Rat Cytomegalovirus: Studies on the Viral Genome and the Proteins of Virions and Nucleocapsids

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SUMMARY

The nucleocapsids (N-capsids) isolated from the nuclei of rat cytomegalovirus
(RCMV)-infected rat embryo fibroblasts (REF) are composed of three major proteins:
142 × 10^3 (142K), 40K and 32K mol. wt. Nucleocapsids isolated from the cytoplasmic
fraction (C-capsids) are composed of proteins found in N-capsids and five major and
seven minor new protein species. Most of the proteins present in C-capsids are found in
the extracellular enveloped virions, although the ratios vary. Proteins that are
abundantly present, particularly in virions (mol. wt. 125K, 116K, 87K, 79K, 71K, 68K,
62K, 50K, 43K and 28K), are probably the major constituents of the viral envelope.
The DNA recovered from extracellular virions was purified to homogeneity and by
equilibrium centrifugation in CsCl one density class of 1.716(±0.001) g/ml was found.
Contour length measurements showed one size class of a linear double-stranded DNA
corresponding to an average mol. wt. of 144(±9) × 10^6 which is in good agreement
with data obtained by restriction endonuclease analysis (REA), which yielded mol. wt.
values of 132(±9) × 10^6 (HindIII), 138(±2) × 10^6 (EcoRI) and 137 × 10^6 (BglII). The
REA patterns also revealed the presence of 0.25 M and 0.5 M fragments, which might
indicate, in analogy with other cytomegalo- and herpesviruses, the existence of four
different configurations of the RCMV genome. The infectivity of RCMV DNA was
determined in subconfluent REF monolayers. A cytopathic effect characteristic of
RCMV was observed 6 days post-transfection and up to 60 plaques/μg DNA were
obtained. Using DNA-DNA filter hybridization the degree of homology between the
genomes of RCMV and murine or human CMV was examined. Under stringent
conditions (50% formamide) values of 12(±2) and 3(±1)% were found whereas
under non-stringent conditions (20% formamide) values of 21(±2) and 6(±1)% were
obtained, respectively.

INTRODUCTION

The role of cytomegaloviruses (CMVs), members of the herpesvirus group, in establishing
persistent and latent infections in man and animals has been studied extensively in the last
decade. In the human immune-compromised host a wide range of disorders has been associated
with CMV, of which the most important are interstitial pneumonia in transplantation patients,
the post-perfusion syndrome, infectious mononucleosis and congenital and neonatal infections
(Weller, 1980). Furthermore, in recent years evidence has been obtained for a possible
oncogenic potential of human CMV (HCMV) (Albrecht & Rapp, 1973; Geder et al., 1976;
Boldogh et al., 1981; Nelson et al., 1982). Experimental animal models have been developed in
recent years to study CMV persistence, latency and reactivation under defined conditions.
Mouse CMV (MCMV) and the murine model (Hudson, 1979) have been extensively studied,
whereas a guinea-pig model has recently been developed particularly for studying congenital
CMV infections (Choi & Hsiung, 1978; Griffith & Hsiung, 1980). It has been our objective to develop an experimental rat model in which CMV persistence, latency and reactivation in relation to immunomodulation and organ transplantation can be studied.

Recently, we have reported the isolation of a cytomegalo-like virus from the salivary glands of wild rats (Bruggeman et al., 1982). The virus, provisionally designated as a rat CMV (RCMV) causes acute and latent infection in laboratory rats and its (cyto)pathological and morphological properties are strongly indicative of a CMV (Bruggeman et al., 1983). We have further characterized this virus regarding its structural elements. The first part of this paper reports the isolation and the protein composition of extracellular virions and nucleocapsids recovered from the cytoplasm and the nucleus of RCMV-infected cells.

One of the most characteristic physicochemical parameters of a CMV is the size of its genome, which in contrast to other herpesviruses has a mol. wt. in excess of 100 × 10^6, as illustrated by the findings for HCMV DNA (150 × 10^6 mol. wt.: Geelen et al., 1978; DeMarchi et al., 1978), MCMV DNA (132 × 10^6 mol. wt.: Mosmann & Hudson, 1973) and equine CMV DNA (126 × 10^6 mol. wt.: Wharton et al., 1981). Hence, another part of this study presents determination of the molecular weight of RCMV DNA by restriction endonuclease analysis and contour length measurements. In addition, the infectivity of the viral DNA as assayed in subconfluent monolayers of rat embryo cells and the buoyant density of the DNA, as determined by equilibrium CsCl centrifugation, are reported. Finally, using filter DNA–DNA hybridization the degree of homology between the genomes of RCMV, MCMV and HCMV is evaluated.

**METHODS**

*Virus and cell culture.* Rat embryo fibroblast cell cultures (REF) were prepared from 16-day-old Lewis rat embryos essentially as described by Bruggeman et al. (1982), and were used for virus propagation at the third passage. Cells were grown in Eagle's minimum essential medium (MEM) containing 10% newborn calf serum (NCS) and maintained in Eagle's MEM with 5% NCS. All media contained penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml). For virus growth the NCS concentration was reduced to 2% Stock RCMV was obtained by infecting REF cells with twice-plaque-purified RCMV at an m.o.i. of 0.01 p.f.u./cell. Supernatant fluid was harvested when cells showed an advanced cytopathic effect (c.p.e.), usually between 6 and 8 days post-infection, and was freed from particulate debris by centrifugation for 10 min at 4000 rev/min. The virus pool was divided into suitable aliquots and stored at −70 °C. Virus titres were determined by plaque assay as previously described (Bruggeman et al., 1982), reaching titres of 5 × 10^6 p.f.u./ml.

*Purification of extracellular virus.* RCMV was propagated on REF grown in roller bottles (1350 cm^2) or Roux flasks (150 cm^2) at an m.o.i. of 0.01 p.f.u./cell. The infection was allowed to proceed until advanced c.p.e. was observed (6 to 8 days). Cells were loosened from the culture vessels and collected by centrifugation for 10 min at 2000 rev/min and 4°C. The cell pellet was used to isolate nuclear and cytoplasmic nucleocapsids whereas the supernatant fluid (after a further clarification by centrifugation for 10 min at 4000 rev/min and 4°C) was used to isolate extracellular virions.

Virus was pelleted from the supernatant by centrifugation for 45 min at 40000 rev/min and 4°C in a Beckman Ti 45 rotor or for 3 h at 18000 rev/min and 4°C in a Spinco Ti9 rotor. The pellet was resuspended in 1 ml TBS (0.05 M-Tris–HCl, 0.15 M-NaCl, pH 7.4) and particulate aggregates were removed by centrifugation for 1 min in an Eppendorf centrifuge. The clarified virus suspension was layered on a 10 to 40 (w/v) linear CsCl gradient in TBS and centrifuged for 1 h at 25000 rev/min and 15 °C in a Beckman SW27 rotor. Gradients were monitored at 280 nm and 0.4 ml fractions were collected using a Beckman gradient collector. Peak fractions were examined by electron microscopy after negative staining. Fractions containing virions or nucleocapsids were pooled, diluted with 10 vol. TBS and pelleted by centrifugation for 1 h at 25000 rev/min and 15 °C in a Beckman SW27 rotor. Virus pellets were prepared for SDS–polyacrylamide gel electrophoresis (SDS–PAGE) or DNA extraction as described below.

MCMV (Smith strain) and HCMV (AD169) were propagated and purified identically from the supernatant fluids of MCMV-infected mouse embryo fibroblasts (passage number 2 and 3) or HCMV-infected human embryo fibroblasts (passage number 15 to 20), respectively.

*Purification of nucleocapsids from the cytoplasm and nucleus of RCMV-infected cells.* The RCMV-infected cell pellet was washed once with 10 vol. ice-cold phosphate-buffered saline (PBS), collected by low-speed centrifugation and resuspended in 5 vol. ice-cold TBS. Nonidet P40 (NP40; 10%, w/v) was added dropwise to a final concentration of 0.5%. After incubation on ice for 30 min and occasional shaking, the suspension was centrifuged for 5 min at 12000 rev/min through a cushion of 0.25 M-sucrose in TBS. The cytoplasmic fraction, remaining on top of the sucrose, was freed from particulate debris by centrifugation for 10 min at 5000 rev/min.
The pellet remaining after centrifugation (pure nuclei) was resuspended in 5 vol. TBS and was lysed with deoxycholate, DNase, urea and Brij 58 as described by Gibson (1981). The lysate was centrifuged for 10 min at 5000 rev/min. Isolation of nucleocapsids from the clarified cytoplasmic and nuclear fractions was performed as described for extracellular virions.

**Recovery of nucleocapsids from virions.** Purified virions were treated with NP40 (0.5%) for 15 min and 4 °C. Nucleocapsids were pelleted by ultracentrifugation for 1 h at 25000 rev/min and 15 °C in a Beckman SW50 rotor through a cushion of 20~ (w/v) sorbitol.

**SDS-PAGE.** This was performed on 10% or 12% polyacrylamide gels essentially as described by Laemmli (1970). Calibration proteins (obtained from Pharmacia) and virus preparations were prepared for electrophoresis at 100 °C for 2 min in 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 50 mM-Tris-HCl pH 7-0. Gels were electrophoresed for 16 h at 70 V and 20 °C and were stained overnight in 0-04% Coomassie Brilliant Blue, 27% ethanol and 10% acetic acid and finally destained in 12% ethanol and 7% acetic acid.

**Isolation and purification of viral DNA.** Purified virions or nucleocapsids in 300 μl TBS + 5 mM-EDTA (STE) were heated for 10 min at 68 °C to destroy DNase activity and were gently lysed in 1% SDS and 200 μg/ml proteinase K for 3 h at 37 °C. The lysate was centrifuged for 4 h at 35000 rev/min and 19 °C in a Beckman SW40 rotor in a 10 to 30% (w/v) linear sucrose gradient in STE. Gradients were monitored at 254 nm and 0.2 to 0.5 ml fractions were collected as described previously. Fractions containing homogeneous and high molecular weight DNA, as indicated by ethidium bromide–agarose gel electrophoresis by comparison with standard phage T4 DNA concentrations, were pooled and dialysed against STE at 4 °C. For contour length measurements and transfection experiments the DNA was stored at 4 °C. For other experiments, the DNA was concentrated by ethanol precipitation, dissolved in 10 mM-Tris-HCl pH 7-4, 1 mM-EDTA, and stored at −70 °C. MCMV DNA and HCMV DNA were purified in a similar way.

**Preparation of radiolabelled RCMV DNA.** REF cells, grown in 150 cm² Roux bottles, were infected with RCMV at an m.o.i. of 0-01 p.f.u./cell. At 48 h post-infection the medium was replaced by Eagle’s basal medium + 2% NCS containing 5 μCi/ml [3H]thymidine (sp. act. 20 Ci/mmol). The infection was allowed to proceed until advanced c.p.e. was observed. Virus and viral DNA were isolated as described above for extracellular virions. Of each fraction (0.2 ml) of the sucrose gradient, an aliquot of 20 μl was spotted onto small Whatman GF/C glass fibre filter discs and the trichloroacetic acid (TCA)-insoluble radioactivity was determined. Gradient fractions containing high molecular weight DNA were used for further analysis.

**Buoyant density of RCMV DNA.** CsCl was added to purified [3H]-labelled RCMV DNA and [14C]-labelled T4 DNA to a final density of 1.700 g/ml. The DNA was centrifuged to equilibrium for 48 h at 34000 rev/min and 20 °C in a Beckman SW60 rotor. After centrifugation, 80 μl fractions were collected using a Beckman gradient collector. Of each fraction, 10 μl aliquots were withdrawn for density measurement using a refractometer (Atago) which was calibrated and temperature-stabilized at 25 °C. Twenty μl samples were withdrawn to determine the TCA-insoluble radioactivity.

**Restriction endonuclease analysis of RCMV DNA by agarose gel electrophoresis.** The restriction enzymes EcoRI, BglII, HindIII, BamHI and BamHI were obtained from Boehringer Mannheim and used according to the manufacturer’s instructions. The restriction enzyme digestions were stopped by the addition of 1/10 vol. of 0-1 M-EDTA, 50% sucrose, 0-2% bromophenol blue, pH 7-6. The DNA fragments were electrophoresed on horizontal 0-65% agarose (SeaKem, type ME) slab gels, for 18 to 42 h at 1-5 V/cm at room temperature in a buffer system described by Sambrook. After electrophoresis the gel was photographed using a Polaroid Land MP-3 camera equipped with a Kodak 23A filter and type 55 film.

Negatives were scanned in a Shimadzu dual wavelength TLC scanner (type C-5910). The molecular weights and molarities of the RCMV DNA fragments were calculated as described by Weststrate et al. (1980). From the photographs the contour lengths were measured with a digitizer (Summagraphic) equipped with a Digital (VT 105, Minc II) data processor.

**Contour length measurements of RCMV DNA by electron microscopy.** The RCMV DNA was spread by the aqueous method of Davis et al. (1971), using SV40 DNA (3-28 × 10⁶ mol. wt.; Wellauer et al., 1974) as internal marker. The carbon-coated grids were rotary shadowed with platinum at an angle of 9° and examined in a Philips EM-201 electron microscope.

The molecular weights of the RCMV DNA molecules were estimated from the ratio of the RCMV DNA length to that of the SV40 DNA.

**Infectivity of RCMV DNA.** The infectivity of RCMV DNA was determined by plaque titration in subconfluent monolayers of REF grown in 6 cm diam. Petri dishes, employing the calcium phosphate method as described by et al.
Graham & Van der Eb (1973), modified by treatment of the cells with 15% glycerol for 4 min at 4 h post-transfection. Each dish of cells was incubated with either 0.2 µg or 0.5 µg of the viral DNA plus 20 µg/ml of the carrier DNA or only carrier DNA. Cell cultures were examined for the appearance of plaques up to 2 weeks post-transfection.

DNA–DNA hybridization on nitrocellulose filters. RCMV DNA was 35S-labelled in vitro by nick translation using dATP (α-35S; 800 Ci/mmol) and other chemicals according to the reaction protocol supplied by New England Nuclear.

One µg of the purified DNA of RCMV, MCMV, HCMV or bacteriophage λ was applied to 1 cm² nitrocellulose filters and alkali-denatured in situ as described by Brandsma & Miller (1980). Filters were baked for 2 h at 80 °C and prehybridized in 1 × Denhardt’s solution for 4 h at 60 °C. Hybridization mixtures (0.3 ml for each filter) containing 10⁶ ct/min of the heat-denatured and sonicated 35S-labelled RCMV DNA probe (sp. radioact. 5 × 10⁷ ct/min/µg) were incubated at 37 °C in 1 M NaCl, 0.01 M Tris–HCl pH 7.4, 0.2% Ficoll, 0.2% polyvinylpyrrolidone and 0.2% bovine serum albumin (Fraction V, Boehringer Mannheim) and the indicated percentage of formamide for various periods of time. Filters were then extensively washed in 6 × SSC at 68 °C or 50 °C following hybridization in 50% or 20% formamide, respectively (Howley et al., 1979) and counted in Econofluor.

RESULTS

Isolation of RCMV particles from infected cells and extracellular fluid

Lewis REF cell cultures infected with RCMV and the respective maintenance medium were processed, as described in Methods, to recover and purify intracellular (cytoplasmic and nuclear) as well as extracellular viral particles. As shown in Fig. 1(a to c) rate velocity sedimentation in 10 to 40% CsCl of the extracellular, cytoplasmic and nuclear fraction, respectively, resulted in single peaks (light-scattering bands). Inspection by electron microscopy showed that fractions 11, 12 and 13 (Fig. 1a) contained enveloped virions, free from cellular material, which tended to aggregate upon concentration (Fig. 2a). The structure of one particular virion is shown in detail in Fig. 2(d). Occasionally, some spike-like structures are visible on the surface of the viral envelope, the meaning or function of which is not clear at present. Sedimentation of the cytoplasmic fraction resulted in a sharp peak (Fig. 1b, fraction 12), exclusively consisting of nucleocapsids (referred to as C-capsids) which were surrounded by

![Fig. 1. Sedimentation profiles in 10 to 40% CsCl gradients of RCMV particles recovered from concentrated extracellular fluid (a), cytoplasmic fraction (b) and nuclear fraction (c) of RCMV-infected cells. RCMV-infected cells and supernatant were processed and prepared for ultracentrifugation in CsCl gradients as described in Methods. Gradient fractions were continuously monitored at 280 nm and peak fractions were examined by electron microscopy as shown in Fig. 2.](image-url)
Fig. 3. SDS-polyacrylamide gel electrophoretic patterns on a 12% gel of highly purified N-capsids, C-capsids and extracellular RCMV virions. The three protein preparations (50 µg), together with the molecular weight marker proteins were run on the same gel, in close proximity to each other. Inset: for a better resolution and derivation of the high molecular weight proteins, the same samples were run on a 10% gel. v, virions; n, N-capsids; c', C'-capsids (remaining after removal of the viral envelope); c, C-capsids.

an undefined protein matrix (Fig. 2b). Sedimentation of the nuclear fraction in CsCl also generated a sharp band (Fig. 1c, fraction 12) consisting of conglomerates of pure nucleocapsids, referred to as N-capsids (Fig. 2c). Both C-capsids and N-capsids were not infectious in REF in contrast to the extracellular virions (recovery of infectivity about 5%).

Treatment of purified enveloped virions with NP40 (see Methods) yielded nucleocapsids (referred to as C'-capsids) which were often slightly damaged, but of which the particular configuration of the capsomer proteins was clearly visible (Fig. 2e).
Structural proteins of virions and nucleocapsids

The proteins of extracellular virions, intracellular nucleocapsids (C-capsids and N-capsids), and the virion-derived C'-capsids were analysed by SDS–PAGE. Protein patterns characteristic of the different viral particles are shown in Fig. 3. As can be seen the N-capsids are composed of three major proteins with estimated molecular weights of 142K, 40K and 32K. The 142K protein was present most abundantly. In addition, four minor proteins were found (74K, 57K, 38K and 28K). C-capsids contained all N-capsid proteins in approximately the same relative proportions as well as five new major protein species (219K, 100K, 55K, 42K and 15K) and seven new minor proteins (116K, 87K, 71K, 62K, 50K, 35K and 34K). Although most of the proteins present in C-capsids could also be identified in virions, the relative amounts of these proteins appeared to differ considerably. Protein species clearly under-represented in virions were the 142K, 100K, 74K and in particular the 40K protein. The 116K, 87K, 71K, 62K and in particular the 50K and 28K proteins were more abundant in virions. Finally, six additional proteins were found in virions: 125K (abundant), 79K, 77K (faint), 68K, 46K (faint) and a 43K protein. In the virion-derived C'-capsids the 142K protein predominated, although some minor C-capsid proteins could also be seen.

Characterization of RCMV DNA

Purified extracellular virions and C-capsids were gently lysed in SDS and proteinase K and the lysates were submitted to rate velocity ultracentrifugation in 10 to 30% sucrose gradients as described in Methods. As can be seen in Fig. 4, the DNA, obtained from extracellular virions,
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sedimented within fractions 16 to 18 (Fig. 4a). Analysis of these fractions by electrophoresis in 0.65% agarose gels indicated that they contained homogeneous and high mol. wt. DNA of more than $110 \times 10^6$, compared to the molecular weight of T4 DNA (Freifelder, 1970). Fractions 17 and 18 were pooled, dialysed and subsequently processed for contour length measurements and restriction enzyme analysis. As can be seen from Fig. 4(b) the sedimentation profile of the DNA recovered from C-capsids differed from that of extracellular virions. The DNA, sedimenting within fractions 16 to 18, was found to be less homogeneous. In shoulder fraction 16 the DNA had a mol. wt. less than $110 \times 10^6$ whereas fractions 17 and 18 consisted of DNA with a mol. wt. of more than $110 \times 10^6$. Fractions 6 to 8 and 10 to 13 were found to contain cytoplasmic RNA (RNase-sensitive), which apparently had not been eliminated during the purification of C-capsids. Based on these results it was decided to perform all further experiments exclusively with DNA obtained from extracellular virions in spite of the fact that the yield of viral DNA was rather low (2 to 3 µg/roller bottle).

**Restriction endonuclease cleavage of RCMV DNA**

Cleavage of RCMV DNA with EcoRI, BglII, HindIII and BamHI resulted in 23, 35, 9 and more than 40 fragments, respectively (Fig. 5). The molecular weights and the molar ratios of the fragments are summarized in Table 1. In the EcoRI restriction patterns fragment C is clearly present in a 0.25 molar ratio, whereas the molarity of fragments A and B could be the result of co-migration of 0.25 M and 0.5 M fragments. Even in overloaded gels it was not possible to determine the molarities of fragments T, U, V and W (not visible in Fig. 5). From the molecular weights and the molarities of the EcoRI fragments a mol. wt. of $138(+2) \times 10^6$ for RCMV DNA was calculated. In the BglII restriction patterns one 0.25 M and two 0.5 M fragments were easily recognized. All BglII fragments totalled a mol. wt. of $137 \times 10^6$.

HindIII cleavage resulted in only nine fragments, of which three could be easily recognized as 0.25 M fragments. It should be noted that the pattern observed was not due to incomplete digestion (partials), since different batches of enzymes, viral DNA or incubation conditions yielded identical patterns. Due to overlap it was not possible to calculate the molarities of the 38 $\times 10^6$ and 32 $\times 10^6$ mol. wt. fragments accurately. The HindIII fragments totalled a mol. wt. of $132(+9) \times 10^6$. Cleavage of RCMV DNA with BamHI resulted in more than 40 fragments, of which five were present as 0.5 M fragments. The total mol. wt. of fragments A to n was $125 \times 10^6$. It should be noted that this molecular weight is a minimal estimate as on overloaded gels some additional diffuse bands were seen of which neither the molecular weights nor the molarities could be determined properly (not visible in Fig. 5).

**Contour length measurement of RCMV DNA**

An independent size determination of RCMV DNA was obtained by contour length measurements of high molecular weight DNA (derived from the previously described fractions 17 and 18; Fig. 4a). Nine RCMV DNA molecules and 24 SV40 molecules were measured. A representative RCMV DNA and SV DNA molecule is shown in Fig. 6. As can be seen the viral DNA is linear and there are no indications of nicks, single-stranded regions or other features. Based on the average length of SV40 DNA [1.70(±0.05) µm], the lengths of the nine RCMV DNA molecules varied between 69.0 and 80.0 µm with an average of 74.8(±5.0) µm, indicating an average mol. wt. of $144(+9) \times 10^6$.

**Buoyant density of RCMV DNA**

A representative centrifugation profile of [3H]thymidine-labelled RCMV DNA and 14C-labelled T4 DNA ultracentrifuged in CsCl to equilibrium, is shown in Fig. 7. Sharp and symmetrical bands emerging at densities of $1.716(±0.001)$ g/ml and $1.700(±0.001)$ g/ml (mean values of eight determinations) respectively, indicate one density class of these DNAs which closely resemble the previously published densities of T4 DNA (1.7005 g/ml, Szybalski, 1968) and other cytomegaloviruses (HCMV DNA: 1.716 g/ml, Huang et al., 1973; equine CMV DNA: 1.7165 g/ml, Wharton et al., 1981).
Fig. 5. Restriction endonuclease patterns of RCMV DNA. Highly purified RCMV DNA recovered from extracellular virions was digested with HindIII, EcoRI, BgIII and BamHI and the fragments generated were analysed by electrophoresis in horizontal 0.65% agarose gels as described in Methods.

Infectivity of RCMV DNA

Subconfluent monolayers of REF, transfected with high molecular weight RCMV DNA (>110 × 10⁶), showed a c.p.e. characteristic of RCMV at approx. 6 days post-transfection. Up to 12 plaques per 0.2 µg (= 60 plaques/µg DNA) were obtained. Higher concentrations of viral DNA (0.5 µg/dish) gave relatively fewer plaques. No c.p.e. or plaques were observed after DNase treatment of the viral DNA.

Homology between the genomes of RCMV, MCMV and HCMV

The degree of homology detectable under stringent and non-stringent hybridization conditions (in 50% and 20% formamide, respectively), between RCMV DNA and MCMV DNA or HCMV DNA was examined using nitrocellulose filters containing either unlabelled RCMV DNA, MCMV DNA or HCMV DNA incubated at 37 °C with sonicated heat-denatured ³⁵S-labelled RCMV DNA (Table 2). The amount of homology under each of the
<table>
<thead>
<tr>
<th>HindIII</th>
<th>EcoRI</th>
<th>BglII</th>
<th>BamHI</th>
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<tr>
<td>Fragment</td>
<td>Mol. wt. ($\times 10^{-6}$)</td>
<td>Molarity</td>
<td>Fragment</td>
</tr>
<tr>
<td>A, B, C</td>
<td>32.38</td>
<td>3 (3.18)*</td>
<td>A</td>
</tr>
<tr>
<td>D</td>
<td>16.2</td>
<td>0.25 (0.21)</td>
<td>B</td>
</tr>
<tr>
<td>E</td>
<td>11.0</td>
<td>1 (1.04)</td>
<td>C</td>
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<tr>
<td>F</td>
<td>6.5</td>
<td>1 (0.96)</td>
<td>D</td>
</tr>
<tr>
<td>G</td>
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<td>E</td>
</tr>
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<td>H</td>
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<td>F</td>
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<td>G</td>
</tr>
<tr>
<td>J</td>
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<td>1 (0.89)</td>
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<td>K</td>
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<td>J, K</td>
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<td>L</td>
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<td>U</td>
<td>1.1</td>
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<td>V</td>
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<tr>
<td>V</td>
<td>0.8</td>
<td>ND†</td>
<td>W</td>
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<tr>
<td>W</td>
<td>0.7</td>
<td>ND†</td>
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<td>Total = 132(±9) × 10⁶</td>
<td>Total = 138(±2) × 10⁶</td>
<td>Total = 137.2 × 10⁶</td>
<td>Total = 125 × 10⁶</td>
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</table>

* Numbers in parentheses represent the actual molarities.
† ND, Not done.
conditions used was quantified by taking the ratio of counts binding to the MCMV DNA or HCMV DNA filters to the counts bound to the RCMV DNA filters. In 50% formamide plateau levels of $^{35}$S-labelled RCMV DNA hybridization to RCMV DNA, MCMV DNA or HCMV DNA were obtained between 68 and 116 h. Under these stringent conditions $12(\pm 2)\%$ of the RCMV DNA was homologous to MCMV DNA whereas only $3(\pm 1)\%$ of the RCMV DNA was homologous to HCMV DNA. The hybridization of the $^{35}$S-labelled RCMV probe to λ-DNA

Fig. 6. Electron micrograph of a representative RCMV DNA and SV40 DNA molecule (inset). The circular SV40 DNA ($3.28 \times 10^6$ mol. wt.) was included as an internal marker. Bar marker represents 1 μm (ultimate magnification).
Fig. 7. Isopycnic CsCl gradient centrifugation of radioactively labelled RCMV DNA. [3H]Thymidine-labelled RCMV DNA (●) and [14C]thymidine-labelled T4 DNA (○) were prepared and submitted to isopycnic CsCl gradient centrifugation as described in Methods. Following centrifugation, 80 μl fractions were collected and suitable aliquots were assayed both for TCA-insoluble radioactivity (3H and 14C) and for determination of the refractive index (▲).

Table 2. Hybridization of RCMV 35S-labelled DNA to DNA bound on nitrocellulose filters*

<table>
<thead>
<tr>
<th>Immobilized DNA</th>
<th>Formamide concn.</th>
<th>Percent of input counts bound in ct/min × 10^4</th>
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<tr>
<td></td>
<td>20 h 44 h 68 h 92 h 116 h 140 h</td>
<td></td>
</tr>
<tr>
<td>RCMV</td>
<td>50</td>
<td>39·0 43·6 47·6 53·7 48·3 35·7</td>
</tr>
<tr>
<td>MCMV</td>
<td>50</td>
<td>3·7 4·2 4·5 7·7 5·6 4·4</td>
</tr>
<tr>
<td>HCMV</td>
<td>50</td>
<td>ND 0·7 1·7 1·4 1·2 ND</td>
</tr>
<tr>
<td>Phage λ</td>
<td>50</td>
<td>0 0·1 0·1 0·1 0·1 0·1</td>
</tr>
<tr>
<td>RCMV</td>
<td>20</td>
<td>21·4 40·6 57·4 67·0 71·3 68·2</td>
</tr>
<tr>
<td>MCMV</td>
<td>20</td>
<td>3·4 8·7 11·8 13·1 14·8 15·6</td>
</tr>
<tr>
<td>HCMV</td>
<td>20</td>
<td>ND 3·1 3·4 4·1 3·8 ND</td>
</tr>
<tr>
<td>Phage λ</td>
<td>20</td>
<td>0·6 0·8 0·8 1·0 1·2 1·1</td>
</tr>
</tbody>
</table>

* Each filter containing 1 μg alkali-denatured immobilized unlabelled DNA of RCMV, MCMV, HCMV or bacteriophage λ was incubated with 1 × 10^4 ct/min of sonicated, heat-denatured 35S-labelled RCMV DNA as described in Methods. Following hybridization in 50% or 20% formamide, filters were extensively washed in 6 × SSC at 68 °C or 50 °C, respectively. From each determination the number of ct/min bound to a blank filter has been subtracted. ND, Not done.
was negligible (0.2%). Although the rates of hybridization in 20% formamide were slightly slower, plateau levels were also reached between 92 h and 140 h. Under these low-stringency hybridization conditions, the homology between RCMV and MCMV or HCMV was considerably higher, being 21(±2)% and 6(±1)%, respectively.

DISCUSSION

The results presented show that intracellular RCMV nucleocapsids could be effectively released and recovered from the cytoplasm and nuclei of RCMV-infected REF by treatment of the cells with a sequence of the detergents NP40 and deoxycholate followed by DNase, urea and Brij 58. It was found that the integrity of N-capsids and C-capsids was not affected by careful treatment with deoxycholate or NP40 which is in agreement with the results of Gibson (1981) for the Colburn strain of CMV. Alternative procedures for recovering virions and nucleocapsids such as freeze-thawing, Dounce homogenization or ultrasonication proved to be less satisfactory due to the persistence of host cell contamination (results not shown).

Extracellular virions of RCMV, MCMV or HCMV as well as C-capsids and N-capsids could be effectively purified by one ultracentrifugation step in 10 to 40% CsCl gradients with a recovery of 20% and an infectivity of about 5% (virions).

Ultracentrifugation in 20 to 50% sucrose gradients or in 20 to 50% potassium tartrate gradients, as described by Kim et al. (1976) and Chantler & Hudson (1978), resulted in lower virus yields and persistent impurities in the various virus preparations (results not shown).

Purified RCMV N-capsids were found to be composed of three major proteins (142K, 40K and 32K) of which the 142K protein is the most abundant one. Gibson (1981) demonstrated three proteins (145K, 34K and 28K) in a molar ratio of 7:3:1 to be the constituent elements of nuclear nucleocapsids from strain Colburn CMV. These findings strongly suggest that only the three major RCMV N-capsid proteins contribute to the basic structure of the simplest RCMV nucleocapsid.

The C-capsids contained all the proteins also present in N-capsids and, in addition, five major and seven minor proteins. The 219K protein, which is also detectable in extracellular virions, is the largest structural protein present in RCMV and might be analogous to the 205K protein of Colburn CMV (Gibson, 1981) or the 225/207K protein of MCMV (Kim et al., 1976).

It should be noted that the protein composition of C-capsids does not reflect a well-defined nucleocapsid species since C-capsids as isolated from the cytoplasm probably consist of a mixture of immature and mature virions from which the envelope has been stripped off upon treatment with NP40. This is supported by the fact that the envelope of RCMV virions can be removed upon treatment with NP40, yielding 'C-capsid'-like nucleocapsids. This has also been observed by Shiraki et al. (1982) and Sarmiento & Spear (1979) employing NP40 as a means of recovering nucleocapsids and envelope proteins from the respective virions of varicella zoster and herpes simplex viruses.

Extracellular RCMV virions have been shown to be composed of proteins mostly present in C-capsids, although the relative proportions appear to differ considerably. Proteins that were absent or hardly detectable in C-capsids but abundantly present in virions (125K, 116K, 87K, 79K, 71K, 68K, 62K, 50K, 43K and 28K) are probably the components of the viral envelope. The reason why the 74K and in particular the 40K protein are largely underrepresented in virions is not clear at present.

We have demonstrated that homogeneous and high molecular weight RCMV DNA can be obtained from purified extracellular virions, using one ultracentrifugation step in 10 to 30% sucrose gradients. Contour length measurements showed that RCMV DNA preparations consist of linear molecules of one definite size class [69 to 80 μm, 144(±9) × 10⁶ mol. wt.].

Although occasionally DNA molecules with lengths between 30 and 70 μm were found, there were no indications of other (lower) size classes of the viral DNA as has been reported for human and murine CMV DNA (Kilpatrick & Huang, 1977; Stinski et al., 1979; Lakeman & Osborn, 1979). This can be explained by the fact that we only employed low multiplicity passaged RCMV (0·01 p.f.u./cell) in all experiments.

The mol. wt. of RCMV DNA as determined by restriction endonuclease analysis yielded
values of 132(±9) × 10^6 (HindIII), 138(±2) × 10^6 (EcoR1), 137 × 10^6 (BgII) and more than 125 × 10^6 (BamHI) which is in good agreement with the value obtained by contour length measurement [144(±9) × 10^6 mol. wt.]. Therefore, a mol. wt. value of 138(±2) × 10^6 is suggested for the DNA of RCMV. Cleavage of RCMV DNA with the above-mentioned restriction endonucleases has also shown the presence of 0·25 M and 0·5 M fragments, which might indicate the existence of four different orientations of the viral DNA as discussed by Clements et al. (1976). The general plan of RCMV DNA, therefore, might resemble the structural organization of HCMV DNA (La Femina & Hayward, 1980; Weststrate et al., 1980) and other herpesviruses (Wilkie, 1976; Cortini & Wilkie, 1978).

We have employed the DNA infectivity assay as a criterion for the biological integrity of the RCMV DNA. It was found that RCMV DNA with a mol. wt. of more than 110 × 10^6 was infectious in REF yielding up to 60 plaques/μg DNA. It should be noted that these results suggest a low efficiency of transfection compared to the values previously obtained for HCMV DNA (480 plaques/μg DNA; Geelen et al., 1978). This could be because of the particular sensitivity of the REF to calcium treatment or glycerol shock. Although not shown, viral DNA preparations with mol. wt. of less than 110 × 10^6 were considerably less infectious (up to 15 plaques/μg DNA). These results indicate that the replicative form of the RCMV genome requires the full DNA size of 138 × 10^6 mol. wt.

Using filter DNA–DNA hybridization under various conditions of stringency, we have examined the degree of homology between the genomes of RCMV, MCMV and HCMV. Under stringent conditions, in 50% formamide and a wash temperature of 68 °C, where only those DNA segments with a mismatch of more than one out of six bases (> 13·3%) will be thermally unstable (Howley et al., 1979), the degrees of homology between RCMV DNA and MCMV DNA or HCMV DNA were found to be 12(±2)% and 3(±1)%, respectively. Huang & Pagano (1974), using reassociation kinetic analysis under stringent hybridization conditions, have also shown that the homology between the genomes of HCMV, MCMV or simian CMV was less than 5%. We found that, even under less stringent conditions, in 20% formamide and a wash temperature of 50 °C, where DNA hybrids with 33% base mismatch are supposed to be thermally stable (Howley et al., 1979) the amount of homology between RCMV DNA and HCMV DNA increased to only 6(±1)% whereas the homology towards MCMV DNA moderately increased to 22(±2)%.

Our results again emphasize the phenomenon of the narrow host range observed among most of the CMVs investigated up to now. Even among ecologically related animal species such as rat and mouse, where virus adaptation might readily occur, the homology between the CMV genomes is surprisingly low. In order to gain more insight into the group relatedness of animal and human CMVs it will be necessary to map the regions of DNA homology using cross-blot hybridization of restriction endonuclease fragments.

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