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Characterization of Guinea Pig Cytomegalovirus DNA

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The genome of guinea pig cytomegalovirus (GPCMV) was analyzed and compared with that of human cytomegalovirus (HCMV). GPCMV and HCMV DNAs were isolated from virions and further purified by CsCl centrifugation. Purified GPCMV DNA sedimented as a single peak in a neutral sucrose gradient and was infectious when transfected into guinea pig embryo fibroblast cells. The cytopathology was characteristic of that seen after infection with GPCMV. Virus DNA purified from virions isolated from infected GPEF or 104C1 cells had a CsCl buoyant density of 1.713 g/cm^3, which corresponds to a guanine plus cytosine content of 54.1%. The CsCl buoyant density of GPCMV DNA was slightly less than that of HCMV DNA (1.716 g/cm^3), but sufficiently different so that the two virus DNA peaks did not coincide. GPCMV DNA cosedimented with T_4 DNA in a neutral sucrose gradient. Restriction endonuclease cleavage of GPCMV or HCMV DNAs with *Hin*dIII, *Xba*I, or *Eco*RI yielded fragments easily separable by agarose gel electrophoresis and ranging from 1.0×10^6 to 25.8×10^6 daltons. The number, size, and molarity of GPCMV DNA fragments generated by restriction enzymes were determined. Hybridization of restriction endonuclease-cleaved GPCMV DNA with radioactively labeled HCMV DNA and, conversely, hybridization of restriction endonuclease-cleaved HCMV DNA with radioactively labeled GPCMV DNA indicated sequence homology between the two virus DNAs.

Human cytomegalovirus (HCMV) causes a variety of diseases (67). Infection of the immunocompromised adult (48, 54) and transplacental transmission of the virus to the fetus (29, 65) result in high mortality and morbidity rates. In addition, HCMV has been associated with neoplastic diseases including cervical carcinoma (44), prostatic carcinoma (23, 55, 56), and adenocarcinoma of the colon (35). Interest in HCMV has recently increased because of its possible role in Kaposi's sarcoma (25, 26, 37) and acquired immunodeficiency syndrome (13, 17).

HCMV can productively infect cells in culture, but infection is strictly species specific (5, 16, 18, 66), and replication is slow and inefficient (12, 20). Infection can also lead to persistence (21, 45, 51) or to oncogenic transformation (1, 6, 22). Recent studies have defined specific fragments of HCMV DNA that can transform mouse and rat cells in culture (47).

To understand HCMV-host cell interactions in permissive and persistent infections and to determine the role of the virus genome in the initiation and maintenance of transformation, HCMV DNA has been extensively characterized. The HCMV genome is a linear DNA molecule with a size of approximately 150×10^6 daltons (11, 24, 41, 42, 60), a CsCl buoyant density of 1.716 g/cm^3, and a guanine plus cytosine (G+C) content of 57%. The genome structure contains long and short regions of unique DNA sequences bounded by a set of repeated and inverted sequences. The unique segments can invert with respect to one another, enabling four DNA sequence orientations (isomers).

Because HCMV demonstrates strict species specificity, experimental studies are limited. What is required is an animal cytomegalovirus-host system that closely resembles the human system. Guinea pigs also have a species-specific guinea pig cytomegalovirus (GPCMV), and the similarities between GPCMV and HCMV with regard to clinical manifestations have been clearly shown. GPCMV, like HCMV, causes a short-lived viremia followed by chronic, persistent infection (31, 63) in salivary gland, pancreas, and lymphoid tissues (28). GPCMV can also cross the placenta (9, 40), can be transmitted by blood transfusion (2), and is immunosuppressive (71).

GPCMV can productively infect guinea pig cells in culture, and the replicative cycle, like that for HCMV, is slow (32). GPCMV can persistently infect guinea pig embryo fibroblast (GPEF) cells and transform guinea pig liver cells (36). Taken together, these findings indicate that GPCMV-host cell interactions for permissive and persistent infections and for oncogenicity in vitro parallel HCMV-human cell interactions. Although the in vivo pathogenicity, in vitro replication, and oncogenic potential of GPCMV have been studied, the molecular biology of GPCMV has not been investigated. We report here preparation of purified GPCMV DNA, measurement of infectivity of GPCMV DNA, and characterization of GPCMV with regard to G+C content, size, and restriction endonuclease cleavage patterns. We also report sequence homology between the GPCMV and HCMV genomes. This is the first study characterizing GPCMV DNA; in addition, since GPCMV was examined as a model for HCMV, properties of both virus DNAs were investigated in parallel.

MATERIALS AND METHODS

Cells and viruses. GPEF cells, established by trypsin dissociation of whole guinea pig embryos taken from pregnant randomly bred Hartley guinea pigs after 35 days of gestation, were subcultured at a ratio of 1:2 with trypsin and grown in Dulbecco medium supplemented with 10% tryptose phosphate broth, 10% fetal calf serum (FCS), 0.075% $NaHCO_3$, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Line 104 clone 1 (104C1) cells, a benzo(a)-pyrene-transformed and cloned line derived from strain 2 guinea pig embryo cells (15) (kindly provided by C. H. Evans, National Cancer Institute, Bethesda, Md.), were grown in RPMI 1640

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medium supplemented with 5% FCS, 0.075% $NaHCO_3$, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Human embryo lung (HEL) cells were grown and maintained as previously described (10). GPCMV strain 22122, generously provided by G. D. Hsiung (Veterans Administration Hospital, West Haven, Conn.) was propagated in GPEF cells; HCMV strain AD169, obtained from F. Rapp (The Pennsylvania State University College of Medicine, Hershey, Pa.), was grown in HEL cells.

Plaque titration of GPCMV DNA. Infectivity of GPCMV was measured by tissue culture infective dose, with stocks averaging 10^6 to 10^7 50% tissue culture infective doses per ml, and infectivity of virus DNA was determined by plaque titration. GPEF cells were grown to confluence in 60-mm plates and allowed to remain confluent for an additional 2 to 4 days before use. At this time, medium was removed, and cells were infected with virus or transfected with virus DNA and overlaid with 0.5% methylcellulose. Plaques were easiest to score when early passage GPEF cells were used.

Radiolabeling of virus DNA. GPEF or 104C1 monolayers (at 90% confluence) were infected with GPCMV, and HEL monolayers were infected with HCMV, at a multiplicity of 0.01 PFU/cell. After a 1-h adsorption period at 37°C, cultures were fed medium containing 2% FCS and refed with fresh medium at 3-day intervals. For routine purification of virus DNA, infected cultures were labeled with 3 μCi of [^{3}H]thymidine (80.1 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml of medium when 50 to 60% of the cells demonstrated cytopathic effects (CPE). For GPCMV-infected GPEF cells, labeling was usually initiated 7 to 10 days postinfection, whereas for HCMV-infected HEL cells, the labeling period began 10 to 14 days postinfection. Cultures were labeled for 48 to 72 h and harvested when 95% CPE was demonstrated. The procedure for preparation of in vivo-labeled virus DNA for autoradiography was the same, except that the amount of radiolabel was increased to 10 μCi of [^{3}H]thymidine per ml.

Purification of virus DNA. Herpesvirus DNAs were purified by three different procedures: (i) directly from the lysate of infected cells, (ii) from virions precipitated with zinc acetate, and (iii) from virions pelleted by centrifugation. Procedure (i) was developed for purification of polyomavirus DNA (30) and later used for the purification of herpes simplex virus (HSV) DNA (50). Purification of GPCMV DNA by procedure i yielded intact DNA that migrated as a single peak in neutral sucrose gradients; however, the yield, averaging 5 to 10 μg of GPCMV DNA per 10^8 GPEF cells, was not adequate for preparing virus DNA for molecular analysis. In procedure ii, GPCMV-infected cells were frozen and thawed, and the lysate was separated into supernatant and pellet fractions by centrifugation at 7,900 × *g* for 10 min. Intracellular virus DNA was purified by treatment of the pellet with 0.1% Nonidet P-40 and 1% sodium deoxycholate. Extracellular virions were precipitated with 1.0 M zinc acetate, and virus DNA was extracted as described previously for purification of rabies virus DNA (58). Virus DNA was separated from cell DNA by CsCl buoyant density gradient centrifugation as previously described for purification of HSV DNA (10, 69, 70). HSV type 1 (HSV-1) and HSV-2 DNAs used for velocity and buoyant density centrifugation were purified from HSV-1- and HSV-2-infected HEL cells by the zinc acetate precipitation procedure. An average of 60 μg of GPCMV DNA per 10^8 infected GPEF cells was obtained when GPCMV DNA was purified from zinc acetate-precipitated virions. The GPCMV DNA contained all identified large DNA fragments when cleaved with *Hin*dIII or *Eco*RI restriction endonucleases. However, GPCMV DNA, in contrast to HSV-1 or HSV-2 DNA purified by this procedure, failed to migrate as a single peak in neutral sucrose. Procedure iii was used for the purification of GPCMV and HCMV DNAs. In this procedure, infected cells were frozen and thawed, and the lysate was separated into supernatant and pellet by centrifugation at 4,000 × *g* for 10 min. Intracellular virions were obtained by mechanical disruption of the cell pellet followed by centrifugation at 3,020 × *g* for 10 min to remove virions in the supernatant from cell debris in the pellet. The supernatants containing intracellular and extracellular virions were pooled and centrifuged at 13,200 × *g* for 30 min, washed, and suspended in 10 mM Tris (pH 8.0) supplemented with 1 mM EDTA buffer. Sodium dodecyl sulfate was added to a final concentration of 0.5%. Proteinase K was added to a final concentration of 200 μg/ml, and the mixture was incubated at 37°C for 30 min. An additional 200 μg of proteinase K per ml was added and incubated at 37°C for 30 min. The proteinase K step was repeated one more time, and the DNA was then extracted twice with phenol, precipitated with alcohol, and subjected to CsCl buoyant density gradient centrifugation. The DNA was dialyzed against Tris-EDTA buffer.

Preparation of phage T_4 and T_4 DNA. *Escherichia coli* phage T_4 was obtained from Charles Hill (The Pennsylvania State University College of Medicine, Hershey, Pa.). *E. coli* strain B/5 (5 ml) in log phase growth was added to 95 ml of M-9 salts medium containing 0.11 M KH_2PO_4, 40 mM NaCl, 90 mM NH_4Cl, 1.0 mM $MgSO_4$, 0.50% glucose, 0.20% Casamino Acids, and 0.50 mg of tryptophan per ml and incubated in a shaking water bath at 37°C. When the absorbance reached 0.5 optical density units at 590 nm, deoxyadenosine (250 g/ml) and 0.25 μCi of [^{14}C]thymidine (53.4 mCi/mmol; New England Nuclear Corp.) were added to the culture. After 10 min the culture was infected with T_4 phage (multiplicity, 5) and incubated for 2 h at 37°C with shaking. The culture was lysed by the addition of 2.0 ml of chloroform and placed at 4°C overnight. The chloroform was removed by bubbling nitrogen through the culture at 37°C followed by filtration through Whatman no. 1 filter paper. The filtered lysate was centrifuged at 4,000 × *g* for 15 min at 4°C, the supernatant was removed and placed on ice, and the salt concentration was adjusted to 0.5 M with 5.0 M NaCl. Polyethylene glycol (PEG 6000; J. T. Baker Chemical Co., Philipsburg, N.J.) was added to a final concentration of 0.9% while stirring gently at 4°C for 1 h. The phage were pelleted at 4,000 × *g* at 4°C for 30 min and suspended in 5 ml of M-9 medium containing 0.01 M $MgSO_4$. After overnight storage at 4°C, the polyethylene glycol was removed by centrifugation at 4,000 × *g* at 4°C. The supernatant was centrifuged at 17,300 × *g* for 90 min at 4°C, and the pelleted phage were suspended in M-9 medium containing 0.01 M $MgSO_4$. The phage were lysed, and the DNA was extracted with phenol.

Preparation of DNA from recombinant DNA clones. HCMV Towne *Xba*I fragments cloned into the bacterial plasmid pACYC 184 (64) were kindly provided by M. F. Stinski (University of Iowa, Iowa City, Iowa). Bacteria containing viral DNA were grown in NZYM Medium (1% N-Z-amine A [Humko Sheffield Chemical {Kraft, Inc.}, Memphis, Tenn.], 0.5% yeast extract, 0.5% NaCl, and 0.2% $MgCl_2$) containing 25 μg of chloramphenicol per ml. Plasmid replication was amplified by adding 300 μg of spectinomycin per ml. Plasmid DNA was extracted by the alkaline method of Birnboim and Doly (4) and further purified as described previously (61).

Preparations of GPEF cell DNA. GPEF cell DNA was purified for use as carrier DNA in calcium phosphate precip-

itations of GPCMV DNA. Ten flasks (150 cm^2) of confluent GPEF cells were washed, and the cells were removed from the flasks after incubation with versene (1.4 mM EDTA containing 1.37 M NaCl, 7.7 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, and 2.7 mM KCl) in the absence of trypsin. The washed pellet was resuspended in 10 volumes of 10 mM Tris (pH 8.0) supplemented with 150 mM NaCl and 10 mM EDTA. The cell suspension was brought to 100 μg of proteinase K per ml and 0.2% sodium dodecyl sulfate and incubated overnight at 37°C. DNA was extracted with phenol and dialyzed against isotonic *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline (27). The DNA concentration was determined by the diphenylamine assay (7, 52).

Transfection with GPCMV DNA. GPEF monolayers were transfected with calcium phosphate-precipitated GPCMV DNA or GPEF carrier DNA as described previously (27). GPCMV and GPEF carrier DNAs were sterilized by adding 15 μl of chloroform to the DNA and holding overnight at 4°C. Residual chloroform was removed or allowed to evaporate. GPEF cells were subcultured 1:2 in 60-mm plates and maintained for 8 to 10 days before transfection experiments. Cells were fed fresh medium every 3 to 4 days. Medium was removed from each plate, and 0.5 ml of suspension containing calcium phosphate-precipitated GPCMV or carrier GPEF (negative control) DNA was added to each monolayer. Cultures were held at room temperature for 20 min, overlaid with 5 ml of fresh 2% FCS-containing medium, and incubated at 37°C for 4 h. Medium was then removed and the cultures were washed with medium and treated with 2 ml of 25% dimethyl sulfoxide in isotonic HEPES-buffered saline for 3 min at room temperature. The dimethyl sulfoxide was removed, and the cultures were washed with medium and either fed 5 ml of fresh 2% FCS-containing medium or overlaid with medium containing 0.5% methylcellulose. Parallel cultures were infected with a known amount of infectious GPCMV as a positive control for the plaque assay. Cultures fed medium were observed for CPE; those overlaid with methylcellulose were fixed and stained 14 days after transfection, and the plaques were counted.

Analysis of DNA by CsCl and sucrose gradient centrifugation. The buoyant density of GPCMV DNA was determined by centrifugation to equilibrium (87,000 × *g* for 72 h) at 20°C in 5-ml gradients of CsCl (initial average density of 1.71 g/cm^3) in 0.01 M Tris-hydrochloride (pH 8.0) and 0.001 M EDTA. Fractions were collected by bottom puncture and analyzed for trichloroacetic acid-insoluble radioactivity by precipitation on filter paper disks. All gradients contained 112 to 114 fractions and were plotted as fractional distance from the bottom of the gradients. Neutral sucrose gradients (10 to 30% sucrose in 0.02 M Tris-hydrochloride [pH 8.0], 1.0 M NaCl, and 0.001 M EDTA) contained in an 11-ml volume were overlaid with 1 to 5 μg of ^3H- or ^{14}C-labeled DNA and centrifuged at 174,000 × *g* in a Beckman SW41 rotor for 3 h at 20°C. Fractions were collected by bottom puncture and analyzed.

Restriction endonuclease digestions. Purified virus DNA was incubated twice at 37°C for 2 h with sufficient endonuclease (total of 5 to 10 U/μg of DNA) in the buffer to ensure complete digestion, and the reaction was stopped by the addition of 5 μl of 0.25 M Tris-hydrochloride (pH 8.0), 30% (wt/vol) Ficoll, 0.5% sodium dodecyl sulfate, 60 mM EDTA, and 0.2% bromphenol blue. The sample was then heated for 5 min at 70°C. *Hin*dIII, *Eco*RI, and *Xba*I were purchased from Boehringer Mannheim Corp. (Indianapolis, Ind.); *Bgl*II was purchased from New England Nuclear Corp.

Agarose gel electrophoresis. DNA fragments were separated by electrophoresis through submerged 0.5% agarose (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) horizontal slab gels that were prepared and run in 40 mM Tris–4 mM sodium acetate–1 mM EDTA (pH 7.9). Electrophoresis was carried out at 3.1 V/cm for 16 to 24 h. After staining with ethidium bromide (2 μg/ml) for 1 h at room temperature, gels were photographed under UV illumination on Polaroid type 667 film. Gels to be used for determining the molecular weights and molarities of GPCMV DNA fragments contained DNA radiolabeled in vivo and were enhanced by fluorography with 1.0 M sodium salicylate (Mallinkrodt) as described previously (8, 14), dried on filter paper at 37°C, and exposed on Kodak XAR-film at −76°C. Fluorographs were scanned at 550 nm with a Beckman DU-8 spectrophotometer, and the areas under the peaks were quantitated. Multiple exposures were used to obtain bands at intensities that were within the linear range of the film.

Blot hybridization. After GPCMV and HCMV DNA fragments were electrophoretically separated in agarose gels, the DNA was denatured, neutralized in situ, and transferred to nitrocellulose sheets as described by Southern (59). The nitrocellulose sheets were then baked (80°C, in vacuo), cut into strips matching the gel lanes preannealed, and then hybridized in 50% (vol/vol) deionized formamide in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 42°C for 20 h with HCMV or GPCMV DNA labeled with ^{32}P by nick translation (53). The filter strips were washed, dried, and subjected to autoradiography in the presence of an intensifying screen at −76°C. For the homology studies reported, GPCMV and HCMV DNAs were labeled in vitro to a specific activity of 10^8 cpm/μg, and 2.5 × 10^6 counts were used for each strip.

RESULTS

Purification of GPCMV DNA. Purification of GPCMV DNA (procedure iii, above) yielded an average of 80 μg of GPCMV DNA per 10^8 GPEF cells; this DNA migrated as a

TABLE 1. Infectivity of purified GPCMV DNA

Source of DNA	DNA added (μg)	Pretreatment of DNA[a]	Calcium phosphate precipitation	Detection of infectivity	
				Cytopathology[b]	Plaques (no.)[c]
GPEF	10		+	−	
	10		+		0
GPCMV	0.1[d]		+	+	
	0.2		+	+	
	0.5		+	+	
	0.5	DNase	+	−	
	0.5		−	−	
	0.5	DNase	+		0
	0.5		−		0
	0.5		+		21

[a] DNA was incubated with DNase I (10 μg DNase per μg of DNA) at 37°C for 1 h.

[b] GPEF cells were transfected with calcium phosphate-precipitated DNA, treated with dimethyl sulfoxide, and overlaid with 2% FCS medium. Plates were scored for CPE 10 days postinfection.

[c] GPEF cells were transfected with calcium phosphate-precipitated DNA, treated with dimethyl sulfoxide, and overlaid with medium containing 0.5% methylcellulose. Cultures were fixed and stained for plaque formation 14 days after transfection.

[d] GPCMV DNA was supplemented with GPEF carrier DNA to a final concentration of 10 μg DNA for each plate, e.g., 0.1 μg of GPCMV DNA plus 9.9 μg of GPEF DNA.

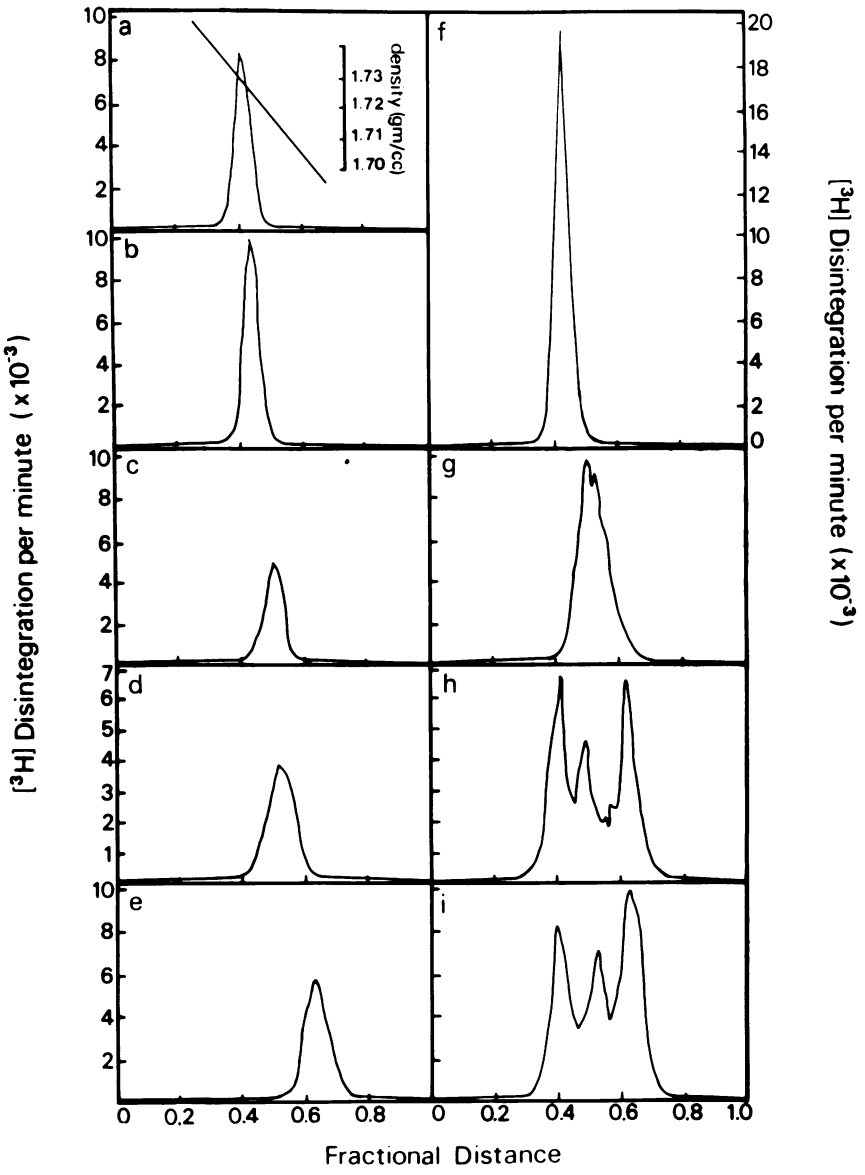

FIG. 1. Buoyant density of GPCMV DNA. CsCl equilibrium buoyant density centrifugation of purified [^{3}H]thymidine-labeled virus DNAs was carried out as described in the text. Refractive index readings were taken to establish linearity of the gradient through the region where the DNA sedimented and the corresponding density is shown. (a) HSV-2 DNA; (b) HSV-1 DNA; (c) HCMV DNA; (d) GPCMV DNA; (e) simian virus 40 DNA; (f) HSV-2 and HSV-1 DNAs; (g) HCMV and GPCMV DNAs; (h) HSV-2, HCMV, simian virus 40 DNAs; (i) HSV-2, GPCMV, and simian virus 40 DNAs.

single peak in neutral sucrose gradient centrifugation. Since GPEF cells can only be subcultured 1:2 every 6 to 7 days, we wanted to find a cell type that grew more efficiently and from which GPCMV DNA could be purified. When 104C1 cells, a benzo(a)pyrene-transformed and cloned line derived from strain 2 guinea pig embryo cells (15), were infected with GPCMV, the virus replicated with a time course and yield similar to that seen in infected GPEF cells. DNA was purified by procedure iii from 104C1 cells at average yields of 110 μg/10^8 cells. No detectable differences were observed in the restriction endonuclease cleavage patterns for DNAs purified from 104C1 or GPEF cells.

Infectivity of purified GPCMV DNA. When confluent GPEF cell monolayers (1.0×10^6 to 1.5×10^6 cells per 60-mm dish) were transfected with 0.1 to 0.5 μg of GPCMV DNA, treated with dimethyl sulfoxide, and overlaid with fresh medium, typical GPCMV CPE became apparent 7 to 9 days post-transfection (Table 1). Quantitation of infectivity of GPCMV DNA was determined by plaque titration in confluent GPEF monolayers overlaid with 0.5% methylcellulose. When GPEF cells were transfected with 0.5 μg of GPCMV DNA, 21 plaques were obtained. Because no CPE or plaques were obtained when cells were (i) infected with GPCMV DNA in the absence of calcium phosphate precipitation, (ii) transfected with GPEF carrier DNA alone, or (iii) transfected with GPCMV DNA preincubated with DNase, we assumed that plaque formation was caused by GPCMV DNA and not by residual virus.

Buoyant density and G+C content of GPCMV DNA. The buoyant density of GPCMV DNA in CsCl was measured in

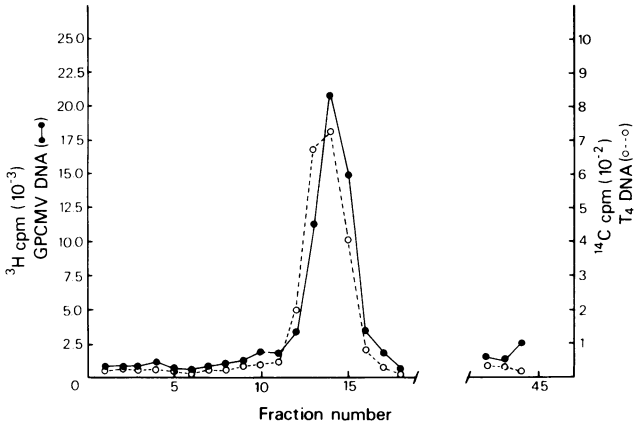

FIG. 2. Sedimentation of purified GPCMV DNA in a neutral sucrose gradient. GPCMV DNA was labeled with [^{3}H]thymidine, and T_4 DNA was labeled with [^{14}C]thymidine.

two independently purified GPCMV DNA preparations. Comparison of the sedimentation profile of GPCMV DNA with DNAs from other herpesviruses showed that the buoyant density of GPCMV DNA (Fig. 1d) was considerably less than that of HSV-2 (Fig. 1a) or HSV-1 (Fig. 1b) DNAs, but only slightly less than that of HCMV DNA (Fig. 1c). When GPCMV and HCMV DNAs were in the same gradient, two separate peaks were observed (Fig. 1g). However, when HSV-1 (density, 1.726 g/cm^3) and HSV-2 (density, 1.728 g/cm^3) DNAs were analyzed in the same gradient, the peaks were not separable (Fig. 1f). Utilizing HSV-2 and simian virus 40 DNAs as markers in the same gradient (Fig. 1i), the CsCl buoyant density of GPCMV DNA was calculated as 1.713 g/cm^3. As a control, the same markers were used (Fig. 1h) to calculate the buoyant density of HCMV DNA; a value of 1.716 g/cm^3 was attained, which is in agreement with values in the literature (33). Because the buoyant density of DNA in CsCl is directly proportional to its G+C content (57), the value for the buoyant density of GPCMV was introduced into the formula (buoyant density, 1.660 + 0.098 [G+C]), yielding a G+C content for GPCMV of 54.1%, compared with 57.1% for HCMV.

Velocity sedimentation of GPCMV DNA. The migration of GPCMV DNA in a low-percentage (0.2%) agarose gel was measured relative to several DNAs of known size to determine the most appropriate DNA to use as an internal marker in neutral sucrose gradient centrifugation. Electrophoresis was carried out at 40 V for 30 min at 4°C and continued at 20

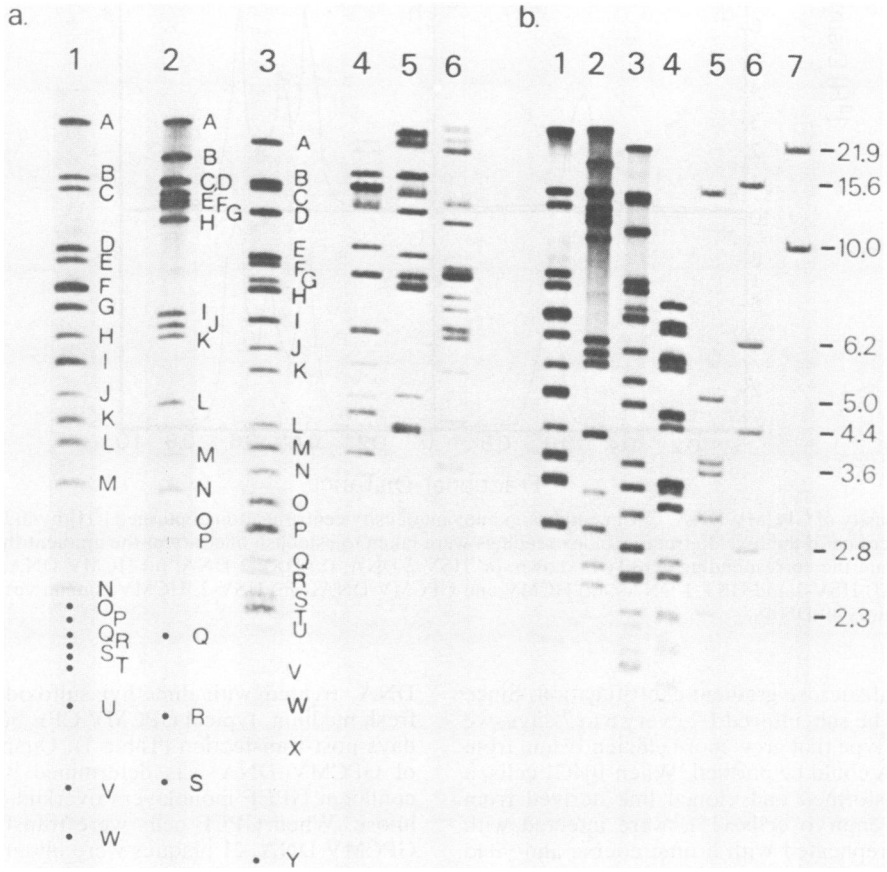

FIG. 3. Fluorographs of restriction endonuclease fragments of GPCMV DNA relative to λ and HCMV DNA size markers. GPCMV, HCMV, and λ DNAs were labeled in vivo, digested with restriction endonucleases, subjected to electrophoresis in 0.5% agarose gels, incubated with sodium salicylate, dried at 37°C, and exposed to XAR-5 film at −76°C. Unique letters were assigned to each fragment in the GPCMV *Hin*dIII, *Eco*RI, and *Xba*I digests (a; lanes 1, 2, 3). (a) GPCMV DNA digested with *Hin*dIII (lane 1), *Eco*RI (lane 2), or *Xba*I (lane 3); HCMV DNA digested with *Hin*dIII (lane 4), *Xba*I (lane 6), or *Bgl*II (lane 6). (b) GPCMV DNA digested with *Eco*RI (lane 1), *Hin*dIII (lane 2), *Xba*I (lane 3), or *Bam*HI (lane 4); λ DNA digested with *Eco*RI (lane 5), *Hin*dIII (lane 6), or *Sal*I (lane 7). Measurements made from these fluorographs were used to determine molecular weights and molarities of lettered GPCMV DNA fragments (Table 1). Dots represent the positions of bands more easily detectable in longer exposures.

V for 48 h at 4°C. GPCMV, T_4, and HSV-2 DNAs migrated to similar positions in the gel and in all cases migrated more rapidly than HCMV DNA (data not shown). GPCMV DNA migrated slightly more rapidly than T_4 DNA and less rapidly than HSV-2 DNA. T_4 DNA was selected as marker DNA. GPCMV DNA labeled with [^{3}H]thymidine was purified by procedure iii and cosedimented in a neutral sucrose gradient with [^{14}C]thymidine-labeled T_4 DNA (Fig. 2). Based on a value of 61.85 S for T_4 DNA (19), the size of GPCMV DNA was estimated at approximately 120×10^6 daltons. However, since the use of T_4 DNA as a marker to determine the molecular mass of an unknown virus DNA has its limitations, we can only state that 120×10^6 daltons is a gross approximation of the size of GPCMV DNA.

Restriction endonuclease cleavage of GPCMV DNA. Cleavage of GPCMV or HCMV DNAs with *Hin*dIII, *Eco*RI, or *Xba*I yielded fragments easily separable by agarose gel electrophoresis and ranging in size from 25.5 to 0.95×10^6 daltons. *Hin*dIII digestion of GPCMV DNA produced at least 19 fragments, digestion with *Eco*RI produced at least 23 fragments, and digestion with *Xba*I produced at least 25 fragments. Fragments smaller than 1.0×10^6 daltons were not studied. Digestion of GPCMV DNA with *Bgl*II generated a large number of fragments that were not easily separable by gel electrophoresis and were less than 5.2×10^6 daltons (data not shown). This contrasted with the pattern seen for *Bgl*II digestion of HCMV DNA, in which numerous easily separable fragments ranging from 32.0×10^6 to 1.3×10^6 daltons were observed.

Molecular weights and molarities of GPCMV *Hin*dIII, *Eco*RI, and *Xba*I fragments. We assigned unique letters to each fragment in the GPCMV *Hin*dIII, *Eco*RI, and *Xba*I digests (Fig. 3). The molecular masses of the GPCMV DNA fragments were determined by scanning the bands in fluorograms of restriction endonuclease digests of GPCMV DNA labeled in vivo. Both HCMV (Fig. 3a) and λ (Fig. 3b) DNA fragments were used as size markers. Table 2 lists the molecular weights and molarities of GPCMV *Hin*dIII, *Eco*RI, and *Xba*I fragments. Comparison of the digestion patterns for GPCMV and HCMV DNAs showed that each virus DNA had its own distinct electrophoretic profile. The *Hin*dIII and *Xba*I cleavage products were most similar; the *Hin*dIII fragments ranged up to 25.8×10^6 daltons for GPCMV DNA and 23×10^6 daltons for HCMV DNA (49), and the *Xba*I fragments ranged up to 21.3×10^6 daltons for GPCMV DNA and 23.4×10^6 daltons for HCMV DNA (49). In comparing only these two digests, the largest fragment for GPCMV DNA was a *Hin*dIII fragment, whereas the largest HCMV DNA fragment was an *Xba*I fragment. Scanning of autoradiograms of GPCMV DNA fragments from three different restriction endonuclease fragments yielded 0.5-molar but no 0.25-molar fragments.

Homology between GPCMV and HCMV DNAs. Extensive genetic relatedness (80% DNA homology) exists between various strains of HCMV (39), but no detectable DNA sequence homology has been reported between human isolates and simian or murine strains (34). When HCMV *Hin*dIII fragments (obtained by restriction endonuclease cleavage of DNA purified from HCMV AD169) were immobilized on nitrocellulose paper by the method of Southern (59) and ^{32}P-labeled GPCMV DNA was added to the reaction mixture, hybridization to HCMV *Hin*dIII-E, -R, -S, -P, and -L occurred (Fig. 4). Similarly, when HCMV *Xba*I fragments (obtained by restriction endonuclease cleavage of DNA

TABLE 2. Molecular weights and molarities of *Hin*dIII, *Eco*RI, and *Xba*I digests of GPCMV DNA

*Hin*dIII fragment	M_r ($\times 10^6$)[a]			Molar ratio[b]	*Eco*RI fragment	M_r ($\times 10^6$)			Molar ratio	*Xba*I fragment	M_r ($\times 10^6$)			Molar ratio
	1	2	Avg			1	2	Avg			1	2	Avg	
A	25.5	26.0	25.8	0.9	A	24.9	25.3	25.1	2.0	A	20.9	21.6	21.3	1.0
B	18.0	17.2	17.6	1.4	B	14.2	15.0	14.6	1.0	B	13.9	14.5	14.2	1.0
C	14.1	13.8	14.0	1.0	C	13.0	13.8	16.8	1.0	C	13.3	14.0	13.7	0.8
D	14.1	13.8	14.0	1.0	D	8.6	9.1	8.9	1.0	D	10.9	11.5	11.2	1.4
E	12.7	12.5	12.6	1.4	E	8.0	8.6	8.3	1.0	E	8.2	8.7	8.5	1.1
F	12.1	12.1	12.1	1.2	F	6.9	7.2	7.1	3.2	F	7.8	8.4	8.1	1.1
G	11.6	11.5	11.6	0.9	G	6.3	6.7	6.5	1.0	G	7.1	7.6	7.4	0.5
H	10.4	10.5	10.5	1.0	H	5.5	5.5	5.5	0.5	H	6.8	7.3	7.1	1.2
I	6.2	6.3	6.3	1.1	I	4.9	5.2	5.1	1.2	I	5.9	6.2	6.1	1.1
J	5.9	5.9	5.9	0.9	J	4.3	4.5	4.4	0.4	J	5.2	5.5	5.4	1.0
K	5.6	5.6	5.6	0.9	K	3.9	4.1	4.0	0.9	K	4.7	5.0	4.9	1.1
L	4.2	4.3	4.3	0.8	L	3.6	3.7	3.7	1.1	L	3.8	4.0	3.9	0.7
M	3.4	3.5	3.5	0.5	M	3.0	3.2	3.1	0.8	M	3.5	3.7	3.6	1.0
N	3.0	3.1	3.1	1.3	N	2.0	2.2	3.1		N	3.2	3.4	3.3	0.6
O	2.7	2.8	2.8	0.8	O	1.9	2.1	2.0		O	2.8	3.0	2.9	1.0
P	2.5	2.6	2.6	0.4	P	1.9	2.0	2.0		P	2.6	2.7	2.7	1.3
Q	1.7	1.9	1.8		Q	1.8	2.0	1.9		Q	2.3	2.5	2.4	
R	1.3				R	1.7	1.9	1.8		R	2.2	2.4	2.3	
S	1.1				S	1.5	1.7	1.6		S	2.0	2.2	2.1	
					T	1.5	1.6	1.6		T	1.9	2.1	2.0	
					U	1.3	1.5	1.4		U	1.7	2.0	1.9	
					V	1.1				V	1.5	1.7	1.6	
					W	1.0				W	1.3	1.5	1.4	
										X	1.2	1.3	1.3	
										Y	1.0			

[a] Molecular weight was calculated from electrophoretic mobility of GPCMV DNA fragments in 0.5% agarose gels relative to HCMV DNA fragments (value 1) or relative to λ DNA fragments (value 2) run in the same agarose gel as the GPCMV DNA. Values obtained for GPCMV DNA from either of the two standard DNAs were quite similar, and an average value was calculated.

[b] Molar ratios were obtained by scanning autoradiograms of gels containing restriction endonuclease digests of GPCMV DNA radioactively labeled in vivo.

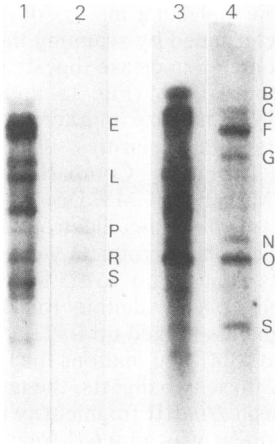

FIG. 4. Hybridization between GPCMV and HCMV AD169 DNAs. HCMV DNA was cleaved with *Hin*dIII (lanes 1 and 2) and *Xba*I (lanes 3 and 4). DNA fragments were immobilized on nitrocellulose paper and hybridized to ^{32}P-labeled GPCMV DNA (lanes 2 and 4) or ^{32}P-labeled HCMV DNA (lanes 1 and 3). Letter designations are for HCMV strain AD169 DNA (49).

purified from HCMV AD169 virions) were reacted with radiolabeled GPCMV DNA, hybridization to *Xba*I-C, -N, -S, -F, -G, -B, and -O was seen. Examination of the published map for the HCMV AD169 genome (49) showed that the sequences of HCMV DNA that indicated some homology with GPCMV DNA are located between 0.06 and 0.33 map units on the HCMV AD169 genome.

When HCMV Towne *Xba*I fragments (obtained by cleavage of fragments cloned into the pACYC bacterial plasmid) were immobilized on nitrocellulose paper and ^{32}P-labeled GPCMV DNA was added to the reaction mixture, hybridization to HCMV Towne *Xba*I -C, -E, -N, and -R was seen (Fig. 5). Examination of the published map for the HCMV Towne genome (64) showed that the sequences of HCMV DNA that indicated some homology with GPCMV DNA are located between 0.5 to 0.77 map units on the HCMV Towne genome. We have detected cross-hybridization between GPCMV DNA and fragments of HCMV DNA located between 0.06 and 0.33 map units on the HCMV AD169 genome and between 0.5 and 0.77 map units on the HCMV Towne strain genome (Fig. 6). These apparent differences in map position are not real, but simply reflect the fact that the prototype orientation chosen for the long unique segment for HCMV Towne strain DNA is inverted relative to that published for HCMV AD169 DNA (43, 60).

When GPCMV *Hin*dIII DNA fragments were immobilized on nitrocellulose paper and ^{32}P-labeled HCMV AD169 DNA was added to the reaction mixture, hybridization to two GPCMV DNA fragments (25.8×10^6 and 14.0×10^6 daltons) was observed (Fig. 7). In patterns obtained from either ethidium bromide-stained GPCMV DNA or fluorograms of in vivo-labeled GPCMV DNA, the 14.0×10^6-dalton fragment from the *Hin*dIII digest appeared to be present at a higher concentration than the remaining fragments. In the process of cloning the *Hin*dIII fragments into bacterial plasmids (Gao and Isom, unpublished data), we found two distinct fragments (each with a size of 14.0×10^6 daltons) that were designated *Hin*dIII-C and -D. HCMV DNA hybridized to both *Hin*dIII-C and -D (14.0×10^6-dalton band) as well as to *Hin*dIII-A (25.8×10^6-dalton band). When GPCMV *Eco*RI fragments were reacted with radiolabeled HCMV DNA, hybridization to *Eco*RI-B, -E, -F, -K, -M, -O, and -S was seen. When GPCMV *Xba*I fragments were reacted with radiolabeled HCMV DNA, hybridization to *Xba*I-A, -D, and -J was observed.

DISCUSSION

We have previously shown that guinea pig cells can be malignantly transformed after exposure to inactivated GPCMV (36). When guinea pig hepatocyte monolayers were infected with UV-irradiated GPCMV, epithelioid cell lines that grew in soft agarose and formed tumors in nude mice were established. We have not determined whether the transformants or tumor cells (or both) contained GPCMV DNA sequences and whether transformation by GPCMV DNA or DNA fragments could be accomplished; that is, the portions of the GPCMV genome necessary for maintenance or initiation (or both) of transformation. To accomplish these goals, it was necessary to purify and characterize GPCMV DNA. Studies on GPCMV DNA were limited to one report (3) demonstrating that the restriction endonuclease cleavage patterns for the guinea pig herpesvirus DNA differed from those for GPCMV.

In previous studies, GPCMV has been propagated in vitro in GPEF cells or passaged in vivo in the salivary gland (3, 31, 63). In this paper, we report that GPCMV can also be propagated in chemically transformed guinea pig cells, 104C1 cells. Infection of either GPEF or 104C1 cells was carried out at low multiplicity (0.01 PFU/cell) to prevent generation of defective virus and the possibility of defective virus DNA (see below). Whether GPCMV DNA was purified from GPEF or 104C1 cells, the restriction endonuclease cleavage patterns (with five different enzymes) were the same. However, it is important to realize that all studies

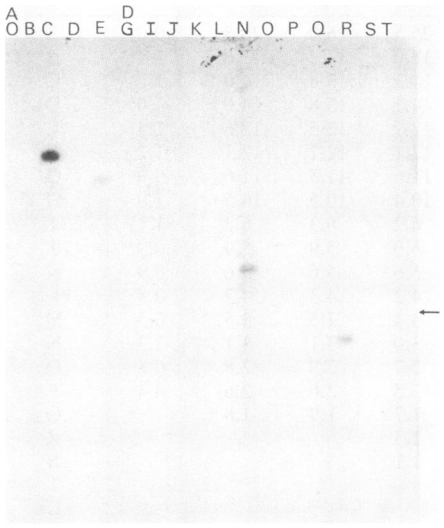

FIG. 5. Hybridization between GPCMV and HCMV Towne *Xba*I fragments. Purified HCMV Towne *Xba*I fragments cloned in the pACYC 184 bacterial plasmid (64) were cleaved with *Xba*I, and the virus DNA fragments were electrophoretically separated from the plasmid DNA on an agarose gel. A total of 17 different recombinant plasmids were examined. DNA fragments were immobilized on nitrocellulose paper and hybridized to ^{32}P-labeled GPCMV DNA. The arrow (←) indicates the location of the pACYC 184 DNA band.

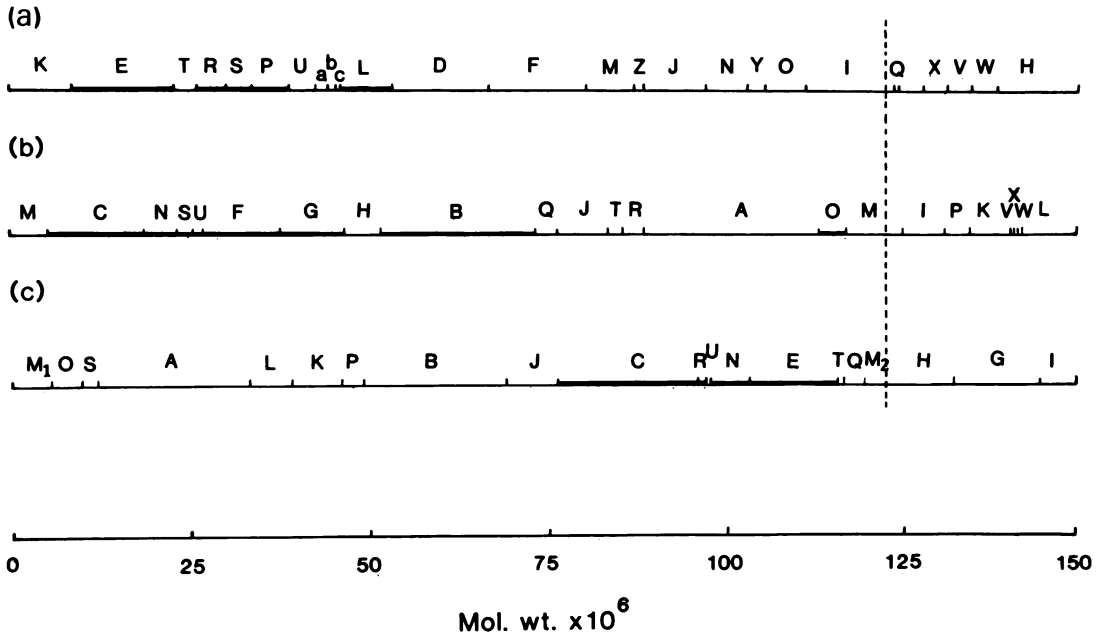

FIG. 6. Restriction endonuclease cleavage maps for HCMV. (a) *Hin*dIII digest of HCMV AD169 as per reference 49; (b) *Xba*I digest of HCMV AD169 as per reference 49; (c) *Xba*I digest of HCMV Towne as per reference 64. Dark bars indicate fragments showing some homology with GPCMV DNA.

were carried out with GPCMV passaged in vitro. We are currently in the process of investigating whether virus DNA from the salivary glands of infected animals (in vivo-passaged virus) has the same size and restriction endonuclease cleavage patterns. Although GPCMV DNA can be prepared more economically from virions than from infected 104C1 cells, it is necessary to be aware of its source of isolation. GPCMV DNA to be used for transformation should be purified from virions isolated from infected GPEF cells to eliminate any possibility that contaminating transformed 104C1 cell DNA has a role in the transformation event.

Characterization of GPCMV DNA has shown that the genome is in many ways very similar to that of HCMV. For example, purification procedures adequate for yielding intact HSV-1 or HSV-2 DNA were not satisfactory for purification of HCMV or GPCMV DNA, which have greater molecular weights and lower G+C contents than HSV DNA. The CsCl buoyant density of GPCMV DNA (1.713 g/cm^3) is just slightly less than that for HCMV DNA. The density of equine CMV DNA is the same as HCMV DNA (68), but the density for murine CMV DNA is greater (1.718 g/cm^3) (46). The reported density for simian CMV (1.710 g/cm^3) (33) is the lowest. The size of HCMV DNA (150 $\times$ 10^6 daltons) is the largest of the herpesviruses. Murine CMV DNA is next in size at 132 $\times$ 10^6 daltons (42, 46), followed by equine CMV and GPCMV DNAs, which both comigrate with T_4 in neutral sucrose gradients.

Generation of infectious DNA was important for two reasons. First, we want to use this DNA for transfection with the ultimate goal of studying transformation. Before cleaving the DNA with restriction endonucleases to eliminate infectivity, we wanted to determine that the transfection procedure was being carried out under conditions in which intact DNA could be expressed. Second, we felt that if the DNA was infectious, there were at least some full-length molecules present.

Early reports analyzing HCMV DNA by sedimentation in

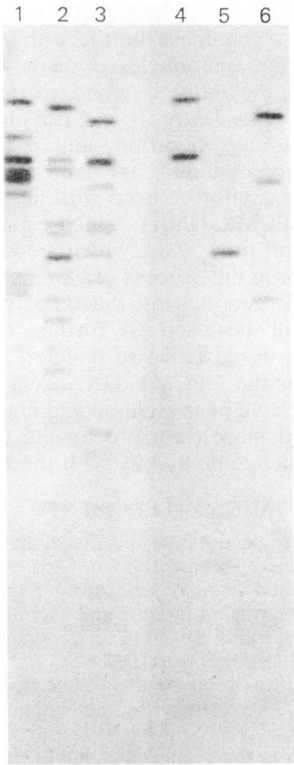

FIG. 7. Hybridization between HCMV and GPCMV DNAs. GPCMV DNA was cleaved with *Hin*dIII (lanes 1 and 4), *Eco*RI (lanes 2 and 5), or *Xba*I (lanes 3 and 6), and the fragments were electrophoretically separated on an agarose gel. DNA fragments were immobilized on nitrocellulose paper and hybridized to ^{32}P-labeled GPCMV DNA (lanes 1, 2, and 3) or ^{32}P-labeled HCMV DNA (lanes 4, 5, and 6).

sucrose gradients (33) indicated that the size was approximately 100×10^6 daltons. It later became apparent that there were two size classes of HCMV DNA, one class of 100×10^6 daltons and another of 150×10^6 daltons (11, 24, 38) and that the larger DNA molecules were infectious. The smaller 100×10^6-dalton molecule can be isolated from defective virions generated by serial high passage of virus (62). GPCMV DNA used in this study was purified from virions generated by passage of virus at 0.01 PFU or less per cell. Since GPCMV DNA that migrated as a single peak in neutral sucrose contained infectious molecules, we concluded that DNA with a size of approximately 120×10^6 daltons most likely represents the correct size for GPCMV DNA. However, because the efficiency in the transfection assay was low (42 plaques per μg of DNA), we cannot completely exclude the possibility that the infectious molecules represented a minor population larger than 120×10^6 daltons. (This is, however, unlikely as no peaks or even shoulders of radioactivity were observed below the major peak in the sucrose density gradient profile.) The low efficiency for transfection by GPCMV DNA probably results from a combination of large size of the DNA, inevitable damage to such a large DNA molecule, and the effects of calcium phosphate-precipitated DNA and dimethyl sulfoxide on GPEF monolayers. Reasonably low efficiencies have also been seen for transfection with DNAs from other CMV strains. For example, a value of 100 PFU/μg of murine CMV DNA was reported (42), and the maximum efficiency reported for HCMV DNA was 480 PFU/μg (24). It will be necessary to map the genome of GPCMV before an accurate size for GPCMV DNA can be calculated.

Determination of genome structure with regard to orientation and restriction endonuclease maps was difficult to accomplish before the ability to clone restriction endonuclease fragments of virus DNA. It was possible through fragment isolation, double digestion, and partial cleavage to construct restriction endonuclease maps for small genomes. These procedures cannot be used with large genomes such as HCMV and GPCMV DNAs, which cannot be cleaved a limited number of times and which contain overlapping fragments. We are in the process of cloning DNA fragments from the GPCMV genome into bacterial plasmid vectors. These clones will be used to further characterize the GPCMV genome, in particular to develop restriction endonuclease maps for the GPCMV DNA. When clones of the GPCMV genome have been isolated and mapped, we will be able to verify and more clearly delineate the location and extent of the sequence homology with the HCMV genome.

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