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Isolation and Structural Characterization of Monomeric and Dimeric Forms of Replicative Intermediates of Kilham Rat Virus DNA

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Two virus-specific species of newly synthesized DNA were isolated from rat fibroblast cell cultures infected with the Kilham rat virus (RV). These two DNA species were purified; their behaviour on hydroxyapatite chromatography and their sedimentation coefficients in sucrose gradients were determined. One of the two species corresponds to the linear double-stranded form of the RV DNA, and the other corresponds to the dimeric duplex form. After denaturation, a fraction of both species showed an intramolecular renaturation; these molecules are composed of viral strand covalently linked to complementary strand. Models for the structure of both species are proposed. Both species may be considered as double-stranded replicative intermediates of the single-stranded RV DNA.

Kilham rat virus (RV), one of the small icosahedric single-stranded DNA (ssDNA) viruses of the parvovirus group, was first isolated from a rat sarcoma by Kilham (6). (The recognized notation RV will be used here, although there are at least three other known parvoviruses of the rat.) RV DNA has been demonstrated to be linear (15) and single stranded (8, 11, 13), and to have a molecular weight of about 1.6×10^6 (8, 11, 13).

Vertebrate parvoviruses fall into two subgroups: (i) parvoviruses, including RV, H_1 virus, and minute virus of mice, which have been shown to replicate autonomously in rapidly dividing cells (21) and to be dependent for their replication upon a cellular event(s) in the late S phase (9, 16); and (ii) adeno-associated viruses, which cannot replicate in cells unless an adenovirus "helper" is present (12). Plus and minus strands of adeno-associated virus DNA have been shown to be coated separately (2).

The study of parvovirus replication may provide a model for the replication of ssDNA in a mammalian system and for the replication of a linear DNA molecule. It is known that ssDNA of several parvoviruses is converted into a double-stranded form after infection (10, 14, 20).

In this paper, two species of double-stranded forms of RV DNA found in infected cells are described. The length and the structure of these molecules were studied, and their possible role in the RV DNA replication is briefly discussed.

MATERIALS AND METHODS

Cell strains and virus. The Kilham RV used in these experiments was obtained from R. Tournier and originally derived from W. Rowe's strain. RV was produced in Wistar AF rat embryo primary culture. Rat embryos (17 days old) were trypsinized and seeded in bottles of a tissue culture Rollacell (New Brunswick Scientific Co.) in 120 ml of Eagle medium supplemented with 10% tryptose phosphate broth and 10% calf serum (Sorga). Infected cells (multiplicity of infection, 0.1 to 1 PFU/cell) were collected 2 days after infection and frozen and thawed three times. Cellular debris was then treated twice in hypotonic medium (double-distilled H_2O). The supernatants collected by low-speed centrifugation were combined and made isotonic by addition of double-concentrated culture medium. This viral suspension was used as a stock virus after titration by the plaque assay technique on secondary cultures of rat embryo cells.

Intracellular synthesis of viral DNA was studied in a rat fibroblast strain (RT cells) obtained from a rat embryo cell culture. (Some RT cells have been found [4] to be contaminated by a new parvovirus, the "RT virus." For the experiments described here, different passages of routinely cultured RT cells were tested by the technique of Hallauer et al. [4; HA titer of glycine buffer extracts] and found free of virus. In some rare cases a contaminant virus was found in degenerating cultures, but this was always identified by inhibition hemagglutination tests as the RV virus. We cannot rule out the possibility that this contaminant virus comes from the RV virus used in this laboratory.) RT cells were maintained in the culture medium supplemented with 5% calf serum in Falcon plastic bottles (75 cm^2). Studies of viral DNA synthesis were carried out in petri dishes (60-mm diameter) with the same medium.

Virus purification. Infected cells were lysed with 1% sodium deoxycholate and treated for 18 h at 37°C with 50 μg of DNase I per ml and 5 μg of RNase III (Sigma) per ml in 10 mM $MgCl_2$. The mixture was adjusted to 0.01% trypsin (Choay) and incubated for

30 min at 37°C. The virus was then purified by isopycnic centrifugation in CsCl (1.4 g/cm³) at 35,000 rpm for 18 h at 25°C in a Spinco SW50.1 rotor.

Viral DNA extraction. Viral DNA was extracted by sodium dodecyl sulfate (SDS) and purified according to the procedure of May and May (8). Viral DNA was also easily prepared by extraction in 0.1 M NaOH for 20 min at room temperature.

Purification of DNA from infected cells. Growing RT cells (1.5×10^5 to 1.8×10^5 cells/cm²) were infected with RV at a multiplicity of infection of 5 PFU/cell. At various times postinfection (p.i.), cells were labeled with tritiated thymidine ([³H]Tdr, 5 μCi/ml, 20 Ci/mM; Saclay) for periods of 1 to 3 h. Low-molecular-weight ³H-labeled DNA was then extracted by an SDS-selective procedure according to Hirt (5), except that the lysate was incubated in the presence of 100 μg of pronase per ml for 30 min at 37°C. Similar results were obtained when pronase was added before or after removing the high-molecular-weight DNA by centrifugation. The ³H-labeled DNA from the Hirt supernatant was deproteinized by two chloroform treatments and then purified in an equilibrium cesium chloride density gradient (CsCl, 1.72 g/cm³) at 35,000 rpm at 25°C for 48 h in a Spinco 65 rotor, either directly or after an ethanol precipitation overnight at −20°C. Fractions from the major peak of radioactivity (Fig. 1) were collected and dialyzed against tris(hydroxymethyl)aminomethane (Tris)-EDTA buffer (0.01 M Tris, 0.001 M EDTA, pH 8). The purification was completed by a phenol extraction at room temperature, and DNA was dialyzed against the same buffer.

Alkaline denaturation of DNA. The ³H-labeled DNA was incubated for 10 min at room temperature in 0.1 N NaOH and then neutralized with 0.1 N Na H_2PO_4 unless specified otherwise.

Hydroxyapatite chromatography. Hydroxyapatite chromatography of purified ³H-labeled DNA, either native or denatured, was performed according to the method of Tapiero et al. (19). Portions of ³H-labeled DNA preparations were diluted to 4 ml with 0.01 M phosphate buffer, pH 7.85, and mixed with 0.25 g of hydroxyapatite (DNA grade; Bio-Rad) and incubated for 5 min at room temperature. Alkali-denatured DNA (100 μl) was diluted with 4 ml of 0.01 M phosphate buffer before neutralization with NaH_2PO_4. The hydroxyapatite was pelleted by low-speed centrifugation, and the supernatant was collected. Hydroxyapatite was then washed by the same procedure with 0.01 M phosphate buffer, pH 7.85. Stepwise elution of the DNA was performed by resuspending the hydroxyapatite pellet in 4 ml of phosphate buffer of increasing molarities (from 0.1 to 0.7 M). The tubes were incubated in a water bath at 56°C for 8 min with intermittent agitation. Hydroxyapatite was again pelleted by low-speed centrifugation at room temperature, and the supernatants were collected. Carrier DNA (calf thymus; Choay) was added to each step-elution fraction, and cold trichloroacetic acid was added to a final concentration of 5%. After an incubation for 10 min in an ice-water bath, the acid-precipitable material was collected on glass fiber filters (Whatman GF/C). Each tube was washed with 4 ml of 5% trichloroacetic acid, which was poured onto the filters. The filters were dried, and the radioactivity was determined in an Intertechnique liquid scintillation spectrometer.

DNA-DNA hybridization. Purified ³H-labeled DNA was denatured and fragmented into pieces by boiling for 15 min in 0.01× SSC (SSC is 0.15 M NaCl plus 0.015 sodium citrate). At this stage, the DNA sedimented in an alkaline sucrose gradient as a uniform band corresponding to about 9S. The DNA was then reannealed in 100 μl of Tris-EDTA buffer containing 0.3 M NaCl at 65°C with different quantities of unlabeled RV DNA (up to 880 ng). After 48 h of incubation, two 40-μl portions designated A and B were taken and treated separately. Portion A was diluted to 0.4 ml with Tris-EDTA buffer, and the total radioactivity of A was counted in the cold trichloroacetic acid-precipitable material. For portion B, only the radioactivity remaining in a double-stranded form was counted in the cold trichloroacetic acid-precipitable material after digestion of ssDNA by the S_1 nuclease from *Aspergillus oryzae* prepared according to Vogt (22). Portion B was diluted to 0.4 ml in a buffer containing 0.03 M sodium acetate, pH 4.6, 0.3 M NaCl, 0.001 M $ZnSO_4$, and 5% glycerol. Heat-denatured calf thymus DNA (20 μg/ml) and 5 U of S_1 nuclease per ml were added. The mixture was then incubated for 1 h at 45°C. The results are given as the percentage of the radioactivity remaining in portion B as compared to that in portion A.

Velocity sedimentation in sucrose gradients. Portions (up to 0.2 ml) of the purified ³H-labeled DNA samples were layered on 4 ml of a 5 to 20% linear sucrose gradient. Neutral sucrose gradients in 1 M NaCl and Tris-EDTA buffer were centrifuged at 20°C for 150 min at 54,000 rpm in a Spinco SW56 rotor. For alkaline sucrose gradients the 5% sucrose solution was made pH 13 in 0.3 N NaOH, 0.7 M NaCl, and Tris-EDTA buffer, and the 20% sucrose solution was made pH 13 in 0.6 N NaOH, 0.4 M NaCl, and Tris-EDTA buffer. The alkaline sucrose gradients were then centrifuged at 20°C for 180 min at 54,000 rpm. Gradients were fractionated by collecting drops from the bottom of the tube directly onto glass fiber filters. Filters were washed twice with cold 5% trichloroacetic acid and then with ethanol. Radioactivity was determined by counting dried filters in a liquid scintillation spectrometer.

RESULTS

Presence of two new species of ³H-labeled DNA in RV-infected cells. RV-infected rat fibroblasts (RT cells) were labeled with [³H]Tdr (5 μCi/ml) for 1 h p.i. to various lengths of time. After the labeling period, they were lysed by SDS. The radioactivity was then counted in the trichloroacetic acid-precipitable material of the SDS-pronase Hirt supernatants (see Materials and Methods). Under these conditions, incorporation of [³H]Tdr was first detected at 6 h p.i. and then became linear as a function of time between 9 and 18 h p.i. and reached a plateau (data not shown). Under the same conditions, incorporation of [³H]Tdr in the trichloroacetic

acid-precipitable material of the Hirt pellets of infected cells reached a plateau at 6 h p.i. In most of the experiments reported here, DNA was labeled at 12 h p.i. for the indicated period, then extracted, and purified as described in Materials and Methods. As shown later, the DNA of Hirt supernatant labeled at 12 h p.i. was essentially virus specific. The last step of the DNA purification was an isopycnic centrifugation gradient in CsCl (Fig. 1). The bulk of the radioactivity banded as a single peak slightly heavier than that of cellular DNA (the difference in densities was about 0.005 g/cm^3), whereas very little radioactivity was found in the region of the RV ssDNA. The study of the ^{3}H-labeled DNA contained in the major peak will be reported in this paper. Purification of ^{3}H-labeled DNA by a density gradient centrifugation reduced contamination by cellular DNA. The radioactivity remaining at the top of the gradient corresponded to ^{3}H-labeled DNA linked to viral proteins in spite of the SDS-pronase treatment performed as described in Materials and Methods. (Progeny ssDNA was always found in this form.) The study of these DNA-protein complexes will be described in a subsequent paper.

The ^{3}H-labeled DNA thus collected from the preparative density gradient (Fig. 1) was analyzed by velocity sedimentation in a 5 to 20% neutral sucrose gradient. The pattern of sedimentation illustrated in Fig. 2 shows two peaks of radioactivity that were not found in mock-infected cells. The major peak (M) accounts for 80% of the radioactivity, and the minor (D) for 20%. To study these two species of DNA, fractions M and D were separately pooled and purified by an additional sucrose centrifugation.

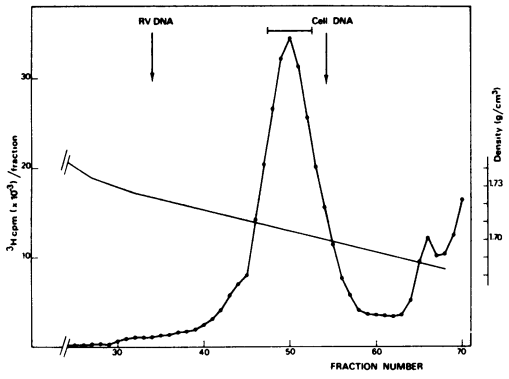

FIG. 1. *Preparative isopycnic centrifugation of newly synthesized DNA in RV-infected cells. RV-infected RT cells (multiplicity of infection, 5 PFU/cell) were labeled with [^{3}H]Tdr (5 μCi/ml) at 12 h p.i. for a period of 1 h. ^{3}H-labeled DNA from the Hirt supernatant (purified as described) was submitted to an equilibrium density gradient centrifugation in 6 ml of CsCl (1.72 g/cm^3) at 35,000 rpm for 48 h at 20°C in a Spinco rotor 65. Fractions were collected from the tube by bottom puncture. Portions (10 μl) from each fraction were spotted on filters, and their radioactivity was counted. Portions were also taken to measure the refractive index. Arrows indicate the positions of cellular DNA and RV DNA added as markers in a parallel gradient.*

Hydroxyapatite chromatography. Elution patterns from hydroxyapatite chromatography of RV DNA and of DNA species M (DNA-M) and D (DNA-D) were studied. Under the experimental conditions used (see Materials and Methods), native dsDNA was eluted from hydroxyapatite with 0.55 M phosphate buffer, pH 7.85, and denatured DNA with 0.15 M phosphate buffer. These characteristics were illustrated in our experiments with rat cellular DNA (Fig. 3a, b). By contrast, RV ^{3}H-labeled DNA was eluted with 0.3 M phosphate, which was unexpected for ssDNA (Fig. 3c). Considering that by increasing concentrations of phosphate buffer the elution from hydroxyapatite mainly depends on the secondary structure of the DNA (1), this result strongly suggests that RV DNA has a special structure. A short palindromic region could account for the value obtained for the molarity of elution. Since minute virus of mice DNA has been found to contain a small duplex region (2 to 5%) of the viral genome (G. J. Bourguignon, P. J. Tattersall, and D. C. Ward, Abstr. Int. Congr. Virol., 3rd, p. 181, 1975), we also studied the behavior of this DNA in hydroxyapatite chromatography and observed an elution pattern similar to that obtained with RV DNA (data not shown). Moreover, a palindromic sequence has also been suggested for the terminal segments of adeno-associated virus DNA strands on the basis of the results of Koczot et al. (7) and Gerry et al. (3).

The DNA species M and D were eluted from hydroxyapatite with 0.55 M phosphate, indicating a double-stranded structure, whereas no radioactivity was found at either 0.15 or 0.3 M phosphate (Fig. 4a and c). Alkaline-denatured DNA of species M and D have similar patterns of elution from hydroxyapatite as shown in Fig. 4 (b and d). Both DNA species, M and D, are resolved into two different peaks. One peak elutes at 0.3 M phosphate, and the other peak at 0.55 M phosphate (about 30 and 50% of the total eluted radioactivity for species M and D, respectively). Similar results were obtained with DNA that was heat denatured in Tris-EDTA buffer at 100°C for 3 min and then quickly cooled. It is important to stress that 0.3 M phosphate is the typical molarity of elution of

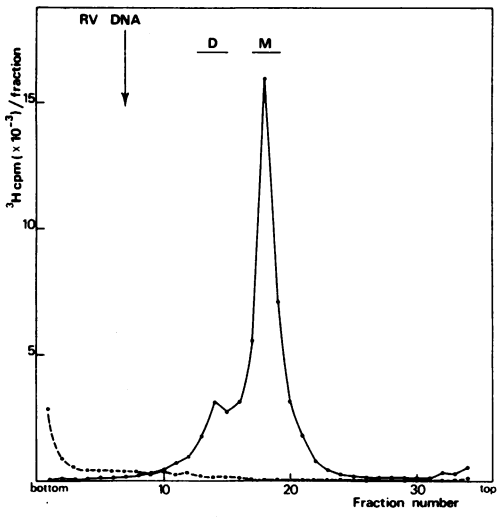

FIG. 2. *Sedimentation in a neutral sucrose gradient of a Hirt supernatant DNA from RV-infected or mock-infected cells. ^{3}H-labeled DNA was layered on the top of a 5 to 20% sucrose gradient in 1 M NaCl and Tris-EDTA buffer and centrifuged at 20°C for 150 min at 54,000 rpm in a Spinco SW56 rotor. Fractions were collected from the bottom of the tube directly on glass fiber filters, and the radioactivity of dried filters was counted. The arrow indicates the sedimentation position of RV DNA added as marker (24S under the conditions used). Symbols: ●, ^{3}H-labeled DNA collected from the major peak, corresponding to the horizontal bar of Fig. 1; ○, ^{3}H-labeled DNA of a Hirt supernatant from mock-infected cells labeled for 1 h and extracted as described for infected cells. Fractions corresponding to DNA-M and -D were collected separately from a similar gradient.*

RV ssDNA and that 0.55 M phosphate is the molarity of elution of dsDNA. These results suggest that ^{3}H-labeled DNA of both species M and D is double stranded. A denaturation treatment converts a fraction of both species to ssDNA that is eluted at 0.3 M phosphate as RV DNA, whereas the remaining molecules are eluted at the molarity corresponding to dsDNA. The experimental conditions of denaturation used are assumed to prevent intermolecular renaturation of DNA (low ionic strength, low DNA concentration, and room temperatures). We will show in the following experiments that the DNA which is eluted at 0.55 M phosphate after denaturation derives from intramolecular "snap-back" renaturation.

Hybridization. To examine the viral specificity of ^{3}H-labeled DNA extracted from RV-infected rat cells, species M and D were heat denatured in 0.01× SSC and reannealed in 0.3 M NaCl at 65°C with increasing quantities of unlabeled RV DNA. After 48 h the DNA remaining ss was degraded by S_1 nuclease, and trichloroacetic acid-precipitable radioactivity was determined (see Materials and Methods). If we assume that species M and D are completely homologous to RV DNA, which contains only the "viral strand," saturating concentrations of unlabeled RV DNA would be expected to displace all viral strands from species M and D (i.e., 50% of the radioactivity, assuming that ^{3}H-labeled DNA is equally labeled on both strands). The ^{3}H-labeled DNA extracted from RV-infected cells could thus be considered as 100% virus specific. As already indicated, a fraction of the alkaline-denatured DNA species

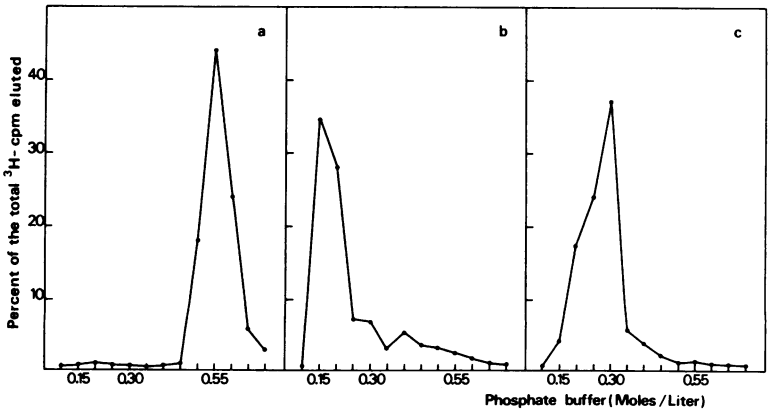

FIG. 3. *Hydroxyapatite chromatography of purified ^{3}H-labeled cellular and RV DNA. ^{3}H-labeled DNA in 0.01 M phosphate buffer, pH 7.85, was adsorbed at room temperature to 0.25 g of hydroxyapatite. Stepwise elution was performed at 56°C by increasing molarities of phosphate buffer, pH 7.85 (from 0.1 to 0.7 M), as described in Materials and Methods, and the radioactivity of the trichloroacetic acid-precipitable material of each eluate was counted. (a) Native cellular DNA; (b) alkali-denatured cellular DNA; (c) RV DNA.*

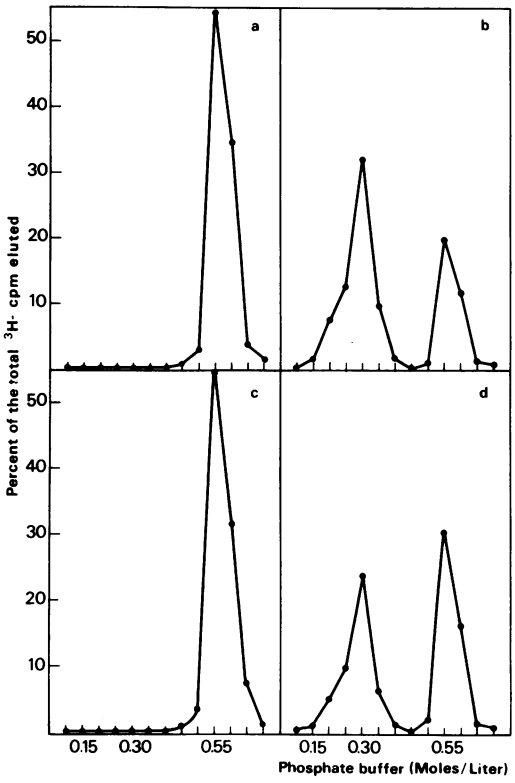

FIG. 4. *Hydroxyapatite chromatography of ³H-labeled DNA-M and -D, by the method described in Fig. 3. (a) Native DNA-M; (b) alkali-denatured DNA-M; (c) native DNA-D; (d) alkali-denatured DNA-D.*

M and D undergoes intramolecular renaturation. The denaturing conditions used for hybridization experiments were chosen to reduce this intramolecular renaturation by reducing the molecular weight. The DNA species M and D were converted to ssDNA of a molecular weight of 4×10^5 (25% of the genome length) by boiling DNA 15 min in 0.01× SSC. Denatured DNA species M and D showed an S_1 resistance of 9% in the absence of reannealing treatment.

After reannealing, ³H-labeled DNA species M showed an S_1 resistance of 92.2 ± 7.5% (mean value ± standard deviation for seven independent determinations). When increasing quantities of unlabeled RV DNA were added, this percentage first decreased and finally leveled off at 47% with 200 ng of unlabeled DNA. This value remained essentially unchanged with addition of increasing amounts of RV DNA up to 880 ng (Fig. 5). When excess heterologous DNA was added to the incubation medium, the amount of reannealing of ³H-labeled DNA-M was about 80% (Fig. 5). This mean value was obtained with unlabeled DNA from RT (rat) or CV1 (monkey) cells and from SV40. To take into account this nonspecific hybridization, the experimental value of renaturation of DNA-M obtained with excess unlabeled RV DNA was multiplied by a correcting factor of 100/80, and we thus obtained a corrected value of 60%. Thus, 40% of the labeled DNA-M was displaced by the unlabeled RV DNA, corresponding to 80% homology for this species. This value is underestimated if we consider that 9% of the DNA is resistant to an S_1 nuclease digestion without reannealing treatment and is probably counted with the DNA remaining double stranded after renaturation. Similar results were obtained with ³H-labeled DNA species D, except that ³H-labeled DNA-D reannealed to the extent of only 75.6 ± 14.7% (mean value ± standard deviation obtained with seven independent determinations). Purified ³H-labeled DNA-D preparations were always in lower concentrations than ³H-labeled DNA-M prepations. Thus, reassociation conditions were probably not optimal, since the standard deviation was relatively high. When unlabeled RV DNA was added in excess to DNA-D, better condi-

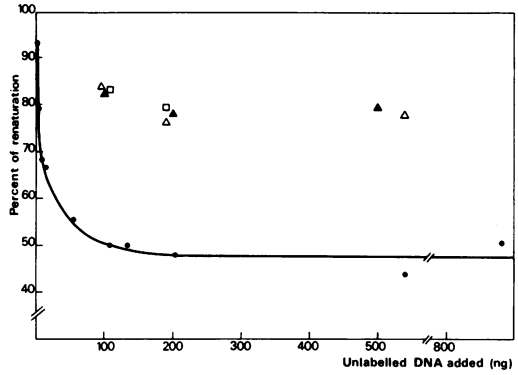

FIG. 5. *Variations of the percentage of renaturation of ³H-labeled DNA-M as a function of increasing quantities of unlabeled RV DNA. ³H-labeled DNA-M labeled from 12 to 15 h p.i. was denatured in 0.01× SSC for 15 min at 100°C and then reannealed in 100 μl of 0.3 M NaCl for 48 h at 65°C in the presence of varying quantities of unlabeled DNA. Each assay was performed with 10 ng of DNA-M. At the end of the reannealing period two equal portions, designated A and B, were taken; the radioactivity was counted directly in the trichloroacetic acid-precipitable material of portion A, and the radioactivity of portion B was counted in the trichloroacetic acid-precipitable material after an S_1 nuclease digestion. All results are expressed as the percentage of the counts per minute remaining in dsDNA after the S_1 nuclease digestion (portion B) as compared to the total counts per minute (portion A). Unlabeled DNAs added: ●, RV DNA; △, RT DNA; ▲, CV1 DNA; □, SV40 DNA.*

tions of concentration were obtained. The percentage of renaturation of DNA-D in the presence of 100 and 200 ng of unlabeled RV DNA was 46 and 45%, respectively. These values are similar to the corresponding values obtained with DNA-M. Both species appear to possess the same extent of virus specificity (over 80%).

Sucrose sedimentation. Species M and D of ^{3}H-labeled DNA were studied separately in sucrose gradients (Fig. 6). Sedimentation coefficients were 14.5*S* for DNA-M and 18.3*S* for DNA-D as compared to the 16*S* sedimentation coefficient of SV40 form II DNA added as a marker (Fig. 6a, d). Since the species M and D have been observed as linear molecules with the electron microscope (M. Gunther, H. Bujard, and G. Hayward, manuscript in preparation), it was possible to calculate the molecular weight of each species. To obtain an accurate evaluation of the molecular weight of DNA-M, we compared, in the same gradient, the sedimentation properties of DNA-M and the linear form of SV40 DNA obtained after Hpa II endonuclease digestion (data not shown). DNA-M sedimented slightly more slowly: the difference in sedimentation coefficients was 0.4*S*, which corresponded to a molecular weight of 3.15×10^6 for DNA-M, with a molecular weight of 3.4×10^6 for SV40 DNA. The ratio (molecular weight of DNA-D/molecular weight of DNA-M)

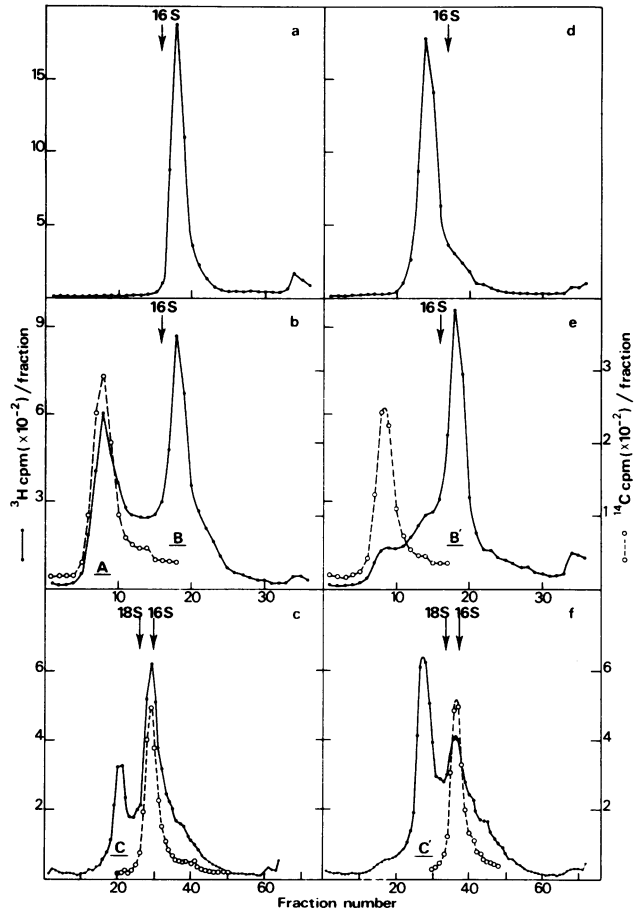

FIG. 6. *Sucrose sedimentations of ^{3}H-labeled DNA-M and -D. Neutral sucrose sedimentations were performed as described in the legend of Fig. 2. Alkaline sucrose gradients (pH 13) were centrifuged at 20°C for 180 min at 54,000 rpm in a Spinco SW56 rotor. The ^{3}H-labeled DNA-M sedimented in (a) neutral, (b) neutral (after an alkaline denaturation), and (c) alkaline 5 to 20% sucrose gradients. The ^{3}H-labeled DNA-D sedimented in (d) neutral, (e) neutral (after an alkaline denaturation), and (f) alkaline 5 to 20% sucrose gradients. In gradients b, c, e, and f, RV ^{14}C-labeled DNA was added as a marker. SV40 ^{14}C-labeled DNA form II was added as a marker in parallel gradients. 16S arrows in neutral sucrose gradients represent the sedimentation position of SV40 form II, and in alkaline sucrose gradients arrows 16S and 18S represent the positions of the circular and linear strands derived from SV40 DNA form II.*

was calculated from the experimental sedimentation coefficients 18.3S and 14.5S by using Studier's formula (18). This ratio was 2 (calculated value, 1.94). Thus, the DNA of species M appears to correspond to the monomeric ds form of RV DNA, and the DNA of species D to a dimer of species M.

Both DNA-M and -D were alkaline denatured and studied separately in alkaline and neutral sucrose gradients (Fig. 6b, c, e, f). An alkaline sucrose gradient of DNA-D (Fig. 6f) showed two peaks whose sedimentation coefficients corresponded to 20.3S and 16S. The 16S peak co-migrated with RV ^{14}C-labeled DNA marker and the 20.3S sedimentation coefficient found for the second peak was close to the coefficient expected (21S) for the corresponding single-stranded dimer. Thus an alkaline sucrose gradient of DNA-D gave essentially two species of ssDNA in a molecular weight ratio of 2 (calculated value, 1.86). An alkaline sucrose gradient of DNA-M showed the same two peaks of sedimentation (Fig. 6c), which will be discussed in detail later. In a neutral sucrose gradient of denatured DNA-M, two peaks were obtained with coefficients of 24S and 14.5S (Fig. 6b). As shown later, the 24S peak, which co-migrates with RV DNA, corresponds to the single-stranded monomer form, and the 14.5S to a double-stranded snap-back DNA of monomer length. Under the same sedimentation conditions, DNA-D gave a major peak at 14.5S and a shoulder of radioactivity at 24S, i.e., the position of RV ssDNA (Fig. 6e). Radioactivity was not found at the position for the single-stranded dimer (whose expected sedimentation coefficient would be ranging about 50S). Thus, in an alkaline sucrose gradient (Fig. 6c, f), the sedimentation patterns of denatured DNA from both DNA-M and -D showed two bands corresponding to the single-stranded monomer and the single-stranded dimer; in a neutral sucrose gradient (Fig. 6b and e) the sedimentation patterns of denatured DNA from both DNA-M and -D showed two bands corresponding to the single-stranded monomer and to an additional component sedimenting at 14.5S.

The fractions (A, B, B', C, and C') indicated in Fig. 6 were pooled and examined on hydroxyapatite and in sucrose gradients. The DNA from pool A was essentially single stranded, as indicated by its molarity of elution (0.3 M phosphate) in hydroxyapatite chromatography (Fig. 7a) and resedimented as the RV DNA in neutral or alkaline sucrose gradients (data not shown). The DNA from pools B and B' was eluted with 0.55 M phosphate from hydroxyapatite (Fig. 7b); i.e., it behaved as dsDNA and sedimented at 20.3S in an alkaline sucrose gradient (Fig. 8a). Thus the DNA from pools B and B' appeared to be double stranded; this DNA in neutral sucrose behaved as a double-stranded monomer and in alkaline sucrose as a single-stranded dimer. DNA from pools C and C', i.e., an "alkaline" single-stranded dimer, renatured when neutralized, and then it was eluted at 0.55 M phosphate from hydroxyapatite (data not shown) and sedimented at 14.5S in a neutral sucrose gradient, as expected for a double-stranded monomer (Fig. 8b). These results show that the rapidly renatured DNA arose from an alkaline single-stranded dimer. To account for these observations, we suppose that in such a single-stranded dimer, both strands, viral and complementary, are linked by an alkali-stable covalent link, giving rise, when neutralized, to a hairpin structure. Molecules which snap back to this hairpin structure have the characteristic monomer length. On the basis of this observation, we propose structures for the monomeric and dimeric species (see Discussion).

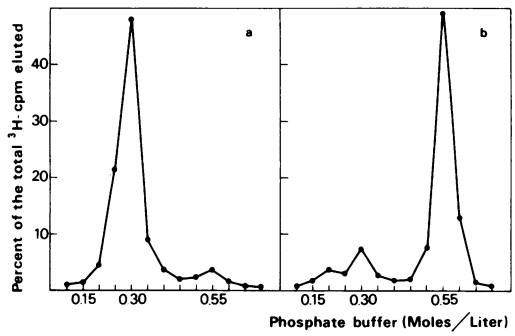

FIG. 7. *Profile of elution from hydroxyapatite of ^{3}H-labeled DNA from pools A and B corresponding to horizontal bars in Fig. 6. The method is as described in the legend of Fig. 3. (a) Pool A; (b) pool B.*

DISCUSSION

In this work, we have shown the presence of two species of DNA in RV-infected rat cells after labeling with [^{3}H]Tdr. The DNA obtained by an SDS-pronase selective extraction from RV-infected rat cells and centrifuged in a CsCl gradient formed a band slightly heavier than that of the rat cellular DNA (Fig. 1). The DNA in this band, when sedimented in a neutral sucrose gradient, was resolved into two distinct peaks, M and D (Fig. 2). Both species were shown to be double stranded by their molarity of elution (0.55 M phosphate buffer) during hydroxyapatite chromatography (Fig. 4) and to be at least 80% virus specific by displacement hybridization experiments with saturating concentrations of unlabeled RV DNA. Analysis of

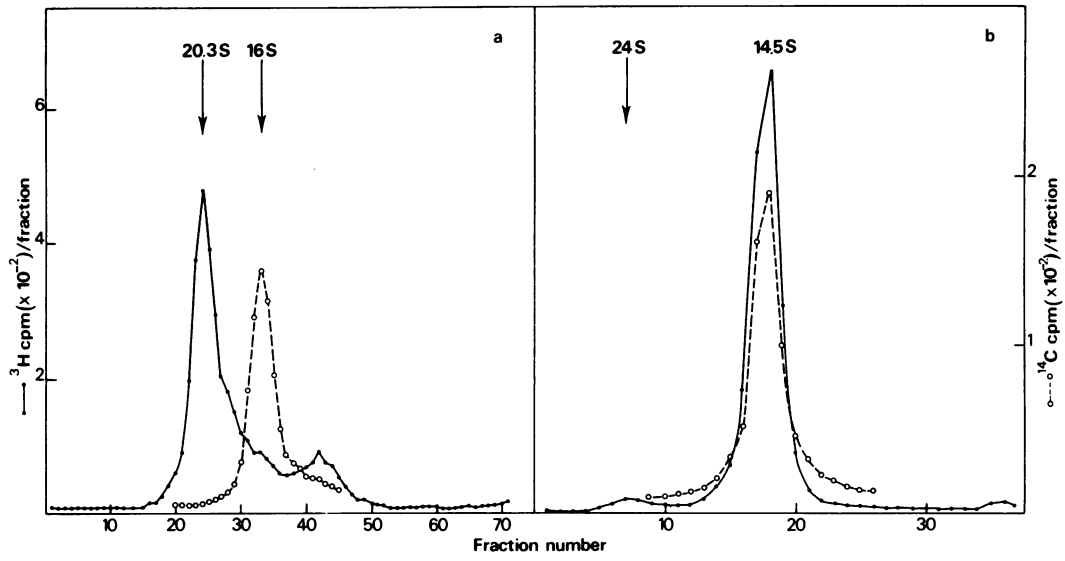

FIG. 8. *Sedimentation of ^{3}H-labeled DNA from pools B and C (corresponding to horizontal bars in Fig. 6). (a) Alkaline sucrose gradient of ^{3}H-labeled DNA from pool B (●); RV ^{14}C-labeled DNA was added as a marker (○); (b) neutral sucrose gradient of ^{3}H-labeled DNA from pool C (●); ^{14}C-labeled DNA-M was added as a marker (○).*

the two species by sucrose gradient centrifugation with various DNA markers (SV40 DNA form II, SV40 DNA linear form III, and RV DNA) showed that species M of ^{3}H-labeled DNA corresponded to the linear duplex form of RV DNA, with a molecular weight of about 3 × 10^6, whereas species D corresponded to the linear dimeric duplex of species M.

The results from sucrose sedimentation analysis (Fig. 6 and 8), when combined with those obtained by hydroxyapatite experiments (Fig. 4 and 7), have shown that a fraction of both species snaps back to a hairpin monomer after denaturation. This property leads us to propose the following structures for the DNA species M and D (see Fig. 9 and 10).

Monomeric DNA preparations probably consist of a mixture of two types (1 and 2) of double-stranded molecules (Fig. 9). These structures are consistent with the behavior of denatured monomeric dsDNA. The monomer gives rise to two kinds of single-stranded molecules in alkaline sucrose gradients: one (of monomer length) comes from structure (1) and the other (of dimer length) comes from structure (2); the latter snaps back when neutralized into a hairpin monomer.

A large proportion (about 50%) of dimeric molecules spontaneously renatured, after denaturation, to hairpin monomeric molecules. Dimeric molecules must have a structure such that viral and complementary strands are

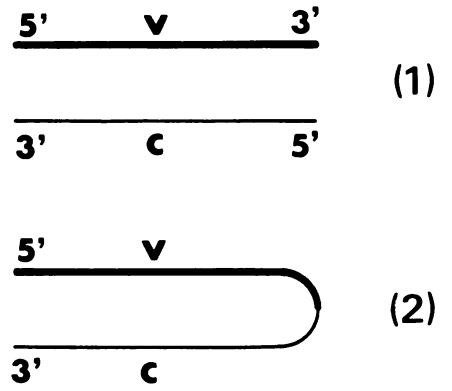

FIG. 9. *Proposed structure for the double-stranded monomeric species (DNA-M) which probably consists of two types (1 and 2). One strand in each duplex in an RV viral strand (v), and the other is the complementary strand (c). In type 2, v and c are covalently linked, forming a hairpin molecule.*

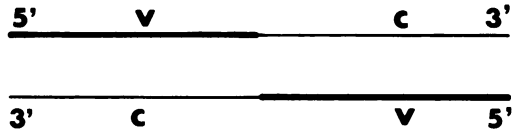

FIG. 10. *Proposed structure for the double-stranded dimeric molecules (species D). Each linear strand in the duplex is composed of one RV viral strand and one complementary strand, which are covalently linked end to end.*

linked by an alkali-stable covalent link, as represented in the scheme in Fig. 10.

This is in accordance with the fact that radioactivity was not detected in neutral sucrose gradients in the region theoretically corresponding to a single-stranded dimer. After neutralization single-stranded dimers were always found in the region of monomeric dsDNA. A fraction of denatured dimeric DNA did not snap back and behaved as single-stranded monomeric molecules in neutral or alkaline sucrose gradients. Thus, it is likely that a fraction of dimeric molecules must have a nick at (or near) the middle of the strand(s).

There is another possible structure for the dimer. Dimeric molecules might arise by the joining by hydrogen bonds of two hairpin monomers, occurring either in infected cells or during the DNA extraction-purification procedure. Such a structure needs a terminal redundancy if the joining occurs between a viral and a complementary strand and a palindromic structure at the ends of the strands if the joining occurs between two viral or two complementary strands. The existence of the latter structure cannot be ruled out, although it seems unlikely if we assume that dimeric molecules play a role in the RV DNA replication.

In conclusion, two new species of virus-specific dsDNA were found in RV-infected rat cells: one species (the more abundant) is monomeric (a one-unit length); the other is dimeric (a double-unit length). Both species contain covalently linked viral and complementary strands (Fig. 9 and 10), which strongly suggests that they are synthesized by a self-priming mechanism. A similar hypothesis was suggested by Tattersall et al., who reported the existence of a hairpin monomer form of intracellular minute virus of mice DNA (20). Recently, Straus et al. (17) identified replicative intermediates in cells infected with adeno-associated virus type 2, which consist of covalently linked plus and minus DNA strands. These authors have proposed a replication scheme that involves a self-priming mechanism. It appears likely that the replication of different parvovirus DNA is a unidirectional and self-priming process. Further studies are necessary to obtain a detailed scheme of RV DNA replication. We are presently studying the relationship between the replicative intermediates and their role in the synthesis of progeny ssDNA. RV DNA replication appears to include different steps: conversion of parental ssDNA to a double-stranded form then synthesis of some other double-stranded replicative intermediates (of monomeric and dimeric length), one replicative intermediate species acting as a template to progeny ssDNA. If every step requires a self-priming process, RV DNA must have a hairpin duplex structure at both ends of the viral strand. The behavior of RV DNA on hydroxyapatite (Fig. 3c) and preliminary results obtained with S_1 nuclease digestion suggest that RV DNA contains a short duplex region(s), which is in accordance with recent results from L. Salzman (Virology, in press). Further studies on RV DNA concerning the structure of the ends of the molecules and the ability of RV DNA to be utilized as a primer template, as has been shown with minute virus of mice DNA (Bourguignon et al., Abstr. Int. Congr. Virol., 3rd, p. 181, 1975), are required.

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