K ($M_r = 6.3 \times 10^6$) all hybridized

to *Bgl*II fragment E (Fig. 7 and Table 6). Since *Eco*RI fragment K also hybridized to *Bgl*II fragment V, we mapped *Eco*RI-K to the far left. To determine the map position of the other *Eco*RI fragments hybridizing to *Bgl*II fragment E, we examined the hybridization of these fragments to partial *Eco*RI digests (Fig. 9) and complete *Hind*III digests (Fig. 8) of HCMV DNA. *Eco*RI fragments f and G both hybridized to *Hind*III fragment I. Since *Eco*RI fragment f hybridized to the same partial *Eco*RI fragment as *Eco*RI fragment h (see Fig. 9), we mapped *Eco*RI fragment f between *Eco*RI fragments G and h. *Eco*RI fragment G also hybridized to one of the *Hind*III fragments N/O (we will designate it O). Consistent with this hybridization data was the observation that *Eco*RI fragment G was cleaved by *Hind*III into two fragments of molecular weights $2.7 \times 10^6 M_r$ (part of *Hind*III-I) and $5.6 \times 10^6 M_r$ (part of *Hind*III-O). Since *Eco*RI-i also hybridized to *Hind*III-O, we mapped *Eco*RI-i to the left of *Eco*RI-G. Both *Eco*RI fragments K and g hybridized to *Hind*III fragment Y; *Eco*RI fragment K, however, also hybridized to one of the *Hind*III fragments N/O (we will designate it N). Since *Eco*RI fragment g was totally included within *Bgl*II fragment E and *Hind*III fragment Y, we mapped the *Bgl*II fragments from right to left as E, V and the *Hind*III fragments I, O, Y, N. Hence, *Eco*RI fragment g mapped between *Eco*RI fragments K and i. We do not believe that *Eco*RI fragment g, however, is immediately adjacent to *Eco*RI fragment i. On the basis of the hybridization of both *Eco*RI fragments i and g to HCMV partially cleaved with *Eco*RI, it seems likely that between i and g are small *Eco*RI fragments of $\sim 0.2 \times 10^6 M_r$, which we have not yet identified. These fragments would span the *Hind*III site separating *Hind*III fragments O and Y and would total $\sim 0.6 \times 10^6 M_r$. *Eco*RI fragment K not only hybridized to *Bgl*II fragments E and V but also to one of the *Bgl*II fragments J/K/L/M (we will designate it *Bgl*II-J). Cleavage of *Eco*RI fragment K with *Bgl*II yielded three fragments with molecular weights of $1.9 \times 10^6 M_r$ (part of *Bgl*II-E), $3.7 \times 10^6 M_r$ (*Bgl*II-V), and $0.72 \times 10^6 M_r$ (part of *Bgl*II-J). On the basis of this information, we mapped *Bgl*II fragment V between *Bgl*II fragments E and J. Since *Eco*RI fragment c ($M_r = 1.65 \times 10^6$) also hybridized to both *Hind*III-N and *Bgl*II-J, we mapped *Eco*RI-c to the left of *Eco*RI-K.

U_L region: *Eco*RI fragments c, Y, S, Q, b, Z, and E. *Eco*RI fragments c ($M_r = 1.65 \times 10^6$), Y ($M_r = 2.5 \times 10^6$), S ($M_r = 3.45 \times 10^6$), and Q ($M_r = 4.35 \times 10^6$) all hybridized to one of the *Hind*III fragments J/K. With long electrophoresis times, it could be determined that they were hybridizing to the larger fragment *Hind*III-J; *Hind*III-K was a terminal fragment flanking the

U_L region. As discussed above, since *Eco*RI fragment c also hybridized to *Hind*III fragment N, we mapped *Eco*RI fragment c to the left of *Eco*RI fragment K. *Eco*RI fragments c, Y, and S also hybridized to one of the *Bgl*II fragments in the group J/K/L/M (we will designate it *Bgl*II-J). *Eco*RI fragment S also hybridized faintly to *Bgl*II fragment O. The presence of a *Bgl*II site in *Eco*RI-S was further indicated by the observation that digestion of cloned *Eco*RI-S with *Bgl*II reduced its size from 3.45×10^6 to 3.25×10^6 (Table 2 of reference 15). Since *Eco*RI fragments Q and b ($M_r = 1.9 \times 10^6$) also shared sequences with *Bgl*II fragment O, we ordered the *Eco*RI fragments from right to left as K, c, Y, S, Q, b. Examination of the hybridization data (Fig. 8) revealed that *Eco*RI fragment Q hybridized to both *Hind*III fragments J and Z, whereas *Eco*RI fragment b hybridized to one of the *Hind*III fragments L/M (we will call it M). Although we could not detect hybridization of *Eco*RI fragment b to *Hind*III fragment Z, we believe that there is some overlap since cleavage of *Eco*RI fragment b with *Hind*III (Table 2 of reference 15) reduced the size of this fragment by about $0.1 \times 10^6 M_r$. Thus, we ordered the *Hind*III fragments from right to left as N, J, Z, M. *Eco*RI fragments Z ($M_r = 2.4 \times 10^6$) and E ($M_r = 10.3 \times 10^6$) both hybridized to *Hind*III fragment M and *Bgl*II fragment U. In addition, *Eco*RI fragment E hybridized to one of the *Hind*III fragments E/F (we will arbitrarily designate it *Hind*III-E) and to *Bgl*II fragments T, g (the hybridization to *Bgl*II-g is not shown in Fig. 7) and J/K/L/M (we will designate it K). On the basis of this information, we mapped *Eco*RI fragment Z between *Eco*RI fragments E and b and *Hind*III fragment M between *Hind*III fragments Z and E. We also ordered the *Bgl*II fragments as J, O, U, g, T, K, but the order of T and g might be reversed. As can be seen in Fig. 9, the hybridization of the cloned *Eco*RI fragments to Southern blots of HCMV DNA partially cleaved with *Eco*RI also supports the order of the *Eco*RI fragments from right to left as K, c, Y, S, Q, b, Z, E.

We have verified that *Eco*RI fragments c, Y, S, Q, b, Z, and E are linked by analysis of a cosmid generously supplied to us by B. Fleckenstein. This cosmid (no. 106) was prepared by inserting an *Eco*RI partial cleavage product of HCMV DNA (strain AD169) into the cosmid pHCT9. When this cosmid was labeled to high specific activity by nick translation with [α^{32} P]dCTP and hybridized to a Southern transfer of *Eco*RI-cleaved HCMV virion DNA, seven fragments could be seen on the autoradiogram (Fig. 10A). These fragments corresponded to *Eco*RI fragments E, Q, S or T, X or Y, Z, b, and c. From the autoradiogram we could not distinguish which fragment of the closely migrating

FIG. 10. Analysis of cosmid no. 106 containing an *Eco*RI partial cleavage product of HCMV DNA. (A) A filter strip from a preparative Southern blot containing the *Eco*RI fragments of HCMV DNA was hybridized with 32 P-labeled cosmid no. 106 DNA. The filter strip labeled "CMV" represents 32 P-labeled HCMV DNA cleaved with *Eco*RI and subjected to electrophoresis in a parallel lane of the gel. (B) Cosmid no. 106 DNA was cleaved to completion with *Eco*RI, subjected to electrophoresis through a preparative agarose gel (0.8%), and transferred to a nitrocellulose filter. Identical strips from preparative Southern blot containing the *Eco*RI fragments of cosmid no. 106 DNA were hybridized with the labeled cloned HCMV *Eco*RI fragment indicated at the top of each strip. The filter strip labeled 106 was hybridized with 32 P-labeled cosmid no. 106 DNA.

fragments S, T, and X, Y the cosmid contained. Therefore, the cosmid DNA was cleaved with *Eco*RI, and the fragments were separated by electrophoresis through agarose and transferred to nitrocellulose filters. We then hybridized our labeled cloned fragments individually to Southern transfers of the cosmid DNA (Fig. 10B). These hybridizations confirmed that fragments E, Q, Z, b, and c as well as fragments S and Y were contained in this cosmid. The small amount of hybridization seen to a fragment comigrating with *Eco*RI fragment Q represented cross-hybridization of some contaminating plasmid pACYC184 with the plasmid portion of the cosmid. In summary, the presence of these seven *Eco*RI fragments in a single cosmid provided additional evidence for their linkage.

***U_L* region:** *Eco*RI fragments E, M, P, U, I, and A. *Eco*RI fragments E ($M_r = 10.3 \times 10^6$), M ($M_r = 5.0 \times 10^6$), and P ($M_r = 4.45 \times 10^6$) all hybridized to *Hind*III fragment E, and *Eco*RI fragments E and M both hybridized to *Bgl*II fragment K. Most of *Bgl*II fragment Z was included within *Eco*RI fragment M, but it did share some sequences with *Eco*RI fragment P. On the basis of these data, we mapped *Eco*RI

fragment M between *Eco*RI fragments E and P and *Bgl*II fragment Z to the left of *Bgl*II fragment K. *Eco*RI fragment P as well as *Eco*RI fragments U ($M_r = 2.85 \times 10^6$), I ($M_r = 7.4 \times 10^6$), and A ($M_r = 11.4 \times 10^6$) all hybridized to *Hind*III-D. We have established that *Eco*RI fragment P contains a *Hind*III site (see Table 2 of reference 15) and thus shares sequences with both *Hind*III-D and *Hind*III-E. To determine the order of the *Eco*RI fragments in *Hind*III-D, we used our *Bgl*II hybridization data as well as some of the cross-blot hybridization data of Westrate et al. (17). *Eco*RI fragment P, in addition to hybridizing slightly to *Bgl*II fragment Z, hybridized to *Bgl*II fragments Y, a, b or c (we will call it c), and Q or R (we will designate it R). Since Westrate et al. (17) have reported that their *Bgl*II fragments T, U, and V (our fragments Y, Z, and a, respectively) cross-hybridize with their *Hind*III fragments B/C (our fragments E/F; we have designated it *Hind*III-E), and *Bgl*II fragments analogous to our fragments a and c cross-hybridize with the *Hind*III fragment analogous to our *Hind*III-D, we established the order of the *Bgl*II fragments from right to left as K, Z, Y, a, c, R, with the *Hind*III cleavage site in *Bgl*II

fragment a. On the basis of the size of the fragments generated by *Bgl*II digestion of *Eco*RI-P (see Table 2 of reference 15), we believe that there is an additional small *Bgl*II fragment less than 0.4×10^6 daltons which is located between *Bgl*II-a and -c. *Eco*RI fragments U ($M_r = 2.85 \times 10^6$) and I ($M_r = 7.4 \times 10^6$) also hybridized to *Bgl*II fragment R. Since *Eco*RI fragment I also hybridized to *Bgl*II fragments X, f, and N, we mapped *Eco*RI fragment U between *Eco*RI fragments P and I. Cleavage of *Eco*RI-I with *Bgl*II yielded four fragments of molecular weights $2.55 \times 10^6 M_r$ (part of *Bgl*III-N), $1.9 \times 10^6 M_r$ (*Bgl*III-X), $0.52 \times 10^6 M_r$ (*Bgl*III-f), and $2.45 \times 10^6 M_r$ (part of *Bgl*III-R). On the basis of these data, we mapped the *Bgl*II fragments in the order (from right to left) R, f, X, N, but the order of f and X might be reversed. The hybridization of the cloned *Eco*RI fragments to partially cleaved *Eco*RI HCMV DNA also supported the *Eco*RI fragment order of E, M, P, U, I (see Fig. 9).

Finally, located to the left of *Eco*RI fragment I is *Eco*RI fragment A, which hybridized not only to *Bgl*II fragment N but also to *Bgl*II fragments S, d, and b. Cleavage of the cloned *Eco*RI fragment A with *Bgl*II yielded four fragments of molecular weights $5.3 \times 10^6 M_r$ (*Bgl*III-S), $4.8 \times 10^6 M_r$ (part of *Bgl*III-N), $0.79 \times 10^6 M_r$ (*Bgl*III-d), and $0.54 \times 10^6 M_r$ (part of *Bgl*III-b). On the basis of this information, we ordered the *Bgl*II fragments as N, (S,d), b, where the order of S and d is uncertain. *Eco*RI fragment A also hybridized to *Hind*III fragments D, L, and c.

To confirm that *Eco*RI fragments E, M, P, U, I, and A were on the same linkage group, we analyzed another cosmid supplied by B. Fleckenstein. This cosmid (no. 1029) contains a *Hind*III partial cleavage product of HCMV DNA which corresponds to the *Hind*III fragments D and E. When this cosmid was labeled to high specific activity by nick translation with [α^{32} P]dCTP and hybridized to a Southern transfer of *Eco*RI-cleaved virion DNA, six bands could be distinguished on the autoradiogram (Fig. 11A). These bands corresponded to an *Eco*RI fragment in the group A/B/C/D, fragment E, a fragment in the group F/G/H/I, a fragment in the group M/N, a fragment in the group O/P/Q, and fragment U. To determine which fragments were contained in the cosmid, we hybridized labeled cloned *Eco*RI fragments individually to a Southern transfer of the cosmid after cleavage with *Hind*III (Fig. 11B). Our hybridization results demonstrated that the cosmid contained the *Eco*RI fragments A, E, I, M, P, and U. Furthermore, we could determine from the autoradiogram that *Eco*RI fragments A, I, and U were hybridizing to the higher-molecular-weight *Hind*III fragment D, *Eco*RI fragments

M and E were hybridizing to the lower-molecular-weight *Hind*III fragment E, and *Eco*RI fragment P was hybridizing to both *Hind*III-D and -E. *Eco*RI-U comigrated with the plasmid pA-CYC184 and therefore could not be separated from the plasmid by gel purification. Thus, our preparations of labeled *Eco*RI-U also cross-hybridized with the plasmid portion of the cosmid which is the fastest-migrating band on the autoradiogram. These data further supported our mapping results and confirmed that *Eco*RI fragments E, M, P, U, I, and A were linked.

U_L region: *Eco*RI fragments A, D, V, C, and R. Both *Eco*RI fragments A ($M_r \approx 11.4 \times 10^6$) and D ($M_r \approx 11.2 \times 10^6$) hybridized to *Bgl*II-b and *Hind*III-c. Hence, we mapped *Eco*RI-D to the left of *Eco*RI-A. *Eco*RI-D also hybridized to *Bgl*III-H and *Hind*III fragments P, U, a, b, and c (see Fig. 8). Digestion of *Eco*RI-D with *Hind*III yielded four distinguishable fragments of M_r 4.4×10^6 (part of *Hind*III-P), 3.9×10^6 (*Hind*III-U), 1.4×10^6 (*Hind*III-a), and 0.88×10^6 (*Hind*III-b). We could not see *Hind*III fragment c or a portion of fragment c in our double-digestion gels mainly due to the large amount of RNA in that region. On the basis of these data, we ordered the *Hind*III fragments as L, c, (b,U,a), P, where the exact order of b,U,a is uncertain. Since *Eco*RI-V ($M_r = 2.8 \times 10^6$) also hybridized to *Hind*III-P and slightly to *Bgl*III-H, we placed *Eco*RI-V to the left of *Eco*RI-D. In addition, *Eco*RI-V hybridized to either *Hind*III fragment S or T (we will arbitrarily designate it S) and *Bgl*II fragment W. Since *Eco*RI fragment C ($M_r \approx 11.2 \times 10^6$) also hybridized faintly to *Bgl*III fragment W and extensively to a *Hind*III fragment in the group S/T, we mapped *Eco*RI-C to the left of *Eco*RI-V. *Eco*RI-C also hybridized to *Hind*III-R. Digestion of *Eco*RI-C with *Hind*III yielded three fragments with molecular weights of $4.6 \times 10^6 M_r$ (fragment R), $4.2 \times 10^6 M_r$ (fragment T), and $2.4 \times 10^6 M_r$ (part of fragment S). On the basis of this information, the order of the *Hind*III fragments from right to left is either P, S, R, T or P, S, T, R. To resolve this point, we used the following information: *Eco*RI fragment C, in addition to hybridizing faintly to *Bgl*III fragment W, hybridized to *Bgl*II fragment J/K/L/M (we will call it M) and *Bgl*II fragment Q or R (we will designate it Q, since *Bgl*II fragment R has already been mapped). Digestion of *Eco*RI fragment C with *Bgl*II revealed that all of *Bgl*III fragment M was included within *Eco*RI fragment C, whereas only part of *Bgl*III fragment Q overlapped with *Eco*RI fragment C. Since Weststrate et al. (17) have reported that both *Hind*III fragments analogous to our *Hind*III-S and -T are cleaved by *Bgl*II, whereas the *Hind*III fragment analogous to our *Hind*III-R is not cleaved, we established that the order of the *Hind*III frag-

FIG. 11. Analysis of cosmid no. 1029 containing a *Hind*III partial cleavage product of HCMV DNA. (A) A filter strip from a preparative Southern blot containing the *Eco*RI fragment of HCMV DNA was hybridized with 32 P-labeled cosmid no. 1029 DNA. The filter strip labeled "CMV" represents 32 P-labeled HCMV DNA cleaved with *Eco*RI and subjected to electrophoresis in a parallel lane of the gel. (B) Cosmid no. 1029 DNA was cleaved to completion with *Hind*III, subjected to electrophoresis through a preparative agarose gel (0.8%), and transferred to a nitrocellulose filter. Identical strips from the preparative Southern blot containing the *Hind*III fragments D and E of cosmid no. 1029 DNA were hybridized with the 32 P-labeled cloned *Eco*RI fragments indicated at the top of each strip. The filter strip labeled 1029 was hybridized with 32 P-labeled cosmid no. 1029 DNA.

ments is P, S, R, T and that the order of the *Bgl*II fragments is W, M, Q. These fragments are arranged such that *Bgl*II fragment M includes all of *Hind*III fragment R and part of both *Hind*III fragments S and T. To the left of *Eco*RI fragment C is located *Eco*RI fragment R ($M_r = 3.9 \times 10^6$), which also hybridized to *Bgl*II fragment Q.

***U_L* region:** *Eco*RI fragments R, d, e, J, and a. *Eco*RI fragments R ($M_r = 3.9 \times 10^6$), d ($M_r = 1.1 \times 10^6$), e ($M_r = 1.0 \times 10^6$), J ($M_r \approx 6.8 \times 10^6$), and a ($M_r = 2.3 \times 10^6$) all hybridized to one of the fragments in the *Hind*III-E/F group. Since we have already mapped *Hind*III-E, we will designate this fragment as *Hind*III-F. *Eco*RI

fragment R, in addition to hybridizing to *Bgl*II fragment Q, also hybridized to *Bgl*II fragment J/K/L/M (we will designate it *Bgl*II-L, since *Bgl*II-J, -K, and -M have already been mapped). *Eco*RI fragments d, e, and J also hybridized to *Bgl*II fragment L. On the basis of the hybridization of *Eco*RI fragments R, d, and e to HCMV partially cleaved with *Eco*RI (see Fig. 9), we determined their order to be (from right to left) R, d, e. Since *Eco*RI fragment J hybridized to both *Bgl*II fragment L and terminal *Bgl*II fragment G, we mapped *Eco*RI fragment J to the left of *Eco*RI fragment e. On the other side of *Eco*RI-J is located *Eco*RI-a, which includes se-

quences from both the terminal repeat and U_L region. *EcoRI*-a hybridized to *HindIII*-F, the terminal fragments *HindIII*-K and *BglII*-G, and faintly to the terminal fragment *HindIII*-I due to homology in the inverted terminal repeat (see Fig. 5 through 8 and Tables 4 through 7). To conclude our map, next to *EcoRI*-a are the *EcoRI* fragments O and W, which are included in the terminal repeat.

DISCUSSION

We have cloned *EcoRI* fragments representing the entire genome and have prepared large amounts of these fragments from the recombinant bacteria. The location of each cloned *EcoRI* fragment was determined by hybridization to Southern blots of fragments generated either by complete cleavage of the genome with the enzymes *BglII* and *HindIII* or by partial cleavage with *EcoRI*. This information, coupled with the data derived from digestion of the cloned *EcoRI* fragments with *BglII* and *HindIII* presented in the accompanying paper (15), has resulted in the detailed physical map of strain AD169 HCMV shown in Fig. 12. From our restriction endonuclease maps, we can estimate

that the AD169 HCMV genome has a molecular weight of 158×10^6 (240 kb). The genome is divided into a long region of 198 kb and a short region of 42 kb. The repeats bounding the U_L segment are approximately 12 kb, whereas those bounding the U_S segment are approximately 2 kb. These values are similar to those reported for the Towne strain of HCMV (8).

In the past, the construction of restriction endonuclease maps involved cross-blot hybridization and comparisons of fragments produced with different enzymes, partial cleavages, and recleavage of isolated fragments with a second enzyme. Although feasible with small genomes, these experiments were inherently difficult with large DNA genomes such as HCMV. It was impossible to find enzymes which cleaved such genomes only a limited number of times and to fractionate and isolate the many comigrating fragments. Most fragments isolated from gels were still contaminated with variable amounts of sequences from the remainder of the genome. This sequence contamination often led to ambiguous results when the isolated fragments were used in hybridization or double-digestion experiments. The use of cloned fragments has eliminated these problems.

FIG. 12. Map of the HCMV genome strain AD169 for the enzymes *EcoRI*, *HindIII*, and *BglII*. Only the prototype orientation is shown. The orientation of the following fragments is unknown: *HindIII*-a, -U, and -b; *BglII*-d and -S; *BglII*-X and -f; and *BglII*-T and -g. The regions in the *EcoRI* map between fragments g and i and in the *BglII* map between fragments c and a likely contain one or more additional small fragments less than 0.4×10^6 daltons. The long and short inverted repeat sequences are indicated by the slanting lines.

The identification of terminal fragments for large genomes has also been a problem for herpesvirus genomes. Traditional methods, which involved processive digestion with or without repair synthesis or end labeling, often led to uninterpretable results, especially if the genome contained many nicks and gaps. The method used in this paper, which involved blunt-end ligation of ^{32}P -labeled *EcoRI* linkers to the ends of the genomes followed by digestion with *EcoRI* and fractionation by electrophoresis through agarose, permitted an unambiguous identification of the terminal *EcoRI* fragments. These results were later confirmed when cloned fragments representing the L-S junction were obtained. These joint fragments represented the termini in the inverted orientation of the genome. Blot hybridization experiments with subfragments from these cloned joint pieces allowed us to identify the *BglII* and *HindIII* terminal fragments and to align them with respect to the *EcoRI* termini. For the enzymes *BglII* and *HindIII*, four terminal fragments were detected, whereas for *EcoRI*, only three major terminal fragments were detected. These results conform to predictions of restriction endonuclease cleavage products assuming the presence of four related genomes with relative inversion of the S and L regions. *BglII* and *HindIII* cleave within the unique regions of L and S, resulting in four terminal fragments, whereas *EcoRI* cleaves within one set of repeats (those bounding the U_L region) and also within the unique regions of S.

From our results, however, we cannot be certain that all four possible orientations are presented equally in the population of HCMV molecules. According to the model originally developed for the herpes simplex virus genome, when an endonuclease cleaves only within the unique sequences, the presence of four 0.25 M joint fragments and four 0.5 M terminal fragments in the electrophoresis pattern indicates that four equimolar orientations of the DNA molecules are possible (1, 4). With HCMV, we can discern submolar fragments. However, their molarity is difficult to determine for two reasons. First, many of the submolar fragments are large and comigrate with other fragments. Second, there appears to be heterogeneity in the sizes of the terminal and the joint fragments such that they form a stepwise pattern on gels.

The heterogeneity in the terminal and joint fragments has also been observed for herpes simplex virus (10, 11, 14, 16) and the Towne strain of HCMV (8). LaFemina and Hayward (8) have presented preliminary data which indicate that the two terminal fragments from the S region of the Towne HCMV genome (in the two possible orientations) can each be resolved into at least three subspecies differing in size by 750

base pairs, with the intermediate-sized subspecies being the most abundant. Our results demonstrate that a similar situation exists for the AD169 strain of HCMV. This pattern of three subspecies for the terminal fragments bounding the S region can be seen most clearly for terminal fragment *HindIII*-Q, although it can also be discerned for the terminal *EcoRI* fragments L and N. These subspecies appear to differ in size by approximately 600 to 700 base pairs. In the case of the AD169 strain of HCMV, we have also observed heterogeneity in the repeats bounding the L region. The terminal *EcoRI* fragment W which maps entirely within the L repeats can be resolved into multiple subspecies differing in size by about 230 base pairs. The origin of this heterogeneity is currently under investigation in our laboratory.

The *BglII* and *HindIII* maps presented in this paper differ in several respects from those published by Westrate et al. (17; see Fig. 10) for the same strain of HCMV. These differences are most notable at the termini and the left half of the U_L segment of the genome. In discussing the following differences, we will use our lettering scheme and refer to their corresponding letters in parentheses. For the *HindIII* map:

(i) *HindIII* fragment K (G) and not J (F) is a terminal fragment.

(ii) Westrate et al. (17) did not detect a *HindIII* terminal fragment comparable in molecular weight to our *HindIII* fragment Q. The *HindIII* fragment which they placed at one of the termini is comparable in molecular weight to our *HindIII* fragment V, which we have mapped in the U_S region of the genome.

(iii) *HindIII* terminal fragments K (G) and I (D) flank the U_L segment and not the U_S segment. Likewise, *HindIII* fragments H (E) and Q flank the U_S segment.

(iv) Our orientation of *HindIII* fragments T (O), R (M), S (N), P (L), a (V), U (P), b (W), c (X), and L (I), which map in the left-hand region of the U_L region, is a direct inversion of the orientation shown by Westrate et al. (17).

(v) Likewise, our order of the *HindIII* fragments D (A) and E (C) in the middle of the U_L region is a direct inversion of orientation shown by Westrate et al. (17).

(vi) *HindIII*-Y (T) maps between *HindIII*-N (J) and -O (K). This fine mapping could be accomplished only with overlapping *EcoRI* fragments.

For the *BglII* map:

(i) Westrate et al. (17) mapped a fragment comparable to our terminal fragment *BglII*-I in the U_L region (their *BglII*-E). Instead, they identified a smaller terminal fragment with molecular weight comparable to that of our *BglII*-J (their *BglII*-H) which we have mapped in the

same region of the U_L sequence in which they mapped their *Bgl*II-E.

(ii) There are four fragments and not three in the group J/K/L/M (F/G/H).

(iii) There is only one *Bgl*II fragment of $M_r 1.9 \times 10^6 = X$ (S/S').

(iv) There is only one *Bgl*II fragment of $M_r 1.35 \times 10^6 = Y$ (T/T').

(v) There are two *Bgl*II fragments of $M_r 0.88 \times 10^6$ to $0.9 \times 10^6 = b, c$ (W).

(vi) There is at least one and probably two additional smaller *Bgl*II fragments of M_r less than 0.5×10^6 which were not detected by Westrate et al. (17).

(vii) As we pointed out for the *Hind*III map, the orientation of *Bgl*II fragments which map in the left-hand region of the U_L region is a direct inversion of the orientation shown by Westrate et al. (17). These fragments correspond to L (G), Q (L), M (F), W (R), H (D), b (W), and d (X). Likewise, the relative orientation of the small *Bgl*II fragments mapping in the middle of the U_L region (colinear with *Hind*III fragment D [A] and E [C]) is inverted. These fragments correspond to S (N), N (I), X (S), f (Z), R (M), c (W), a (V), Y (T), and Z (U). The fine-structure mapping of these small *Bgl*II fragments was accomplished with our overlapping cloned *Eco*RI fragments.

(viii) Westrate et al. (17) mapped the *Bgl*II fragments corresponding to our fragments T (O) and U (P) at the far left-hand end of the U_L region adjacent to the terminal fragment. We have mapped these fragments to the left of *Bgl*III-O (J) in the right end of the U_L region. The fourth fragment in the group J/K/L/M (designated K) also maps just to the left of *Bgl*III-T and -U.

The differences cited above in the *Bgl*II and *Hind*III maps are probably not the result of major differences between our stocks of AD169 HCMV, but rather due to the increased sensitivity afforded by the use of cloned fragments for mapping. We have directly confirmed the linkage of several fragments by using cosmids containing either *Eco*RI or *Hind*III partial cleavage products of the AD169 HCMV genome (provided by B. Fleckenstein). Furthermore, the *Eco*RI and *Hind*III restriction endonuclease maps constructed with these cosmids (B. Fleckenstein, I. Muller, and J. Collins, submitted for publication) are in complete agreement with our maps.

The restriction endonuclease maps presented in this paper should form the framework for fine-structure analysis of the HCMV genome and for further studies on the molecular biology of HCMV gene expression and replication.

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