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Characterization of the Guinea Pig Cytomegalovirus Genome by Molecular Cloning and Physical Mapping

MIN GAO AND HARRIET C. ISOM*

Department of Microbiology and Cancer Research Center, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

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Fragments of guinea pig cytomegalovirus (GPCMV) DNA produced by *Hin*dIII or *Eco*RI restriction endonuclease digestion were cloned into vectors pBR322 and pACYC184, and recombinant fragments representing ca. 97% of the genome were constructed. Hybridization of ^{32}P-labeled cloned and gel-purified *Hin*dIII, *Eco*RI, and *Xba*I fragments to Southern blots of *Hin*dIII-, *Eco*RI-, and *Xba*I-cleaved GPCMV DNA verified the viral origin of cloned fragments and allowed construction of *Hin*dIII, *Eco*RI, and *Xba*I restriction maps. On the basis of the cloning and mapping experiments, the size of GPCMV DNA was calculated to include 239 kilobase pairs, corresponding to a molecular weight of 158 × 10^6. No cross-hybridization between any internal fragments was seen. We conclude that the GPCMV genome consists of a long unique sequence with terminal repeat sequences but without internal repeat regions. In addition, GPCMV DNA molecules exist in two forms. In the predominant form, the molecules demonstrate sequence homology between the terminal fragments; in the minor population, one terminal fragment is smaller by 0.7 × 10^6 daltons and is not homologous with the fragment at the other end of the physical map. The structural organization of GPCMV DNA is unique for a herpesvirus DNA, similar in its simplicity to the structure reported for murine cytomegalovirus DNA and quite dissimilar from that of human cytomegalovirus DNA.

Infection of guinea pigs with guinea pig cytomegalovirus (GPCMV) causes a viremia followed by chronic, persistent infection (27, 62) in salivary gland, pancreas, and lymphoid tissues (23). GPCMV can cross the placenta and infect the fetus (6, 34), can be transmitted by blood transfusion (2), is immunosuppressive (72) and, therefore, is ideal for studying parallel clinical manifestations caused by human cytomegalovirus (HCMV) infection of humans (16, 25, 45, 51, 65, 69). In vitro, GPCMV can persistently infect guinea pig embryo fibroblast cells and can transform guinea pig liver cells (29). Insight into acute, latent, and persistent infections and into the oncogenic potential of GPCMV could be extended if more was known about the GPCMV genome and its gene products. We recently reported that (i) infectious GPCMV DNA can be purified in reasonable quantities, (ii) the virus DNA has a guanine plus cytosine content of 54.1%, (iii) the size of GPCMV DNA is ca. 120 × 10^6 daltons based on its ability to cosediment with T_4 DNA in a neutral sucrose gradient, and (iv) restriction endonuclease cleavage of GPCMV DNA with *Hin*dIII, *Xba*I, or *Eco*RI yields fragments easily separable by agarose gel electrophoresis (28).

The technology for preparation of recombinant plasmids containing HCMV DNA (17, 48, 61, 64) has made it possible to generate restriction endonuclease maps and to understand further the complex structural organization of the large HCMV genome (22, 57, 64). The availability of large quantities of pure subgenomic HCMV DNA fragments has already enabled study of the transcription of the immediately-early genes of HCMV (10, 30, 42, 57, 60, 63, 68) and examination of the sequences involved in transformation (46, 47).

Another animal cytomegalovirus used as a model for HCMV infection, particularly with regard to latency (41) and immunity (24, 31, 38, 39), is murine cytomegalovirus (MCMV). Restriction fragments of MCMV representing most of the genome have been cloned by two groups of investigators (13, 43). Physical mapping has revealed that MCMV DNA is ca. 235 to 240 kilobase pairs in size, and consists of a long unique sequence without large terminal or internal repeat regions. The molecular weight determined by mapping to be 155 × 10^6 is considerably larger than the molecular weights of 132 × 10^6 (44) and 136 × 10^6 (36) reported previously from velocity sedimentation studies.

HCMV DNA also consists of ca. 235 kilobase pairs; however, the molecule contains two unique stretches of DNA (a unique long segment of 195 kilobase pairs and a unique short segment of about 40 kilobase pairs) bordered by a pair of inverted repeats (11, 17, 19, 35, 36, 56, 64). Because of the structural organization of the genome, HCMV DNA molecules exist in four isomeric conformations, whereas MCMV DNA only has one form. The organization of the MCMV genome demonstrates major differences between MCMV and HCMV DNAs even though extensive similarities in the biology and pathogenicity of the two viruses have been demonstrated. Because GPCMV infection of guinea pigs so closely resembles the human system, we wanted to determine the organization of the GPCMV genome so that it could be compared with those of HCMV and MCMV. In this paper, we describe cloning of the *Hin*dIII and *Eco*RI fragments of GPCMV into plasmid vectors pBR322 and pACYC184 and construction of *Hin*dIII, *Eco*RI, and *Xba*I cleavage maps for GPCMV DNA.

MATERIALS AND METHODS

Cells and virus. GPCMV strain 22122, generously provided by G. D. Hsiung (Veterans Administration Hospital, West Haven, Conn.) or purchased from the American Type Culture Collection (Rockville, Md.), was propagated in guinea pig embryo fibroblast or line 104 clone 1 (104C1) cells as previously described (28, 29). 104C1 cells, a benzo[*a*]pyrene-transformed and cloned line derived from strain 2

* Corresponding author.

guinea pig embryo cells (15), were kindly provided by C. H. Evans (National Cancer Institute, Bethesda, Md). Infectivity of GPCMV was measured by plaque assay (28).

Purification of GPCMV DNA. Virus DNA was radioactively labeled in vivo by incubating infected cultures with 3 to 10 μCi of [^{3}H]thymidine (80.1 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml of medium as described previously (28). Virions were purified by centrifugation at 4,000 × *g* for 10 min (28). Virus DNA was extracted from virions by a modification of the method of Stinski et al. (59). The extraction buffer consisted of a 1% Sarkosyl solution pretreated with proteinase K for 30 to 60 min at 37°C. Purified virions were incubated with the extraction buffer supplemented with an additional 150 μg of proteinase K per ml. After incubation for 1 h at 37°C, an additional 200 μg of proteinase K per ml was added, and the mixture was brought to 0.5% with sodium dodecyl sulfate. Incubation at 37°C was continued for 1.5 to 2.0 h. Released viral DNA was extracted with phenol-chloroform-isoamyl alcohol (25:25:1). Sodium acetate was added to the aqueous phase to a final concentration of 0.3 M, and the DNA was precipitated with 2 volumes of isopropanol. The pellet was suspended in TE buffer (10 mM Tris-hydrochloride [pH 8.0] supplemented with 1 mM EDTA), and the DNA was centrifuged in CsCl density gradients (average density, 1.710 g/cm^3). GPCMV DNA peak fractions were pooled and dialyzed against TE buffer.

Exonuclease digestion. GPCMV DNA was incubated with 1.5 U of lambda exonuclease (BioLab, Inc., Beverly, Mass.) per μg of DNA in the recommended buffer at room temperature for various times up to 60 min. The enzyme reaction was terminated by extraction with an equal volume of chloroform-isoamyl alcohol (24:1), and the DNA was precipitated with sodium acetate and isopropanol. The pellet was washed with 70% and then with 100% ethanol, suspended in the appropriate enzyme buffer, and cleaved with a specific restriction endonuclease, and the fragments were separated by agarose gel electrophoresis. Parallel sets of fragments were subjected to electrophoresis on 0.4 and 1.0% agarose gels to visualize the large and small DNA fragments, respectively. Gels were stained with ethidium bromide (2 μg/ml) and photographed under UV illumination on Polaroid type 667 film.

Construction of recombinant plasmids. Recombinant DNA studies were conducted as described in the National Institutes of Health Recombinant DNA Research Guidelines. The pBR322 (3) and pACYC184 (5) plasmids were propagated in *Escherichia coli* HB101 (*recA*) (4). Plasmid DNA was extracted from bacteria and purified by equilibrium cesium chloride-ethidium bromide centrifugation (8). GPCMV, pBR322, and pACYC184 DNAs were independently cleaved with *Hin*dIII or *Eco*RI as previously described for GPCMV DNA (28). After digestion, GPCMV DNA was extracted with phenol-chloroform-isoamyl alcohol (25:25:1). The DNA solution was adjusted to 0.3 M sodium acetate, precipitated with isopropanol, and suspended in TE buffer. Restriction endonuclease-cleaved pBR322 and pACYC184 DNAs were treated with calf intestinal alkaline phosphatase (Collaborative Research, Inc., Waltham, Mass.) at a concentration of 0.28 U/μg of DNA according to the manufacturer's recommended conditions. Calf alkaline phosphatase-treated digested plasmid DNAs were extracted with phenol-chloroform-isoamyl alcohol (25:25:1), precipitated with isopropanol, and suspended in TE buffer in the same manner as for cleaved GPCMV DNA. Just before ligation, virus fragments and plasmid DNAs were heated at 70°C for 3 to 5 min to assure the linearity of the DNAs. GPCMV *Hin*dIII restriction fragments were ligated to the vector by incubating 1 μg of GPCMV DNA, 0.32 μg of pBR322 DNA, and 400 U of DNA ligase (BioLab, Inc.) in 0.05 M Tris (pH 7.8)–0.01 M MgCl–0.02 M dithiothreitol–0.001 M ATP–bovine serum albumin (0.5 mg/ml) at 14°C (±1°C) for 3 to 5 h. GPCMV *Eco*RI restriction fragments were ligated to the pACYC184 vector by the same protocol except that for each 1.0 μg of GPCMV DNA, 0.5 μg of pACYC184 DNA was used. The ligated DNAs were transfected into *E. coli* HB101 by the calcium shock method (40).

To isolate recombinants of pBR322 containing GPCMV *Hin*dIII DNA fragment inserts, transformants were selected on agar plates containing 100 μg of ampicillin per ml and tested for sensitivity to 50 μg of tetracycline per ml. To isolate recombinants of pACYC184 containing GPCMV *Eco*RI DNA fragment inserts, transformants were selected on agar plates containing 50 μg of tetracycline per ml and tested for sensitivity to 10 μg of chloramphenicol per ml. Positive colonies were picked and grown overnight in NZYM medium (10 g of NZ amine, 5 g of NaCl, 5 g of yeast extract, 2 g of $MgSO_4 \cdot 7H_2O$ per liter) with either 100 μg of ampicillin or 50 μg of tetracycline per ml under continuous agitation at 37°C. A portion of these cultures was mixed with an equal volume of glycerol and stored at −20°C. DNA was extracted from the remaining culture by the rapid method (8). This procedure was used as the initial screen for recombinants containing GPCMV DNA fragments.

Gel purification of GPCMV DNA fragments. In some experiments, the GPCMV DNA fragments were fractionated by size before ligation to plasmids. A modification of the method of Dretzen et al. (12) was used for gel isolation of specific DNA fragments. After restriction endonuclease cleavage, the DNA was electrophoretically separated on 0.3 to 1.0% agarose gels depending upon the size of the desired fragment. To recover sufficient quantities of specific fragments, a 26-mm-wide gel lane (instead of 8 mm for analytical gels) was used and fivefold more DNA was loaded than had been used routinely for gel analysis. The gel was stained with ethidium bromide (2 μg/ml) and observed under UV light. Horizontal cuts below and above the band to be recovered were made in the gel with a razor blade. A DEAE-NA45 membrane (Schleicher & Schuell, Inc. Keene, N.H.) was placed into each cut, and the gel was replaced in the electrophoresis apparatus and put in the dark, and electrophoresis was continued for 3 to 4 h. The membrane piece in the cut below the desired band was removed, washed with electrophoresis buffer, placed in a 1.5-ml conical tube containing 0.5 ml of 0.02 M Tris (pH 7.5)–0.002 M EDTA–1.5 M NaCl–0.05 M arginine, and incubated at 70°C for 4 to 5 h. The eluate was extracted with *n*-butanol-saturated TE buffer. The solution was made 0.3 M with sodium acetate, and the DNA was precipitated with isopropanol, washed with ethanol, and suspended in the appropriate buffer.

Fragments purified by gel isolation were used to ligate to plasmid DNA to clone fragments that were not obtained with unfractionated GPCMV DNA. Gel-purified fragments were recleaved with the same restriction endonuclease used initially, placed again in a large well on a second agarose gel, reisolated, and used to prepare in vitro ^{32}P-labeled probe DNA for *Hin*dIII or *Eco*RI fragments that could not be cloned and for specific *Xba*I fragments used for mapping.

Characterization of cloned inserts. Electrophoretically separated restriction endonuclease-cleaved GPCMV DNA was denatured, neutralized in situ, and transferred from the gel to nitrocellulose paper (Schleicher & Schuell, Inc.) by the method of Southern (55) or to 2-aminophenylthioether (APT)

TABLE 1. Size of *Hin*dIII, *Eco*RI, and *Xba*I fragments of GPCMV DNA

Band nomenclature (*Hin*dIII digest)		Size of fragments ($M_r \times 10^6$)	Band nomenclature (*Eco*RI digest)		Size of fragments ($M_r \times 10^6$)	Band nomenclature (*Xba*I digest)		Size of fragment ($M_r \times 10^6$)
New	Original[a]		New	Original		New	Original	
A	A	30.9	A[b]	A	27.9	A	A	22.4
B	B	18.0	B[b]		27.2	B	B	13.9
C	C	14.1	C	B	14.2	C	C	13.6
D	D	14.1	D	C	13.0	D	D	10.9
E	E	12.7	E	D	8.6	E	E	8.2
F	F	12.0	F	E	8.0	F[b]	F	7.8
G	G	11.6	G	F_1	6.9	G[b]	G	7.1
H	H	10.4	H	F_2	6.9	H	H	6.8
I	I	6.2	I	F_3	6.9	I	I	5.9
J	J	5.9	J	G	6.3	J	J	5.2
K	K	5.6	K	H	5.5	K	K	4.7
L	L	4.2	L	I_1	4.9	L	L	3.8
M[b]	M	3.4	M	I_2	4.9	M	M	3.5
N[b]	N	3.0	N	J	4.3	N[b]	N	3.2
O		2.7	O	K_1	3.9	O	O_1	2.8
P	O	2.7	P	K_2	3.9	P	O_2	2.8
Q	P	2.5	Q	L_1	3.6	Q	P_1	2.6
R[b]	Q	1.7	R	L_2	3.6	R	P_2	2.6
S	R	1.2	S	M_1	3.0	S	P_3	2.6
T	S	1.1	T	M_2	3.0	T	Q	2.3
U	T	0.5	U	N_1	2.0	U	R	2.2
			V	N_2	2.0	V	S_1	2.0
			W	O	1.9	W	S_2	2.0
			X	P	1.9	X	T_1	1.9
			Y[b]	Q	1.8	Y	T_2	1.9
			Z	R	1.7	Z	T_3	1.9
			a	S	1.5	a	U_1	1.7
			b	T	1.4	b	U_2	1.7
			c	U	1.3	c	V	1.5
			d	V	1.2	d	W_1	1.3
			e	W	1.1	e	W_2	1.3
			f	X	1.0	f	W_3	1.3
			g	Y_1	0.7	g	X	1.2
			h	Y_2	0.7	h	Y_1	1.2
			i	Y_3	0.7	i	Y_2	1.2
			j	Z	0.6	j	Y_3	1.2
						k	Y_4	1.2
						l	Z_1	0.7
						m	Z_2	0.7
						n	a	0.6

[a] See Isom et al. (28).
[b] Terminal fragment.

paper as described previously (55, 67). APT paper was prepared according to the procedure described by Seed (52). After DNA transfer to nitrocellulose, the filters were baked in a vacuum oven at 80°C for 2 h. Each nitrocellulose filter or APT paper was prehybridized for 18 h at 46°C in a solution containing 43% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt solution (1× Denhardt solution is 0.02% polyvinylpyrrolidone plus 0.02% Ficoll), 0.05 M sodium phosphate (pH 6.5), 1.0% glycine, and salmon sperm DNA (0.5 mg/ml). Cloned fragments to be labeled and used as probe DNA were prepared and purified by a modification of the method of Davis et al. (8). Purified cloned DNA fragments and gel-isolated fragments were ^{32}P labeled in vitro (49) with DNA polymerase I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and [α-^{32}P]dCTP and [α-^{32}P]TTP (3,000 Ci/mmol; Amersham, Arlington Heights, Ill.) to specific activities of 1×10^8 to 4×10^8 cpm/μg of DNA. ^{32}P-labeled DNA fragments were hybridized to DNA bound to nitrocellulose filters or APT paper at 46°C for 16 to 20 h in a solution containing 43% formamide, 5× SSC, 1× Denhardt solution, 0.02 M sodium phosphate (pH 6.5), salmon sperm DNA (0.1 mg/ml), and 10% dextran sulfate. After hybridization, DNA that failed to anneal was removed by washing the filters or papers three times with 2× SSC plus 0.1% sodium dodecyl sulfate at room temperature (5 min per wash) and three times with 0.1× SSC plus 0.1% sodium dodecyl sulfate at 50°C (30 min per wash). The dried filters or papers were subjected to autoradiography with intensifying screens and Kodak XAR-film at −76°C.

RESULTS

Identification and size of GPCMV DNA fragments after restriction endonuclease cleavage. We previously reported gel patterns for GPCMV DNA fragments after cleavage with the restriction endonucleases *Hin*dIII, *Eco*RI, and *Xba*I and assigned letter designations to each fragment (28). These values were obtained with DNA labeled in vivo. Cloning and

FIG. 1. Identification of cloned GPCMV *Hin*dIII fragments. GPCMV DNA and *Hin*dIII recombinant plasmids were digested with *Hin*dIII and subjected to electrophoresis on 0.5% (for fragments B through N, larger than pBR322) and 1.0% (for fragments P through T, smaller than pBR322) agarose gels. The DNA was blot transferred to nitrocellulose filters and hybridized to GPCMV DNA labeled with ^{32}P by nick translation.

mapping of the genome identified comigrating fragments and smaller fragments not found previously. The new and old letter designations for the fragments as well as fragment sizes are listed in Table 1. Cleavage of GPCMV DNA with *Hin*dIII yielded 21 major fragments ranging in size from 0.5 $\times 10^6$ to 30.9 $\times 10^6$ daltons; *Eco*RI cleavage yielded 36 major fragments ranging from 0.6 $\times 10^6$ to 27.9 $\times 10^6$ daltons; and *Xba*I cleavage yielded 40 major fragments ranging from 0.6 $\times 10^6$ to 22.4 $\times 10^6$ daltons. The *Hin*dIII digest was selected as the first to be cloned because it had the fewest fragments and the lowest number of low-molecular-weight fragments with only one fragment smaller than 1 $\times 10^6$ daltons. Because we anticipated difficulty in cloning the larger *Hin*dIII A fragment, we also cloned the *Eco*RI digest to enable construction of an entire GPCMV DNA library and to facilitate genome mapping.

Molecular cloning of GPCMV *Hin*dIII and *Eco*RI restriction endonuclease fragments. Molecular cloning of the GPCMV *Hin*dIII restriction fragments was carried out with unfractionated GPCMV DNA and the pBR322 vector. The cloned fragments were characterized by migration on agarose gels and compared with restriction endonuclease-cleaved virion DNA. Large fragments were subjected to electrophoresis in 0.5% agarose gels, whereas small fragments were analyzed in 1.0% agarose gels. The cloned inserts were determined to be of viral origin by their ability to hybridize with Southern (55) transfers of *Hin*dIII-cleaved virion DNA. A representative set of 15 clones containing 16 of the 21 *Hin*dIII fragments is shown in Fig. 1; the G + K clone contains both the G and K fragments (see below). Four of the remaining fragments (A, M, O, and R) were gel purified before ligation; however, attempts to clone these fragments failed. The O and P fragments migrated as a doublet. Attempts to purify O from gels always yielded O and P. *Hin*dIII-P was repeatedly cloned from the gel mixture, but O was never obtained. P was also cloned from unfractionated GPCMV DNA. The smallest fragment (*Hin*dIII-U) was not cloned. Double digestion with *Hin*dIII and *Eco*RI of a series of clones containing a GPCMV *Hin*dIII fragment (14.1 $\times 10^6$ daltons) showed that two distinct fragments (C and D) comigrated at this position in the gel (Fig. 2). The identity of C and D was confirmed by the inability of ^{32}P-labeled C to hybridize to blot transfers of *Hin*dIII and to *Eco*RI double-digestion products of the D fragment or of D to hybridize to cleavage products of C clones (data not shown). *Hin*dIII-C and -D were the only comigrating fragments of the *Hin*dIII digest of GPCMV.

Of the 36 GPCMV *Eco*RI fragments, 28 were individually cloned into the pACYC184 and pBR322 vectors. *Eco*RI-I and -f were cloned by using gel-isolated fragments. The yield of transformed colonies was lower with gel-purified than with unfractionated DNA, but greater than 50% of the colonies tested contained the desired DNA fragment. Double digestion and subsequent hybridization revealed that the *Eco*RI digest of GPCMV DNA contained several sets of overlapping fragments. The bands at 6.9 $\times 10^6$ daltons (*Eco*RI-G, -H, -I) and at 0.7 $\times 10^6$ daltons (*Eco*RI-g, -h, -i) each contained three comigrating fragments. Multiple attempts at cloning *Eco*RI-D or -E into the pACYC184 vector with unfractionated DNA or gel-purified DNA failed. This was surprising since the larger *Eco*RI C fragment had been

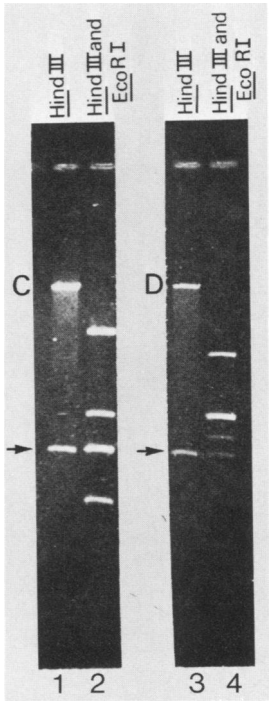

FIG. 2. Double digestion of GPCMV DNA *Hin*dIII fragments C and D. The *Hin*dIII C and D fragments from recombinant plasmids were digested with *Hin*dIII (lanes 1 and 3) and double digested with *Hin*dIII and *Eco*RI (lanes 2 and 4) and subjected to electrophoresis on 0.5% agarose gel. The gel was stained with ethidium bromide and photographed under UV illumination to visualize the DNA bands. The arrows indicate the pBR322 vector.

easily and repeatedly obtained. When, however, the same gel-purified preparations were ligated to pBR322, clones of both fragments were obtained. When *Eco*RI-D was cloned, 19% of the colonies with insert-containing plasmids had the desired fragment; for *Eco*RI-E, the value was 86%. A representative set of the GPCMV *Eco*RI fragments is shown in Fig. 3. *Eco*RI-T could not be cloned, perhaps because of a peculiarity in the virus DNA structure at this position in the genome; it is interesting that *Eco*RI-T is colinear with *Hin*dIII-K, a fragment which has only been cloned along with *Hin*dIII-G. *Eco*RI-A, -B, and -Y could not be cloned by our routine procedures because they are terminal fragments of the genome. The smallest *Eco*RI fragments, g, h, i, and j, also were not cloned.

Identification of GPCMV DNA terminal fragments. GPCMV DNA migrates as a broad band in alkaline sucrose gradients, suggesting that GPCMV DNA, like other herpesvirus DNAs (18, 32, 66), contains nicks and gaps. When GPCMV DNA was treated with lambda exonuclease, incubated with DNA polymerase I and [^{32}P]dCTP and [^{32}P]TTP, digested with *Hin*dIII or *Eco*RI, and subjected to agarose gel electrophoresis, all of the fragments in each digest were radioactively labeled. We wanted to identify the terminal fragments of GPCMV DNA by digestion with lambda exonuclease, which catalyzes the stepwise 5′→3′ removal of mononucleotides from double-stranded DNA with a terminal 5′ phosphate (37). If it is assumed that the nicks and gaps are randomly distributed, then treatment with lambda exonuclease will eventually result in digestion of all *Hin*dIII, *Eco*RI, and *Xba*I fragments; however, the terminal fragments should be digested more rapidly. GPCMV DNA was incubated with lambda exonuclease at a constant concentration (1.5 U/μg of DNA) for differing lengths of time (0 to 60 min). The DNA was then digested with *Hin*dIII, *Eco*RI, or *Xba*I and electrophoretically separated by agarose gel electrophoresis, and the resulting fragments were compared (Fig. 4). Lambda exonuclease treatment resulted in the loss of the following GPCMV DNA fragments: *Hin*dIII-M, -O, and -R; *Eco*RI-A and -Y; and *Xba*I-F, -G, and -N. This result was consistent with the finding that none of these fragments could be cloned.

Construction of *Hin*dIII, *Eco*RI, and *Xba*I cleavage maps of the GPCMV genome. The locations of the restriction endonuclease fragments in the GPCMV genome was determined by hybridizing ^{32}P-labeled fragments to blot transfers of total GPCMV DNA cleaved with *Hin*dIII, *Eco*RI, or *Xba*I. Identical digests of GPCMV DNA were separated on 0.5% agarose gels. The gels were stained and photographed, and the DNA was transferred to nitrocellulose membranes or APT paper. Individual gels included three sets of DNAs for each of the three enzymes. One set was hybridized against ^{32}P-labeled total GPCMV DNA, and the other sets were hybridized against ^{32}P-labeled cloned or gel-isolated fragments (Fig. 5). Each ^{32}P-labeled cloned *Hin*dIII or *Eco*RI fragment hybridized only to a single band (identical to itself in electrophoretic mobility) in its own digest. Cross-hybridization to multiple bands in each of the other two digests made it possible to align many of the fragments (Fig. 5; Table 2).

Use of only ^{32}P-labeled cloned *Hin*dIII and *Eco*RI fragments did not enable us to complete the linkage order. For

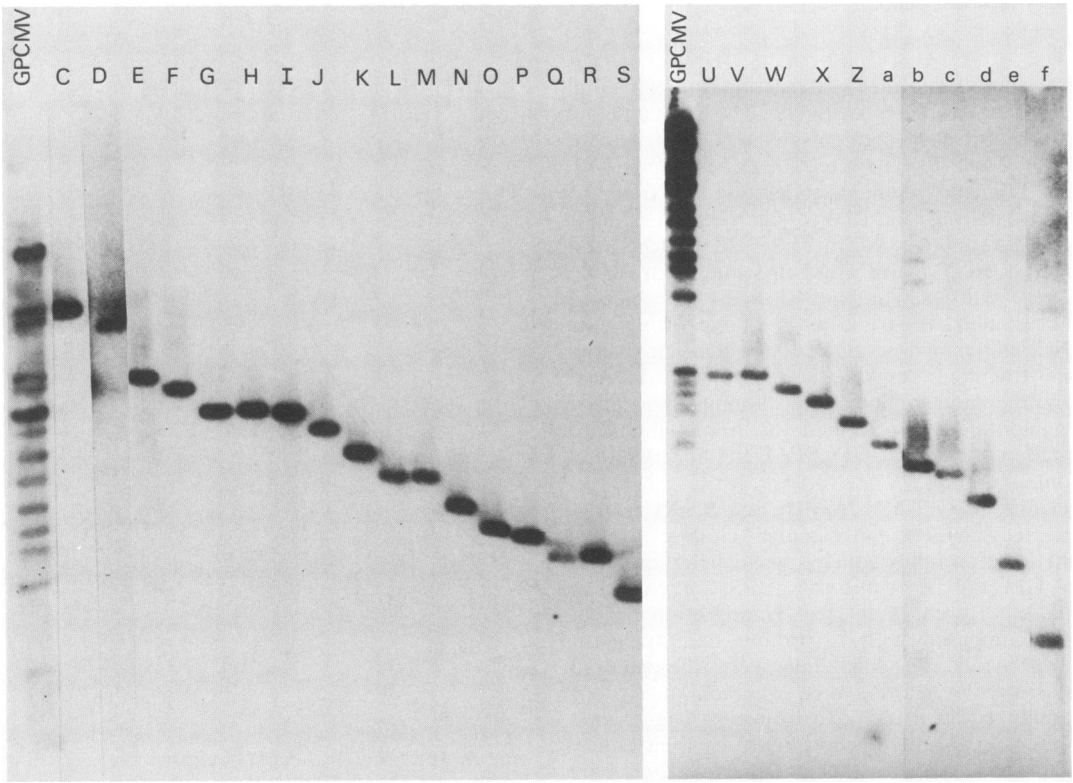

FIG. 3. Identification of cloned GPCMV *Eco*RI fragments. GPCMV DNA and *Eco*RI recombinant plasmids were digested with *Eco*RI and subjected to agarose gel electrophoresis. *Eco*RI fragments B through S, larger than the vector, were subjected to electrophoresis on 0.5% agarose gels. *Eco*RI fragments U through f, smaller than the vector, were subjected to electrophoresis on 1.0% agarose gels. The DNA was blot transferred to nitrocellulose filters and hybridized to GPCMV DNA labeled with ^{32}P by nick translation.

this purpose, specific gel-isolated ^{32}P-labeled *Hin*dIII, *Eco*RI, and *Xba*I fragments were used. To reduce contamination with neighboring fragments and partial digestion products, each uncloned fragment was isolated from the gel, redigested with the same restriction enzyme, rerun on a gel, reisolated, and labeled with ^{32}P to use as a probe. Data obtained with the three gel-isolated *Xba*I fragments appear in Table 3. When ^{32}P-labeled cloned *Hin*dIII-B was used as a probe, it hybridized with *Eco*RI-W, -Q, -G, -M, and -g and weakly with -A. Similarly, when gel-purified *Eco*RI-A was used as the probe, it hybridized with *Hin*dIII-E, -I, -L, -O, and -M and weakly with -B, suggesting that the *Hin*dIII and *Eco*RI restriction sites were very close to each other in this particular region. Because both *Hin*dIII-B and *Eco*RI-A hybridized with *Xba*I-H, labeled *Xba*I-H was used as a probe to verify the linkage order. This same strategy was used for mapping regions involving *Hin*dIII-A and -K and *Hin*dIII-S, -G, and -T.

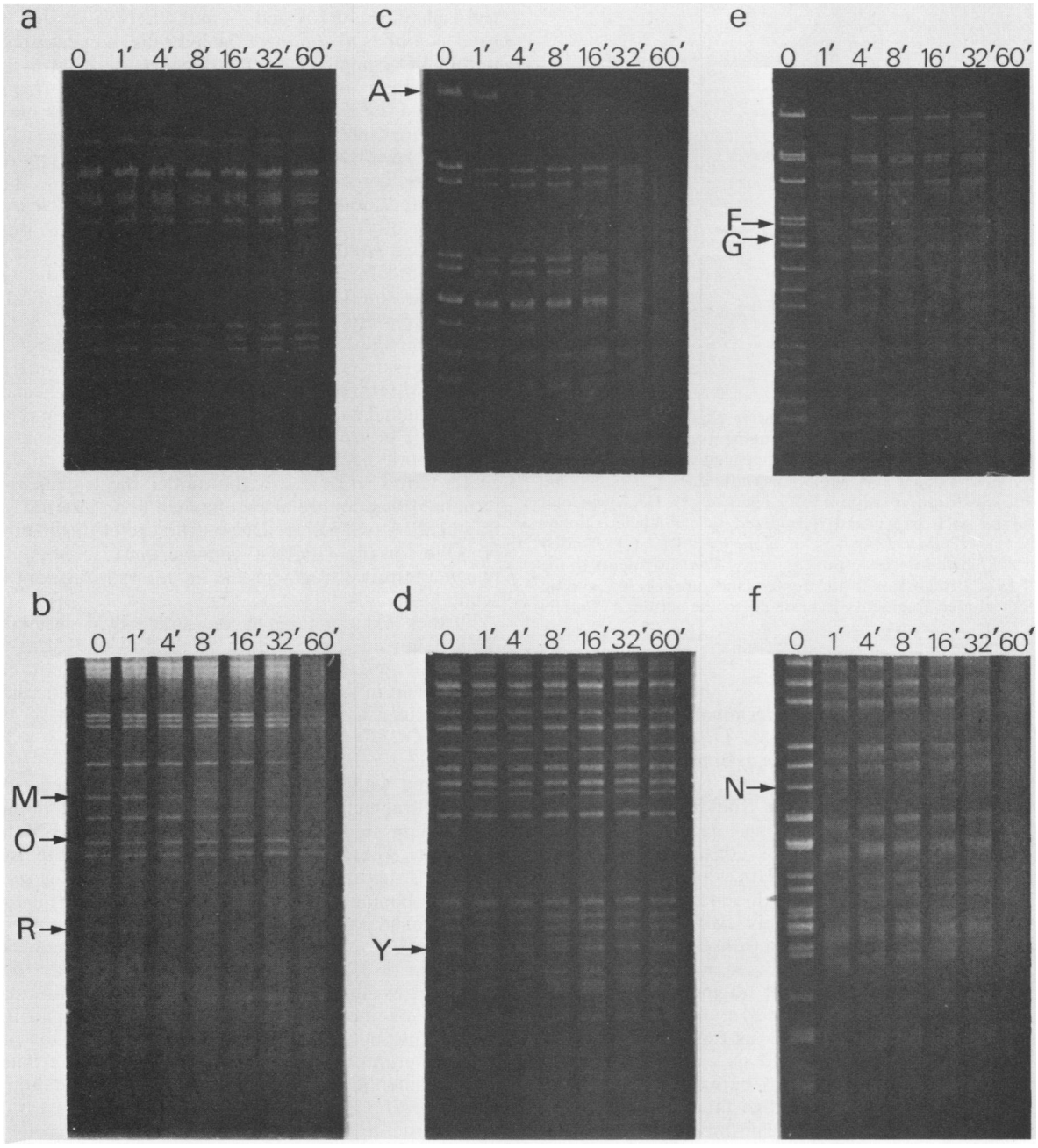

FIG. 4. Identification of the terminal fragments of GPCMV DNA by lambda exonuclease digestion. GPCMV DNA was digested with lambda exonuclease (1.5 U/μg of DNA) for various times (0 to 60 min) and then digested with *Hin*dIII (a, b), *Eco*RI (c, d), or *Xba*I (e, f) as described in the text. DNA fragments were electrophoretically separated on 0.4% agarose gels (a, c, e) to identify the large fragments or on 1.0% agarose gels (b, d, f) to identify the small fragments. The gels were stained with ethidium bromide and photographed under UV illumination to visualize the DNA bands. Lettered arrows indicate the terminal fragments.

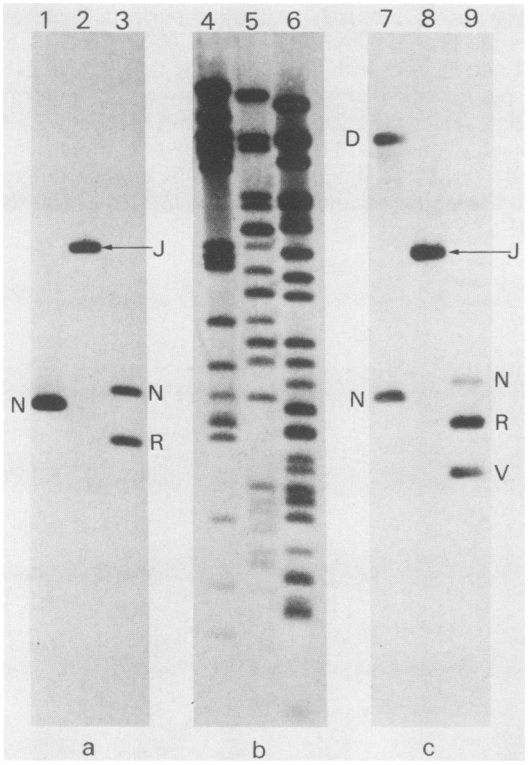

FIG. 5. Autoradiogram of representative Southern blot hybridization used to align GPCMV DNA fragments on the restriction endonuclease maps. GPCMV DNA was cleaved with *Hin*dIII (lanes 1, 4, and 7), *Eco*RI (lanes 2, 5, and 8), or *Xba*I (lanes 3, 6, and 9) and subjected to electrophoresis on a 0.5% agarose gel. DNA was blot transferred to APT paper and hybridized to ^{32}P-labeled cloned *Hin*dIII-N (a), GPCMV DNA (b), or cloned *Eco*RI-J (c). *Hin*dIII fragments are labeled to the left of the lanes, *Xba*I fragments to the right, and *Eco*RI fragments in the center lanes are labeled with an arrow. Both cloned fragments hybridized to the terminal *Xba*I N fragment.

Organization and structure of the terminal fragments of the GPCMV genome. GPCMV *Hin*dIII-M, -O, and -R, *Eco*RI-A and -Y, and *Xba*I-F, -G, and -N were assumed to be terminal fragments from the lambda exonuclease results and because these fragments could not be cloned from unfractionated or gel-purified DNA. Further analysis was necessary to establish a genome structure which was consistent with three terminal fragments and would explain why two restriction endonuclease digests demonstrated three terminal fragments whereas the *Eco*RI digest had only two. Each putative terminal fragment was twice purified from a gel, labeled, and tested as a probe. *Hin*dIII-M hybridized not only with itself but also with the *Hin*dIII-O and -R fragments in the same digest (Fig. 6). That is, cross-hybridization within a digest involved only terminal and not internal fragments. *Hin*dIII-M hybridized with *Xba*I-F, -G, and -N and with *Eco*RI-A and -Y. These cross-hybridizations between fragments in different restriction endonuclease digests involved only the putative terminal fragments and again included three fragments in the *Hin*dIII and *Xba*I digests but only two fragments in the *Eco*RI digest.

To verify the results, *Hin*dIII-R was isolated, labeled, and used as a probe. *Hin*dIII-R hybridized to itself; to *Hin*dIII-M but not to *Hin*dIII-O; to *Xba*I-N and -F but not to *Xba*I-G; and to both *Eco*RI-A and -Y (Fig. 6). It was not possible to isolate *Hin*dIII-O from a gel without simultaneously isolating *Hin*dIII-P. However, since *Hin*dIII-P (an internal fragment) had been cloned, we were able to test cloned *Hin*dIII-P and gel-isolated *Hin*dIII-(P + O) as probes to determine indirectly which fragments hybridized to *Hin*dIII-O (Fig. 7). *Hin*dIII-P hybridized only to itself in the *Hin*dIII digest; to *Eco*RI-P and -c; and to *Xba*I -Q and -e. *Hin*dIII-(P + O) hybridized to *Hin*dIII-P, -O, and -M; to *Eco*RI -P, -c, and -A; and to *Xba*I-Q, -e, -G, and -F. Therefore, we concluded that *Hin*dIII-O hybridized to *Hin*dIII-O and -M but not to -R; to *Eco*RI-A but not to -Y; and to *Xba*I-F and -G but not to -N. Gel-isolated *Eco*RI-A and -Y and *Xba*I-N, -F, and -G were used as probes to complete the hybridization patterns for the terminal fragments (Table 4). Because the *Xba*I-N fragment is larger than the *Hin*dIII-R or the *Eco*RI-Y fragment, it hybridized not only to these terminal fragments but also to neighboring internal fragments *Hin*dIII-N and *Eco*RI-J. Aligning *Hin*dIII-N and *Eco*RI-J next to the terminal fragments was consistent with the finding that these fragments disappeared more rapidly upon lambda exonuclease digestion than other internal fragments. This result, confirmed with cloned *Hin*dIII-N and *Eco*RI-J (Fig. 5) probes, indicated that the fragment order for one end of the GPCMV genome is *Hin*dIII-R, *Hin*dIII-N, *Eco*RI-Y, *Eco*RI-J, *Xba*I-N, and *Xba*I-R.

To complete the *Hin*dIII restriction endonuclease map it was necessary to identify locations for the *Hin*dIII-O and *Hin*dIII-M terminal fragments. One possible explanation for three terminal fragments would be a structure in which one end of the molecule can invert to form two isomers as has been reported for pseudorabies virus (1, 58) and varicella-zoster virus (14) DNAs. In this model, the sequences for one terminal fragment are also contained in an internal repeated fragment. A two-isomer DNA structure of this nature is not possible for the GPCMV genome because homology between a terminal fragment and an internal fragment was not seen.

Further examination of the unassigned terminal fragments showed that (i) both *Hin*dIII-O and *Xba*I-G were present in apparently submolar concentrations; (ii) *Eco*RI-A was present in supramolar concentration; (iii) the difference in molecular weight between *Hin*dIII-M (3.4×10^6) and *Hin*dIII-O (2.7×10^6) and between *Xba*I-F (7.8×10^6) and *Xba*I-G (7.1×10^6) was exactly 0.7×10^6; and (iv) *Hin*dIII-O hybridized with *Hin*dIII-M but not the assigned *Hin*dIII-R terminal fragment, just as *Xba*I-G hybridized with the *Xba*I-F fragment and not with the assigned *Xba*I-N terminal fragment. These data are consistent with the following genome structure for the *Hin*dIII and *Xba*I digests. There are two populations of GPCMV molecules (Fig. 8). The predominant population (ca. 70%) consists of molecules in which both terminal fragments contain repeat sequences of a maximum of 0.7×10^6 daltons (*Hin*dIII-R and *Hin*dIII-M, and *Xba*I-N and *Xba*I-F). The minor population (ca. 30%) consists of molecules in which one terminal fragment (*Hin*dIII-R and *Xba*I-N) is identical to that in the predominant structural form, whereas the remaining terminal fragment is identical except for the 0.7×10^6-dalton repeat sequence (*Hin*dIII-O and *Xba*I-G). We therefore assume that these structures were also present in the *Eco*RI digest; however, a fragment 0.7×10^6 daltons smaller than *Eco*RI-A was not identified because it was not separable from the large *Eco*RI A fragment even with a 0.3% agarose gel (data not shown). However, if *Eco*RI-A is purified from a gel and cleaved with *Hin*dIII it should be possible to identify internal

TABLE 2. Cross-hybridization between GPCMV *Eco*RI and *Hin*dIII fragments

*Eco*RI fragments	*Hin*dIII fragments																			
	R[a]	N	D	F	H	S	G	T	J	P	Q	K	A	C	B	E	I	U	L	M[a]	O[a]
Y[a]	+																			+	
J		+	+																		
e			+																		
O			+																		
a			+																		
K			+	+																	
N				+																	
R				+																	
I				+	+																
Z					+																
L					+	+															
j						+															
U							+														
E							+														
V							+	+	+												
d									+												
h									+												
i									+												
P									+	+											
c										+	+										
T										+	+										
b												+									
X												+									
f												+									
D													+								
C													+								
H													+	+							
S														+							
F														+							
W														+	+						
Q															+						
G															+						
g															+						
M															+						
A[a]	+														+	+	+	+	+	+	+
B[a]															+	+	+	+	+	+	+

[a] Terminal fragment.

fragments *Hin*dIII-E, -I, -U, and -L, and terminal fragments -O and -M. This hypothesis was tested experimentally, and both the M and O fragments were found (Fig. 9), suggesting the existence of an *Eco*RI B fragment (0.7×10^6 daltons smaller than the *Eco*RI A fragment). The data obtained from hybridizations with cloned, gel-purified internal and terminal fragments and from double cleavages (in particular for overlapping fragments) allowed linear arrangement of all the *Hin*dIII fragments (Fig. 8). All but four of the *Eco*RI fragments, *Eco*RI-e and -O and *Eco*RI-h and -i, were aligned. In the *Xba*I restriction endonuclease cleavage map, there are three regions of uncertainty; the *Xba*I-P, -Y, and -g fragments, the *Xba*I-M, -L, -j, and -n fragments, and the *Xba*I-l and -k fragments could not be ordered.

TABLE 3. Cross-hybridization between GPCMV *Xba*I and *Hin*dIII or *Eco*RI fragments

*Xba*I fragments[a]	Hybridized to:	
	*Hin*dIII fragments	*Eco*RI fragments
B	S, G, T	L, j, U[b], E, V[b]
C	K, A	X, f, D
H	B, E	M, A[c]

[a] *Xba*I fragments were twice purified from gels as described in the text and used as probes to complete and confirm mapping results. Each hybridization was carried out two to three times, using independently isolated fragments as probes.

[b] *Eco*RI-U and -V comigrate on the gel.

[c] Hybridization to *Eco*RI-A actually indicates hybridization to *Eco*RI-A and -B.

DISCUSSION

To obtain adequate quantities of DNA, we prepared a cloned library of the GPCMV genome and then constructed recombinant fragments of GPCMV DNA representing ca. 97% of the total genome. Of the 21 fragments obtained after digestion with *Hin*dIII, 5 fragments (the three terminal fragments M, O, and R, the largest fragment A, and the smallest fragment U) could not be cloned. The 16 *Hin*dIII fragments that were cloned represent 77% of the genome. Of the 36 *Eco*RI fragments, 8 fragments (terminal fragments A, B, and Y, the smallest fragments g, h, i, and j, and the T fragment) could not be cloned. The 28 *Eco*RI fragments that were cloned represent 78% of the genome. Failure to clone the large *Hin*dIII A fragment was compensated by our ability to clone *Eco*RI-D, -C, and -H, which are colinear with *Hin*dIII-A.

We used the cloned DNA fragments as well as gel-purified *Hin*dIII, *Eco*RI, and *Xba*I fragments to establish *Hin*dIII, *Eco*RI, and *Xba*I restriction maps of the GPCMV genome. Hybridization of radioactively labeled fragments to Southern (55) blots of *Hin*dIII-, *Eco*RI-, and *Xba*I-cleaved GPCMV DNA verified the viral origin of cloned fragments and allowed construction of the physical maps of the genome. Double digestion of fragments was carried out to eliminate ambiguities. We derived two important conclusions from the restriction enzyme cleavage maps: (i) the size of GPCMV DNA is $158 \pm 4 \times 10^6$ daltons, which is considerably larger than the value based on sucrose gradient sedimentation data that we previously published (28); and (ii) GPCMV DNA

exists in one conformation with no internal repeat sequences.

The particularly unique aspect of GPCMV DNA structure is that one end of the genome assumes two different conformations, one in which the terminal fragment contains a 0.7×10^6-dalton repeat sequence homologous with the other terminus of the molecule and a second in which this sequence is missing. Support for this statement comes from the following data. (i) Lambda exonuclease digestion indicated three terminal fragments for the *Hin*dIII and *Xba*I digests. (ii) The same fragments identified as terminal fragments by lambda exonuclease digestion could not be cloned regardless of whether unfractionated or gel-purified DNA was used. (iii) With 97% of the genome cloned, homology was not seen between an internal fragment and any of the three terminal fragments, indicating that the presence of three terminal fragments is not indicative of a molecule that can assume two isomeric forms. (iv) Only one of the three *Hin*dIII or *Xba*I terminal fragments demonstrated sequence homology with all three termini; the other two fragments each demonstrated homology with only two fragments. (v) Only one of the three *Hin*dIII and *Xba*I terminal fragments was apparently present in submolar quantities. (vi) The third *Eco*RI terminal fragment was not detected only because of the technical difficulty in separating two large fragments (27.9×10^6 and 27.2×10^6 daltons) that differ by only 0.7×10^6 daltons. (vii) The existence of a third terminal fragment, *Eco*RI-B, that comigrates with *Eco*RI-A was verified by

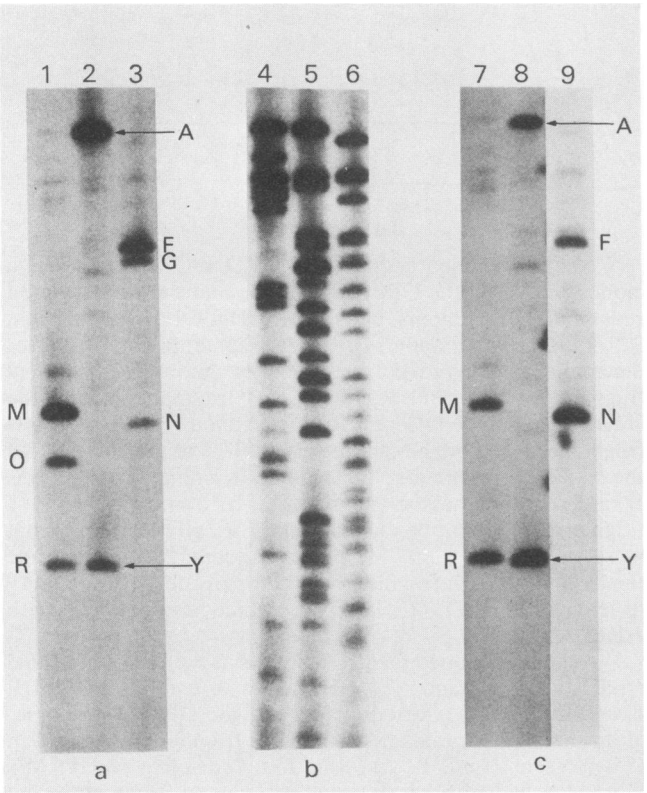

FIG. 6. Hybridization of terminal *Hin*dIII M and R fragments to *Hin*dIII (lanes 1, 4, and 7)-, *Eco*RI (lanes 2, 5, and 8)-, and *Xba*I (lanes 3, 6, and 9)-cleaved GPCMV DNA. Blot transfers of restriction endonuclease-cleaved GPCMV DNA were generated as described in the legend to Fig. 5 and hybridized to ^{32}P-labeled *Hin*dIII-M (a), GPCMV DNA (b), or *Hin*dIII-R (c). *Hin*dIII-M and -R were isolated from gels, as described, before labeling. The fragments are identified as described in the legend to Fig. 5.

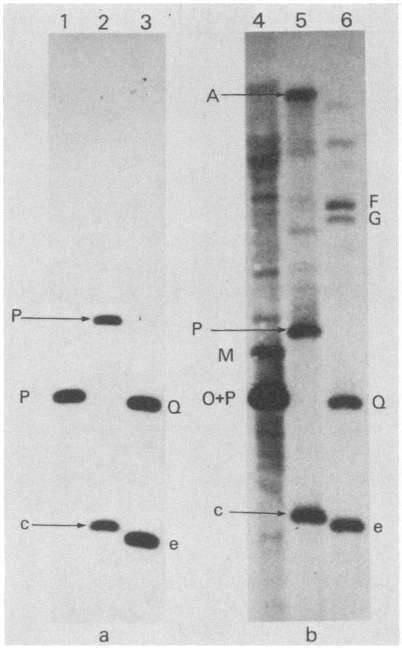

FIG. 7. Hybridization of terminal *Hin*dIII O fragment. GPCMV DNA was cleaved with *Hin*dIII (lanes 1 and 4), *Eco*RI (lanes 2 and 5), and *Xba*I (lanes 3 and 6) as described in the legend to Fig. 5. Hybridization was to ^{32}P-labeled cloned *Hin*dIII-P (a) or gel-isolated *Hin*dIII-(P + O) (b). The fragments are identified as described in the legend to Fig. 5.

double digestion of the gel-isolated band containing both fragments.

We cannot totally exclude the possibility that the minor population of GPCMV DNA represents defective molecules, but this probably is not the case for the following reasons. (i) The conclusions about the termini enumerated above were verified by several independently purified preparations of virus DNA. In all cases, *Hin*dIII-O and *Xba*I-G were apparently present in submolar quantities. (ii) Most of our studies were carried out with GPCMV strain 22122 obtained directly

TABLE 4. Identification of terminal fragments of GPCMV DNA

Probe	Fragments hybridized to probe		
	*Hin*dIII	*Eco*RI	*Xba*I
*Hin*dIII-R	M, R	A, Y	F, N
*Hin*dIII-O	M, O	A[a]	F, G
*Hin*dIII-M	M, O, R	A[a], Y	F, G, N
*Eco*RI-Y	M, R	A, Y	F, N
*Eco*RI-A[b]	M, O, R (E, I, L)[c,d]	A[c], Y	F, G, N (H, M, L, j, n, b, K)
*Xba*I-N	M, R (N)	A, Y (J)	F, N
*Xba*I-F	M, O, R (L)[d]	A[a], Y	F, G, N
*Xba*I-G	M, O (L)[d]	A[a]	F, G

[a] Hybridization to *Eco*RI-A actually indicates hybridization to *Eco*RI-A and -B.

[b] *Eco*RI-A was isolated from a gel, and therefore is actually *Eco*RI-A and *Eco*RI-B.

[c] Hybridization to internal fragment *Hin*dIII-U was not seen because of the size of U. However, when *Hin*dIII-U was used as a probe it hybridized to *Eco*RI-A (and -B) and *Xba*I-F, -G, and -K.

[d] Internal fragments which also hybridized are listed in parentheses.

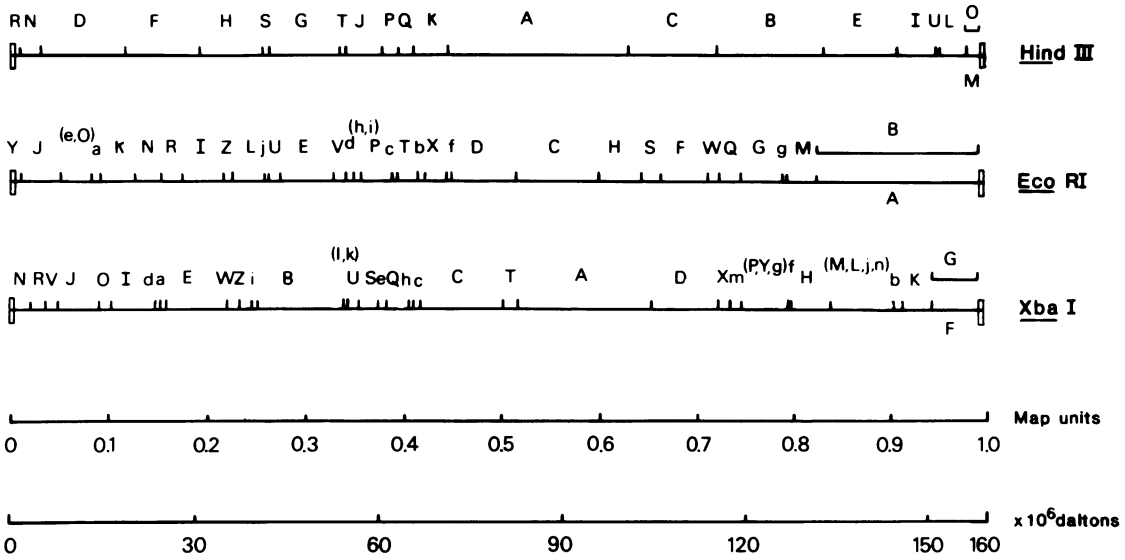

FIG. 8. Schematic diagram of the GPCMV genome with restriction sites for *Hin*dIII, *Eco*RI, and *Xba*I. Parentheses indicate that the order of fragments in this region is unknown. Open rectangles at the termini indicate regions containing sequence homology.

from G. D. Hsiung. We also obtained GPCMV strain 22122 from the American Type Culture Collection and prepared GPCMV DNA with this virus to initiate the infection. Once again *Hin*dIII-O and *Xba*I-G were present in submolar quantities and identical hybridization data for the terminal fragments were obtained. (iii) If the finding reflects the presence of defective GPCMV DNA, we would expect the results to be variable and the difference between defective and nondefective DNA to be greater than 0.7×10^6 daltons.

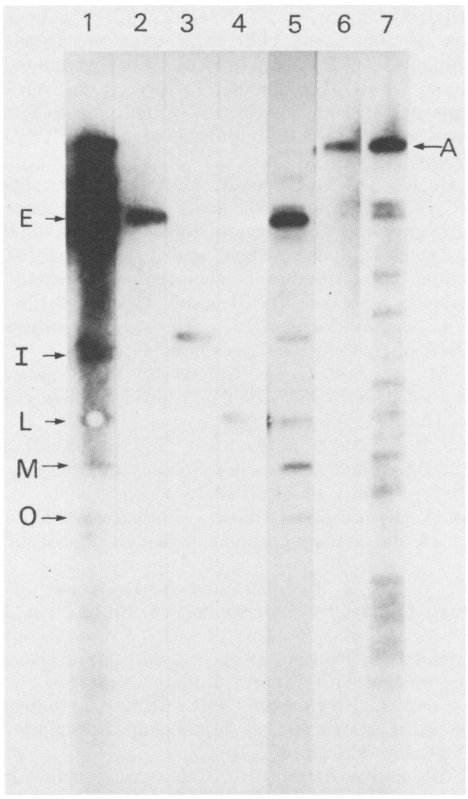

FIG. 9. Double digestion of *Eco*RI-A (and -B) with *Eco*RI and *Hin*dIII. *Eco*RI-A (and -B) was purified from gels. Lane 1, GPCMV DNA cleaved with *Hin*dIII; lane 2, cloned *Hin*dIII-E cleaved with *Hin*dIII; lane 3, cloned *Hin*dIII-I cleaved with *Hin*dIII; lane 4, cloned *Hin*dIII-L cleaved with *Hin*dIII; lane 5, gel-isolated *Eco*RI-A (and -B) cleaved with *Eco*RI and *Hin*dIII; lane 6, gel isolated *Eco*RI-A (and -B) cleaved with *Eco*RI; lane 7, GPCMV DNA cleaved with *Eco*RI. Restriction endonuclease-cleaved GPCMV DNA, cloned GPCMV fragment DNA, and gel-purified GPCMV fragment DNA were subjected to electrophoresis in 0.5% agarose gels, transferred to nitrocellulose filters, and hybridized to ^{32}P-labeled GPCMV DNA.

GPCMV DNA can now be compared with other cytomegalovirus and other herpesvirus DNAs. The molecular weight of the HCMV genome is 150×10^6 to 160×10^6, that of MCMV is 155×10^6, and that of GPCMV is 158×10^6; within error of measurement, the HCMV, MCMV, and GPCMV genomes are the same size. In contrast, the organization of the three genomes is unique for each cytomegalovirus DNA. HCMV DNA exists as four isomers (17, 35, 56, 64, 70) and as such is similar to herpes simplex virus DNA (9, 50, 53, 54, 71). MCMV DNA has a long unique sequence of DNA without large terminal or internal repeat regions and can, therefore, exist only in one conformation. Epstein-Barr virus DNA also does not isomerize but does contain tandem repeats of a 500-base-pair sequence at the termini (21, 33) and a series of internal clusters of 3.1-kilobase-pair repeat units (7, 20, 26). The genomic organization of GPCMV DNA is most like that of MCMV DNA in that it is far simpler than that of its human counterpart and does not isomerize. Further experiments will have to be carried out to establish whether the terminal repeat sequences of GPCMV DNA are direct or inverted repeats.

We have previously reported that GPCMV can malignantly transform guinea pig liver cells (29) and that sequence homology between GPCMV and HCMV DNAs exists (28). The availability of cloned GPCMV DNA fragments will enable us to determine whether GPCMV-transformed cells retain any virus DNA sequences and to identify sequences in the GPCMV genome required for immortalization or trans-

formation or both. The availability of cloned GPCMV DNA fragments and knowledge of the restriction endonuclease cleavage maps will enable us to examine more closely the extent of homology between HCMV and GPCMV DNAs and to locate the regions of homology on the GPCMV genome.

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