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Human Cytomegalovirus

I. Purification and Characterization of Viral DNA

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Two human strains (AD-169 and C87) and one simian strain (GR2757) of cytomegalovirus (CMV) have been purified from the extracellular fluids of virus-infected cultures by sedimentation through a sucrose gradient followed by brief centrifugation in a preformed gradient of CsCl. Enveloped virus particles were located in the density region, 1.219 g/cm^3, and nucleocapsids at 1.263 g/cm^3. Purified viral DNA, both human and simian, sedimented in the region of 55*S* in neutral sucrose gradients with herpes simplex type I DNA as a marker; the molecular weight of the CMV DNA was estimated as approximately 10^8. The density of the viral DNA determined by analytical ultracentrifugation was 1.716 g/cm^3 for the human strains and 1.710 g/cm^3 for the simian strain. Tritiated viral complementary RNA synthesized in vitro with *Escherichia coli* transcriptase has been used for detection and localization of viral genome in membrane hybridization and in situ cytohybridization. Newly synthesized viral DNA appeared 24 h after infection and localized at two acrocentric areas; later the viral DNA distributed in a band resembling intranuclear inclusions 48 to 70 h after infection. Total DNA synthesis began to increase 24 h after infection and reached its peak at 70 h; RNA synthesis increased at 13 h, and reached its peak at 24 to 33 h. The viral DNA was also labeled with ^{3}H-TTP by repair-synthesis in vitro with Kornberg's enzyme in order to analyze the purity of the DNA and for detection of viral DNA by DNA-DNA reassociation kinetics.

Molecular biologic studies of human cytomegalovirus (CMV), increasingly recognized as an important human pathogen (30), have been retarded because purified virus and viral DNA are difficult to prepare. Even specific immune serum for immunofluorescence and complement fixation assays of CMV virion antigen are unavailable because virus preparations are commonly contaminated with cellular antigens. Moreover, the existence of different strains of human cytomegaloviruses although suspected has never been well established from either biologic or physical evidence. We describe here a rapid method for purifying CMV and CMV DNA and characterize two strains of human cytomegalovirus DNA and the DNA of a simian strain with respect to purity, density, and molecular weight. Also, we show the utility of tritiated CMV complementary RNA synthesized in vitro with *E. coli* transcriptase for the detection and localization of viral genome in membrane hybridization and in situ cytohybridization tests.

MATERIALS AND METHODS

Cells. A human fibroblastic cell strain (WI38) cultivated in Eagle minimal essential medium (MEM) with 15% fetal bovine serum and neomycin, 100 μg/ml, in roller bottles (surface area about 1,000 cm^2) was maintained after reaching confluence or after infection in MEM with 2% fetal bovine serum.

Virus. Two human strains of CMV, AD-169 (23) and C87 (1) and a simian strain, GR2757 (5), were obtained from the National Heart and Lung Institute, courtesy of R. M. Pennington. The KOS strain of herpes simplex virus type I (25) was from E. R. Alexander.

The cell monolayers were infected at a multiplicity of 1 to 2 PFU/cell. There was an absorption period of 2 h; the medium was changed 4 days after infection. To label the virus ^{3}H-thymidine, 3 μCi/ml (44.9 Ci/mM, New England Nuclear Corp.), was added 14 h after infection. The virus was harvested exclusively from the extracellular fluid 4 to 6 days after infection, depending upon the MOI and guided by the appearance of cytopathic effect (CPE), usually the third or fourth day after all of the cells showed CPE.

Purification of virus. The infected cells from several roller bottles, some labeled with ^{3}H-thymi-

dine, were shaken into the medium, pooled, and centrifuged at 7,000 rpm in a Sorvall GSA rotor for 20 min. The supernatant fluid containing free virus particles was then centrifuged in a Spinco T-19 rotor at 18,000 rpm for 60 min. The virus pellets were resuspended in 4 ml of Tris-buffered saline (TBS), 0.05 M Tris-hydrochloride, pH 7.4, 0.15 M NaCl, and homogenized vigorously in a Vortex mixer until no particulate matter was visible. Virus was purified by sedimentation through a 10 to 50% sucrose gradient, followed by a brief centrifugation through a preformed gradient of cesium chloride (density, 1.16 to 1.37 g/cm^3) as shown later.

Quantitative electron microscopy: virion counts. The number of virions in both crude and purified preparations was determined by D. Gordon Sharp with the agar pseudoreplica method (24).

Purification of viral DNA. The purified virus was extracted with 1% sodium dodecyl sulfate (SDS) and Pronase, 1 to 2 mg/ml, in the presence of 0.005 M EDTA for 8 to 10 h at 37 C (18). The DNA was purified by sedimentation in a 10 to 30% sucrose gradient and by two cycles of isopycnic centrifugation, as described later.

Synthesis of CMV complementary RNA (cRNA). We prepared and purified *E. coli* transcriptase according to Burgess (2); the activity of the enzyme was 200 U/ml (400 U of protein per mg). The CMV template DNA (1.6 μg in 0.12 ml), *Escherichia coli* transcriptase, 6 U in 30 μliters, and 0.10 ml of reaction mixture containing 0.1 M Tris, pH 7.9, 0.025 M $MgCl_2$, 0.25 mM EDTA, 0.25 mM dithiothreitol, 0.375 M KCl, 1.25 mg of bovine serum albumin per ml, 0.375 mmol of ATP, GTP, and CTP, and 0.5 mCi of ^{3}H-UTP (30 Ci/mmol) were mixed and kept at 37 C for 2.5 h. After reaction the DNA was digested with pancreatic DNAse (RNAse-free, Worthington) at a concentration of 40 μg/ml for 1 h; SDS was added to stop the reaction. The mixture was chromatographed through a Sephadex G-50 column (0.8 by 27 cm) in 0.1 × SSC and 0.01% SDS buffer. The first peak of radioactivity and absorbancy at 260 nm (A_{260}) was collected, extracted with phenol three times, and dialyzed against three changes of 0.1% SSC with 0.01% SDS at 4 C for 24 h. The efficiency of incorporation of ^{3}H-UTP into acid-precipitable RNA was about 6% of its total input. Complementary RNA with a specific activity between 8×10^6 and 10^7 counts per min per μg was obtained, the concentration of RNA being determined by absorption spectrophotometry (28).

Complementary RNA-DNA hybridization: (i) on membrane filters. The hybridization of in vitro virus-specific ^{3}H-cRNA to immobilized denatured DNA was by the method of Gillespie and Spiegelman (10) and as described before (18).

(ii) Cytohybridization in situ. This was carried out according to Gall and Pardue (8) modified as follows. Virus-infected cells on a cover slip were hypotonically treated with either 0.1 × Hanks solution or 0.1 × SSC for 20 min at 37 C and fixed with ice-chilled ethanol and acetic acid (3:1) fixative for 10 min. The cover slip was then dipped in absolute alcohol three times, alkalinized in 0.07 N NaOH for 2 min, and washed by dipping in 70 and 95% alcohol three times each. A 0.1-ml amount of ^{3}H-cRNA solution containing 5×10^5 counts/min of CMV cRNA, 6 × SSC, 0.1% SDS, and 1 mg of yeast RNA was applied; a cover glass was placed and the samples were kept wet with 6 × SSC. The hybridization was carried out at 66 C for 20 to 22 h. After RNAse digestion, complete washing and dehydration with alcohol, the cover slip was dipped in NTB 2 emulsion (Eastman) and air-dried in a darkroom for at least 2 h. The exposure was carried out at −20 C for a period up to 2 weeks, depending on the specific radioactivity of the ^{3}H-cRNA. The cover slip was developed and fixed with Kodak D-19 and rapid fixer and stained with Giemsa stain for 30 min.

Kornberg's enzyme. Kornberg's enzyme was purified according to Richardson et al. (22) as modified by Jovin et al. (14). Sephadex G-100 filtration was used to remove exonuclease III instead of hydroxyapatite chromatography. The enzyme fraction obtained from Sephadex G-100 was designated as fraction VII (14).

Radioisotope labeling of CMV DNA in vitro. The method essentially follows that used to label Epstein-Barr virus DNA (19). CMV DNA (1 to 1.5 μg) in 0.45 ml of TBS, pH 7.4, 0.01 M $MgCl_2$, 0.006 M β-mercaptoethanol was exposed to 0.05 ml of pancreatic DNAse I (0.1 μg/ml at 37 C for 15 to 30 min) and then was brought to 80 C for 10 min to inactivate the DNAse. The polymerization and repair of nicked DNA was carried out in the presence of 0.5 to 1 mCi of lyophilized ^{3}H-TTP (48 Ci/mmol) in 0.1 ml of a 7 × concentration of Kornberg's reaction mixture containing 0.49 M potassium phosphate buffer, pH 7.4, 0.049 M $MgCl_2$, 0.007 M β-mercaptoethanol, 0.0007 M cold dCTP, dGTP, dATP, 0.06 ml (4 U) of Kornberg's enzyme, fraction VII (14), and water to make the volume 0.7 ml. After incubation at 17 to 19 C for 5 h or more until incorporation reached its plateau, the reaction was stopped by the addition of Sarkosyl 97 (final concentration, 1%) and then heated to 80 C for 5 min. The specific radioactivity of the acid-precipitable product was 10^6 to 3×10^6 counts per min per μg. The reaction mixture was passed through Sephadex G-50, equilibrated with Tris buffer 0.01 M, pH 7.4, EDTA, 0.001 M, and Sarkosyl 97, 0.1%. The DNA-containing fractions were extracted with phenol and precipitated with 2.5 volumes of alcohol dissolved in 0.1 × SSC or TBS, then dialyzed and stored at −70 C.

DNA-DNA reassociation kinetics analysis. The in vitro tritium-labeled viral DNAs and unlabeled cell DNA were fragmented by ultrasonic vibrations to about 5 to 6*S* as determined by preparative ultracentrifugation (19). The procedure for DNA-DNA reassociation followed the method of Kohne and Britten (16) modified as described later (7, 19).

RESULTS

Propagation of extracellular virus. At relatively higher multiplicities of infection the number of extracellular viral particles was increased significantly by 48 h and reached a plateau 72 to 96 h after infection. This was true both in AD-169 and GR2757-infected cells; the simian strain gave higher particle numbers. Particle numbers ranged from 10^9 to 5×10^9 and

the infectivity from 10^6 to 10^7 PFU/ml 3 to 4 days after infection.

Virus purification. After the initial processing the virus harvested from the extracellular fluids was purified by a two-step procedure: velocity sedimentation and banding in a preformed gradient of cesium chloride, as shown in Fig. 1A and B. In the sucrose gradient there was a radioactive peak at the virus position (region of 42% sucrose). The material in fraction 5 (Fig. 1A) when stained with phosphotungstate and examined with an electron microscope showed predominantly enveloped virus particles. In the cesium chloride gradient, fraction 10 (Fig. 1B), density 1.263 g/cm^3, contained chiefly nucleocapsids as well as some partially enveloped virions, whereas completely enveloped virus particles predominated at density 1.219. The proportions of naked to partially enveloped virus depended on handling; if virus was frozen and thawed several times, or there was a delay in harvest, then a biphasic peak appeared with an increase in partially enveloped virus and nucleocapsids.

A tabulation of recovery of virions, monitored by quantitative electron microscopy, indicates that between 20 and 30% of the virions in the starting material are recovered at the end of the purification procedure (Table 1). Infectivity drops sharply in the step involving exposure to sucrose. At the same time there was extensive aggregation of virus visible in the EM, and this may explain the loss of infectivity. In an electron photomicrograph of the final product the virus appears distinctly freer of extraneous debris than virus purified only by sedimentation in sucrose (3).

Purification of viral DNA. After sedimentation of the viral DNA in a sucrose gradient, shown in Fig. 2, the viral DNA was subjected to two cycles of centrifugation to equilibrium in cesium chloride (Fig. 3). The host cell DNA in the first CsCl gradient (fractions 20 to 22, ρ = 1.69 ~ 1.70 g/cm^3) was no longer detectable in the second CsCl gradient. The density of the viral DNA was 1.716. The viral DNA (fractions 16 to 18 Fig. 3B) was dialyzed in TBS and stored at −70 C for use as template.

Attempts to purify viral DNA from virus taken directly from CMV-infected cells were not successful. Even after purification of the extracted virus through sucrose and cesium chloride as described in Fig. 1A and B, the radioactively labeled viral DNA was heavily contaminated with cellular DNA.

Velocity sedimentation analysis of human and simian CMV DNA. The analyses were carried out immediately after release of the DNA from the purified virus. The DNA of all

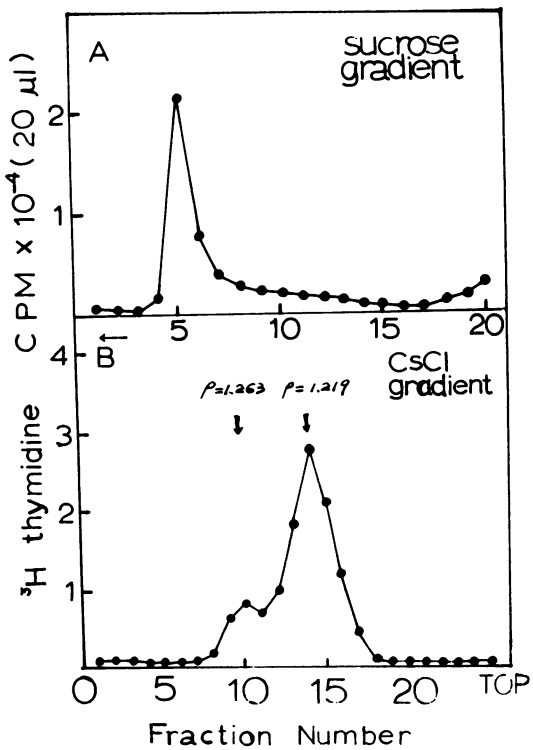

FIG. 1. *Sedimentation of CMV through a sucrose gradient and a preformed gradient of cesium chloride. The virus in 4 ml of TBS was centrifuged through a 10 to 50% sucrose gradient in TBS in the Spinco SW27 rotor at 27,000 rpm for 60 min at 4 C. Fractions (2 ml) were collected from the bottom of the tube. A, The peak of ³H-thymidine-labeled virus is in fraction 5. B, Fractions 4 to 7 from A were pooled, diluted to 14 ml in TBS, and layered without dialysis on 25 ml of a preformed gradient of CsCl, ρ = 1.16–1.37 g/cm³, in TBS and centrifuged in the SW27 rotor at 27,000 rpm for 60 min at 4 C.*

three strains sedimented as homogeneous 55S DNA in neutral gradients. Based on the HSV I DNA marker (11, 15) and the equation of Freifelder (6) the molecular weight of the DNA for the three strains was estimated to be approximately 10^8 as shown in Fig. 4.

Density of human and simian CMV DNA. Determinations of the density of the DNA of strains AD-169, C87 and GR2757 were carried out by analytic ultracentrifugation with results that confirm closely the values obtained by isopycnic centrifugation in the preparative ultracentrifuge (see Fig. 3). As shown in Fig. 5 the density of both human strains of CMV DNA was 1.716 g/cm^3 in contrast to the density of the simian CMV DNA, 1.710 g/cm^3, which indicates a higher guanine-plus-cytosine content for the human strains.

Characterization of the viral DNA: utility

TABLE 1. *Purification of extracellular CMV: recovery of particles and infectivity*

Purification procedures	Expt.[a]	Volume (ml)	Titer/ml $TCID_{50}$	Particle (counts/ml)	Total particles recovered	Particles recovered (%)
Crude harvest material	1	500	2×10^5	1.3×10^9	6.5×10^{11}	100
	2	710		7.9×10^8	5.6×10^{11}	100
Low-speed clarified supernatant fluid	1	500	2×10^5	9.3×10^8	4.6×10^{11}	71
	2	710		7.7×10^8	5.4×10^{11}	96
T-19 high-speed supernatant fluid	1	500	2×10^3	7×10^7	3.5×10^{10}	5
	2	710		$<10^7$	$<7.1 \times 10^9$	<1.3
T-19 high-speed pelleted virus	1	4	2×10^8	5.4×10^{10}	2.0×10^{11}	30
	2	3.8		3.4×10^{10}	1.29×10^{11}	23
Sucrose gradient viral band	1	6	$<10^5$	3.1×10^{10}	1.9×10^{11}	29
	2	6		2.0×10^{10}	1.2×10^{11}	21
Preformed CsCl viral band	1	4	2×10^5	4.6×10^{10}	1.8×10^{11}	27
	2	4		2.7×10^{10}	1.1×10^{11}	20

[a] Experiment 1, WI-38 cells infected with GR2757; Experiment 2, WI-38 cells infected with AD-169.

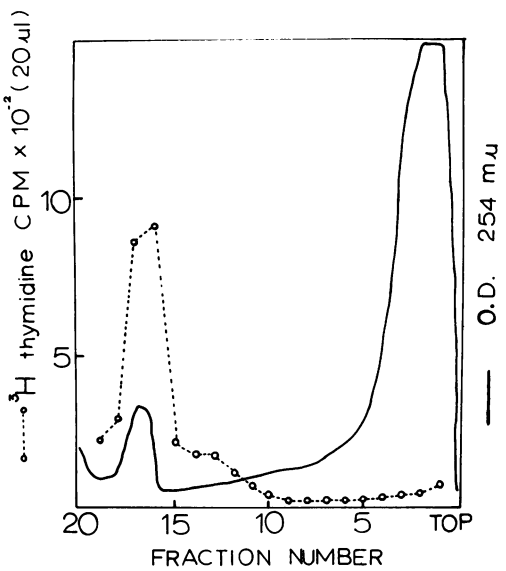

FIG. 2. *Velocity sedimentation of CMV DNA. The purified virus was treated with Pronase, 2 mg/ml, and SDS (final concentration, 1%) at 37 C for 8 h, and then carefully layered on a 10 to 30% sucrose gradient in TBS with 0.005 M EDTA. After centrifugation at 21,000 rpm in a Spinco SW27 rotor for 15 h at 18 C, the gradient was collected from the top by an ISCO fractionator. Sedimentation is toward the left.*

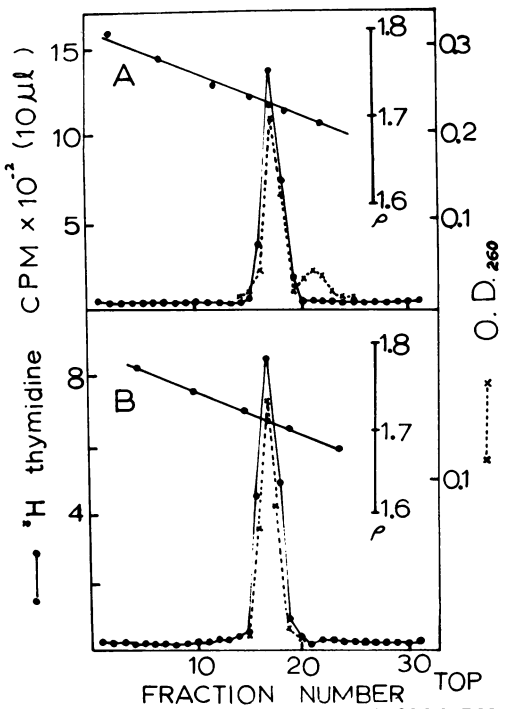

FIG. 3. *Isopycnic centrifugation of CMV DNA. The DNA from fractions 16 to 18 (Fig. 2) was dialyzed against three changes of TBS with 0.005 M EDTA at 4 C. It was then extracted with phenol and reprecipitated by alcohol and purified by two cycles of centrifugation to equilibrium in CsCl in a Spinco T-65 rotor, each time at 35,000 rpm for 68 h at 20 C. Fractions 16 to 18 of the first CsCl gradient (A) were collected and rerun to equilibrium in the second CsCl gradient as indicated in (B).*

as template. cRNA synthesized with the purified CMV DNA as template hybridized specifically to viral DNA and not to host cell DNA as indicated in the reconstruction curve shown in Fig. 6. The increase in the hybridized radioac-

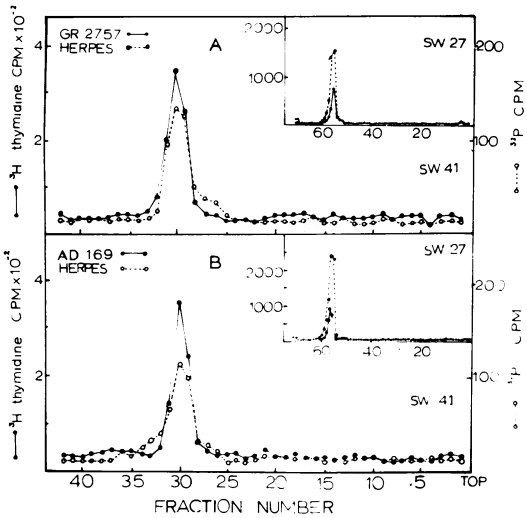

FIG. 4. *Velocity sedimentation analysis of human and simian CMV DNA. Two methods are shown. The viral DNA depicted in the insets (A and B) was released from virions by treatment with Pronase, 2 mg/ml, and SDS, 1%, in TBS with 0.005 M EDTA and then run through a gradient of sucrose, 10 to 30%, in TBS and 0.005 M EDTA in an SW27 rotor at 27,000 rpm for 14 h at 20 C. The larger figures (A and B) show viral DNA extracted by disruption of virions in 0.5% SDS and 2% Sarkosyl in 0.9 M NaCl, 0.001 M EDTA, and 0.05 M TBS, pH 7.4. The virions were heated at 60 C for 3 min (15) and then sedimented in 10 to 30% sucrose in a neutral DNA buffer by centrifugation in the SW41 rotor for 4 h at 35,000 rpm. The CMV DNA is labeled with ^{3}H-TdR, and the herpes simplex DNA is labeled with ^{32}P.*

tivity was directly proportional to the amount of CMV DNA on the filters. Both WI38 (host cell) DNA and Hep2 DNA without CMV DNA gave similar results, 130 and 118 counts/min, respectively, after correction for the background radioactivity.

The CMV cRNA was also tested for its utility for in situ cytohybridization. As shown in Fig. 8A the background nonspecific hybridization of the cRNA to the uninfected cells was negligible, with scattered grains in and between the cells and no concentration in single cells, in contrast to the autoradiograms of the infected cells. These results are another indication of the lack of or very low level of host cell DNA contamination in the viral DNA template material.

DNA synthesis, RNA synthesis, and intracellular localization of viral DNA. Figure 7 shows the time course of ^{14}C-thymidine and ^{3}H-uridine incorporation in CMV-infected cells. DNA synthesis as reflected by the incorporation of thymidine began to increase 24 h after infection, reaching its peak at 72 h (Fig. 7A). Uridine incorporation started earlier and reached its peak 33 h after infection (Fig. 7B). When thymidine incorporation is compared with the results of cytohybridization which shows only viral DNA (Fig. 8) there is a good correlation. Viral DNA can be detected 24 h after infection, and it is found in two acrocentric areas. At 33 h the viral DNA continued to accumulate in these areas; the DNA began to distribute in a band resembling intranuclear inclusions 48 to 70 h after infection (Fig. 8 A–D). At 120 and 144 h heavy concentrations of grains were located outside as well as inside the nuclei.

CMV DNA renaturation kinetics. To characterize the CMV DNA further and to demonstrate its freedom from detectable host cell DNA we carried out renaturation analyses as shown in Fig. 9 according to Kohne and Britten (16) and Frenkel and Roizman (7). Viral DNA with high specific radioactivity was prepared by labeling in vitro. In the presence of 2 mg of *E. coli* DNA the half-Cot value for 0.01 μg ^{3}H-AD-169 DNA was 2.1 × 10^{-1} mol s/liter; for 0.02 μg

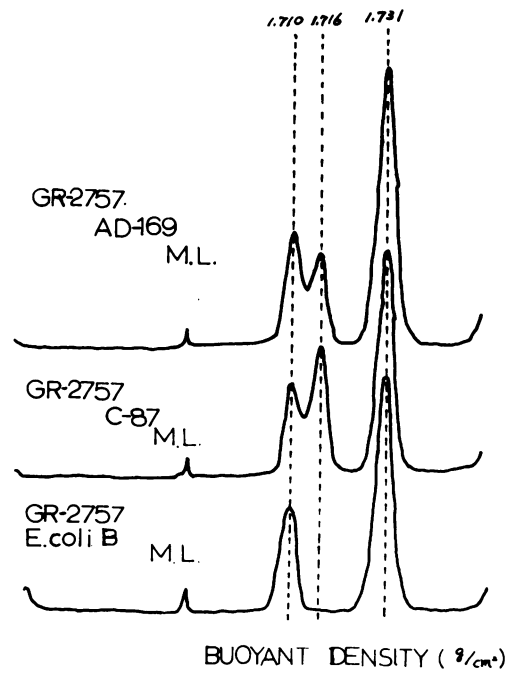

FIG. 5. *Density of human and simian CMV DNA determined by analytic ultracentrifugation. The DNA was purified as before, suspended in 0.01 M Tris-hydrochloride, pH 7.4, and 0.001 M EDTA and mixed with CsCl, final density 1.710 g/cm^3, and centrifuged in the model E, rotor ANF, double sector 12-mm cell, at 34,000 rpm for 67 h at 20 C. Micrococcus lysodeikticus DNA (density, 1.731 g/cm^3) and E. coli DNA (1.710 g/cm^3) were the markers.*

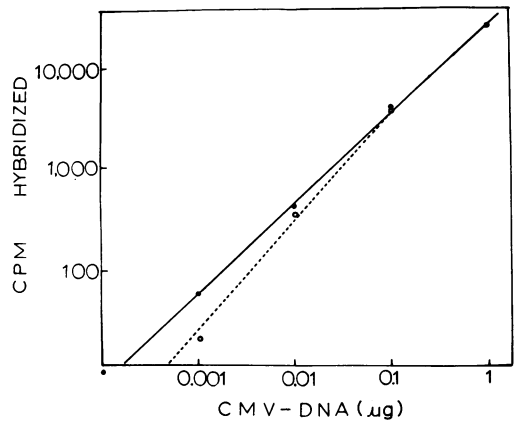

FIG. 6. *Hybridization of CMV cRNA to CMV DNA. Graded amounts of CMV DNA were mixed with 50 μg of HEp-2 DNA (●——●) or WI 38 DNA (O·····O) and denatured in 0.5 N NaOH for 2 h at 37 C. The DNA solution was neutralized with 1.1 N HCl in 0.2 M Tris, adjusted to 6 × SSC, and immobilized on filters. The input cRNA for each filter was 1.5 × 10⁵ counts/min (specific activity is about 10⁷ counts per min per μg). The amount of DNA retained on the filter, determined by a diphenylamine test after hybridization, remained constant. The background hybridized counts for 50 μg of either HEp-2 or WI 38 DNA (130 or 118 counts/min) were subtracted from each value.*

^{3}H-DNA the value was 1.1×10^{-1} mol s/liter. For 0.04 μg ^{3}H-DNA it was 4.8×10^{-2} mol s/liter. The addition of 1 μg of cold AD-169 to the 0.01 μg of ^{3}H AD-169 DNA gave a half-Cot value of 1.8×10^{-3} mol s/liter; that is, the acceleration of the renaturation reaction was proportional to increases in the concentration of the viral DNA in the system. With replacement of the *E. coli* DNA with 2 mg of WI38 DNA or calf thymus DNA there was no acceleration of the reassociation of ^{3}H-AD-169 DNA; this result indicates the lack of detectable host cell DNA contamination in the purified viral DNA.

DISCUSSION

We present in this investigation basic technology needed to proceed with the molecular biologic characterization of human and other species of cytomegaloviruses. The results include the first precise determination by sedimentation analyses of the molecular weight of CMV DNA, as well as determination of the density of three strains of CMV DNA.

The techniques for isolation of herpes simplex virus from infected cells (15) are not suitable for purification of CMV because the unreleased CMV remains associated with nuclear contents so that it is difficult to procure virus free from cellular DNA and protein. The glycine buffer extraction technique (21) does not solve the problem of releasing the virus particles from the infected cells. If we started with crude free virus with a relatively high viral particle number (1 ~ 5×10^9 particles per ml) rather than virus extracted from cells, relatively pure CMV could be rather easily concentrated from the extracellular fluid through velocity sedimentation and a short run on a preformed density gradient. This was despite the unfavorable experience with cesium chloride in the purification of HSV, which is probably explained by 44 h of centrifugation (26).

It is important to use roller bottle cultures for more concentrated extracellular virus, also noted by Chambers et al. (3); to take care to prepare virus seed of relatively high titer for a higher multiplicity of infection; and to delay in the harvesting of the virus until CPE is ad-

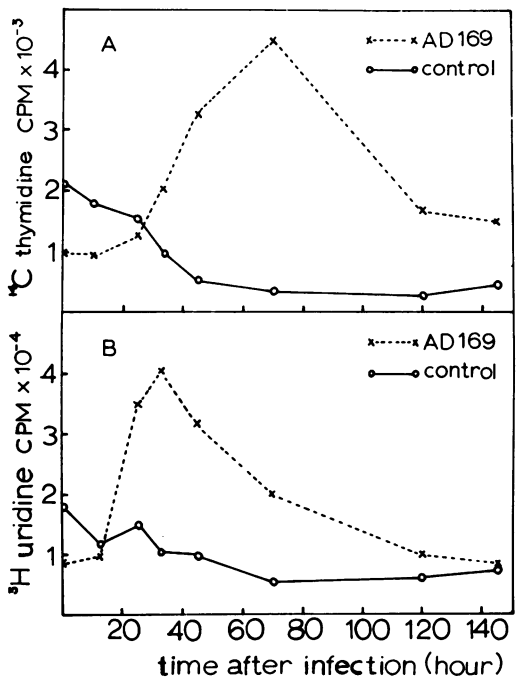

FIG. 7. *Incorporation of ^{14}C-thymidine and ^{3}H-uridine in CMV-infected cells. WI38 cells were infected with AD-169 at a multiplicity of 1 to 2 PFU/cell. At various times after infection 0.4 μCi of ^{14}C-thymidine per ml or 10 μCi of ^{3}H-uridine per ml were added for 1 h. At the end of labeling period the cells were washed with TBS and lysed with 1% SDS in 0.01 M EDTA and 0.01 M Tris-hydrochloride, pH 8.0. The lysates were precipitated in the cold with trichloroacetic acid at a final concentration of 20%, collected on membrane filters, and thoroughly washed with cold 5% trichloroacetic acid. Panel A, incorporation of ^{14}C-thymidine; panel B, incorporation of ^{3}H-uridine.*

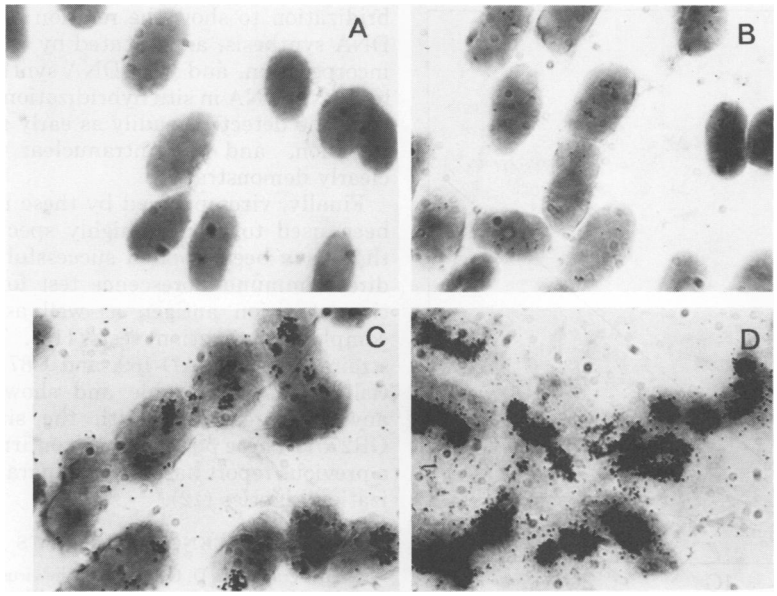

FIG. 8. *In situ cytohybridization for detection of CMV DNA. WI38 cells on cover slips were infected at a high multiplicity (1 to 2 PFU/cell). The nuclei were released by hypotonic treatment and fixed. The DNA was denatured with 0.07 N NaOH for 3 min. After washing and fixation cRNA-DNA hybridization was carried out with 5 × 10^6 counts/min for 22 h at 66 C. The preparations were washed and treated with RNAse, and autoradiography was carried out with NTB2 emulsion. Because of the hypotonic treatment only nuclei are visible. A, Uninfected control cells; B, 24 h after infection; C, 33 h after infection; D, 70 h.*

vanced so that more virus is released into the extracellular fluid.

We can detect no physical differences in the two strains of human CMV that we have completed testing as revealed by virion density, molecular weight of DNA and guanine-plus-cytosine content as indicated by isopycnic centrifugation of the two viral DNAs. However, the simian CMV DNA is distinctly different in density (1.710 g/cm^3) from the human strains (1.718), as noted also by Plummer et al. (20). The evidence that the two human strains are indeed different biologically is scant. It is, however, entirely possible that distinct strains of CMV do in fact exist in view of the broad spectrum of biologic behavior and their varying habitat including the cervix and human semen (9), brain, retina, salivary gland, lung, blood, abdominal viscera, and the kidneys (17, 27, 29, 30, 31). Weller classified the Davis strain of human CMV as type I, the AD-169 as type II, and the Kerr and Esp strains as type III (30, 32).

The molecular weight of both the human and simian strains of CMV DNA are estimated as 10^8, like herpes simplex type I (11, 15), herpes simplex II (11, 15), and Epstein-Barr virus DNA (18). This value is higher than that determined by Crawford and Lee (4): 32 million. However the DNA in those determinations was analyzed by band-width analyses and was taken from CMV-infected cells; the authors thought that this molecular weight was an underestimate.

The best (but not absolute) measure of the purity of the viral DNA obtained by these methods is probably provided by the DNA renaturation kinetics analyses. The reaction was carried out to within 90% of completion, which indicates that the analysis would account for nearly the entire genome if the test is applied to extracts of tissues thought to harbor CMV. Neither *E. coli* DNA, calf thymus DNA nor, especially, WI38 DNA had any effect in accelerating renaturation of the tritiated viral DNA. This result confirms the freedom of the viral DNA from detectable host cell DNA sequences.

This technique has proved to be a valuable tool for determination of interrelatedness between strains and species of CMV and other herpes viruses (manuscript in preparation). For practical purposes there was no detectable host cell DNA contamination in the viral DNA that served as template for synthesis of cRNA used for cRNA-DNA membrane and cytohybridization.

The percentage of the CMV genome that is copied into the CMV-cRNA is unknown. However the cRNA is a useful probe and will allow the gathering of valid comparative data based

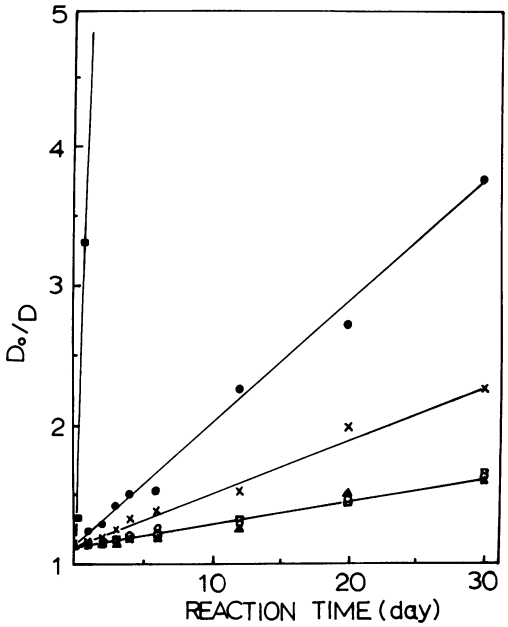

FIG. 9. *DNA-DNA renaturation kinetics analysis of CMV DNA. After the DNAs were denatured by heating at 100 C for 15 min the salt concentration was adjusted to 1.2 M NaCl in 0.01 M Tris-hydrochloride, pH 7.4, and 0.0025 M EDTA, and the DNA was allowed to renature at 66 C. The fraction of unreassociated DNA versus reannealed DNA was determined by hydroxyapatite chromatography (16, 19). The results were plotted according to Frenkel et al. (7); Do/D is the ratio of the concentration of unreassociated DNA at zero-time (Do) and various times (D) after reaction. Symbols: O, WI38 DNA, 2 mg + in vitro labeled ^{3}H-AD-169 DNA, 0.01 μg; □, E. coli DNA, 2 mg + ^{3}H-AD-169 DNA, 0.01 μg; ▲, calf thymus DNA, 2 mg + ^{3}H-AD-169, 0.01 μg; ×, E. coli DNA, 2 mg + ^{3}H-AD-169 DNA, 0.02 μg; ●, E. coli DNA 2 mg + ^{3}H-AD-169 DNA, 0.04 μg; ■, E. coli DNA, 2 mg + ^{3}H-AD-169 DNA, 0.01 μg + cold AD-169 DNA, 1 μg.*

on the number of CMV genome equivalents detectable. If the molecular weight of CMV DNA and diploid host cell DNA are 10^8 and 4×10^{12} (9), respectively, then 0.1 μg of CMV DNA/50 μg of cellular DNA is equivalent to 80 CMV genomes per cell. The counts of cRNA hybridized to 0.1 μg of CMV DNA in the presence of 50 μg of Hep2 or WI38 DNA are 3,852 and 3,512 counts/min, respectively, equal to 48 to 40 counts per min per genome per cell. By this calibration we should readily detect as few as 3 to 4 CMV genome equivalents per cell; detection of fewer genome equivalents would be unreliable because of the usual background level of nonspecific hybridization.

The CMV cRNA was also used for cytohybridization to show the relation between total DNA synthesis, as indicated by ^{14}C-thymidine incorporation, and viral DNA synthesis, shown by cRNA-DNA in situ hybridization. Viral DNA could be detected readily as early as 23 h after infection, and its intranuclear localization clearly demonstrated.

Finally, virus purified by these methods has been used to prepare highly specific antisera that have been applied successfully in an indirect immunofluorescence test for the detection of virion antigen as well as in specific complement fixation tests (13). The human strains of CMV, AD-169, and C87 were essentially indistinguishable and showed little, if any, cross-reactivity with the simian strain GR2757. These observations confirmed those in a previous report based on comparative neutralization kinetics (12).

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