Replication of the Parvovirus MVM

II. Isolation and Characterization of Intermediates in the Replication of the Viral Deoxyribonucleic Acid

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The time course of the appearance of intracellular viral DNA has been studied in mouse L cells infected with the single-stranded DNA virus MVM (minute virus of mice) by using a selective extraction procedure. Approximately half of this DNA elutes from hydroxyapatite as single-stranded DNA. It is sensitive to *Escherichia coli* exonuclease I and shows a sedimentation profile similar to DNA from the virus, suggesting that it is progeny viral DNA. The remainder of the selectively extracted DNA elutes from hydroxyapatite in the position of double-stranded DNA and is resistant to exonuclease I. Most of this DNA has a sedimentation coefficient of 14 to 16S, indicating that its molecular weight is twice that of the viral DNA. Denaturation renders the majority of the double-stranded DNA sensitive to exonuclease I, but a significant fraction renatures spontaneously in a monomolecular fashion, indicating that it has a cross-linked or hairpin structure. Chromatography of the double-stranded DNA on benzoylated diethylaminoethyl cellulose resolves two components, one with duplex structure and one which contains single-stranded regions. A short pulse label late in infection predominantly labels the latter class of DNA, suggesting that it contains replicating intermediates. The possible roles of these various forms of DNA in the replication of the viral genome are discussed.

The minute virus of mice (MVM) (5) is a member of the parvovirus group of small, isometric viruses containing 1.2×10^6 to 2.0×10^6 daltons of single-stranded DNA (6, 17, 19, 20, 24, 32). These viruses fall into two subgroups, the adenovirus associated viruses (AAV) which are dependent upon adenovirus co-infection and those which are autonomous in most cell systems. Viruses of the autonomous subgroup display a striking affinity for rapidly dividing cells, and appear to infect preferentially cells in S phase (9, 25, 29, 30, 31). Such dependence suggests that studies on the replication of these viruses may yield information on the programming of cellular functions during the mitotic cycle.

Although the DNAs of several parvoviruses have been studied in some detail, little is known about the synthesis and structure of intracellular forms of the viral DNA and their roles in the replication of the virus. The DNA of Kilham's rat virus (RV), another parvovirus, has been shown to be sensitive to *Escherichia coli* exonuclease I, indicating that the majority of the molecules are linear (21). Both MVM and RV DNAs appear to be linear by electron micro-

copy (6, 21), and both are converted to a double-stranded form early in infection (P. J. Tattersall, Ph.D. thesis, Univ. of London, 1971; 23). We have used a selective extraction procedure to study the synthesis of intracellular MVM DNA and report the isolation and partial characterization of a double-stranded form and a partially duplex structure which appears to be a replicating intermediate.

MATERIALS AND METHODS

Virus and cells. The plaque-purified strain MVM (T) used in these experiments was assayed and propagated in mouse L cells, strain A-9, as described previously (29). Ethidium bromide-cesium chloride purified, ^{14}C -labeled polyoma virus component I DNA and component II DNA were the kind gift of D. C. Ward.

Virus purification and extraction of viral DNA. Virus was extracted from infected monolayers and purified as described previously (29). Labeled virus was prepared in the same way from infected cells cultured in the presence of [*methyl- ^3H*]thymidine (1 $\mu\text{Ci/ml}$, 5×10^{-6} M). Virus from the dense band (density 1.42 g/ml) in cesium chloride equilibrium gradients was desalted by gel filtration on Sephadex G-25 in 0.01 M Tris buffer, pH 7.4. The virus

suspension was made 0.005 M MgCl_2 and was incubated with 50 μg of DNase I per ml and 100 μg of RNase A per ml for 1 h at 37 C. The suspension was then layered on top of a 25-ml 10 to 30% sucrose gradient in phosphate buffered saline (PBS) (8) without magnesium and calcium, and centrifuged at $63,000 \times g$ (average) for 3.5 h at 10 C in a Spinco SW25.1 rotor. The band of virus was transferred directly to cesium chloride and sedimented to equilibrium as described previously (29). The virus was then desalted by gel filtration in 0.02 M Tris, 0.02 M EDTA, pH 8.6. Pronase was then added to 500 $\mu\text{g}/\text{ml}$. (The Pronase had previously been heated to 80 C for 5 min and self digested for 6 h at 20 mg/ml in the same buffer.) After 30 min of incubation at 37 C, sodium dodecyl sulfate (SDS) was added to 0.5% and the incubation continued for a further 30 min. The suspension was then extracted with 2 volumes of phenol at room temperature (saturated with the same buffer). After removal of the aqueous layer, the phenol layer was re-extracted with buffer. The two aqueous phases were combined and extracted again with phenol as before. The phenol was removed from the aqueous layer by four extractions with ether and the ether was removed by bubbling with nitrogen gas. The viral DNA was then precipitated with 2.5 volumes of ethanol overnight at -20 C, collected by centrifugation, dissolved in 0.01 M Tris, 0.001 M EDTA, pH 7.4, and stored at -20 C.

Labeling and fractionation of infected cell DNA.

A-9 cells, from a rapidly dividing suspension culture, were washed with PBS and suspended in PBS at 2×10^7 cells/ml. The cells were then infected with 5 PFU per cell for 1 h at 37 C. Receptor-destroying enzyme and anti-MVM antiserum were then added to terminate the adsorption and penetration period, and the incubation continued for 30 min. The cells were then washed twice with 5 volumes of PBS, and resuspended at 10^6 cells/ml in DP medium (29) supplemented with 5% fetal calf serum, containing [*methyl*- ^3H]thymidine (5 $\mu\text{Ci}/\text{ml}$, 2.5×10^{-6} M) and incubated in a spinner flask. Control cells were prepared in the same way omitting the virus.

Intracellular DNA was fractionated by a modification of the selective extraction of Hirt (10). Samples of labeled cells were removed from the cultures, washed with 0.02 M Tris, 0.15 M NaCl, 0.01 M EDTA, pH 7.5, resuspended at 2×10^7 cells/ml in the same buffer, and added carefully to an equal volume of 1.2% SDS in 0.02 M Tris, 0.01 M EDTA, pH 7.5. Self-digested Pronase was then added to 2 mg/ml and the lysate was incubated for 1 h at 37 C. After digestion NaCl was added to 1.0 M, and mixed in by gently inverting the tube several times. After standing on ice for at least 8 h, the lysate was centrifuged at 12,000 rpm for 30 min at 0 C in a Sorvall SS-34 rotor. The supernatant fluid was removed for further analysis. The efficiency of extraction into the supernatant fluid of double-stranded polyoma virus DNA was over 80%, and of DNA from MVM particles was between 50 and 60%, as determined by reconstruction experiments. Conversion of polyoma component I (supercoiled) DNA to component II (relaxed circle) DNA was not detected during reconstruction experiments using this SDS-Pronase extraction.

HAP chromatography. Supernatant DNA from the selective extraction procedure was diluted 1:20 into 0.05 M sodium phosphate buffer, pH 6.8, and loaded on to columns (15 by 5 mm) of hydroxyapatite (HAP) (Bio-Rad HT). After being washed with 10 column volumes of 0.05 M buffer, the DNA was eluted with a 15-ml gradient of 0.05 to 0.4 M sodium phosphate buffer, pH 6.8, at room temperature. Fractions (0.5 ml) were collected, and EDTA was added to each fraction to 0.01 M. Samples were dried on to 2.5-cm Whatman no. 1 filter paper squares, which were placed in 10 ml of toluene containing per liter 5 g of 2,5-diphenoloxazole (PPO) and 0.3 g of 1,4-bis-(4-methyl, 5-phenoloxazole) benzene (POPOP), and the radioactivity was determined in a liquid scintillation counter. Essentially all the acid-precipitable label adsorbed to the column and recoveries were greater than 80%.

Benzoylated DEAE cellulose (BDC) chromatography. Fractions from HAP chromatography were pooled and transferred into 0.3 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 7.5, by gel filtration on Sephadex G-25. The sample, in a volume of 5 ml, was introduced at 0.5 ml/min on to a column (1 by 1.5 cm) of benzoylated diethylaminoethyl cellulose (Schwarz/Mann BD; 50 to 100 mesh) previously washed extensively with the same buffer. The DNA was then eluted successively with a 20-ml gradient of 0.3 to 1.0 M NaCl in 0.01 M Tris, 0.001 M EDTA, pH 7.5, a 20-ml gradient of 0 to 1.5% caffeine in 1.0 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 7.5 (prewarmed to 37 C), then 1 ml each of 1.5% caffeine, 2% caffeine, and 8 M urea. Fractions were collected and assayed as before. Recoveries were greater than 80%.

Ethidium bromide-cesium chloride equilibrium gradients. Radioactive DNA was added to cesium chloride in 0.5 M NaCl, 0.05 M Tris, 0.002 M EDTA, pH 7.4, containing 300 μg of ethidium bromide per ml. The final density of the solution was 1.562 g/ml.

Neutral sucrose sedimentation. Radioactive DNA (0.2 ml) was layered on a 4.8-ml 5 to 20% (wt/vol) linear sucrose gradient in 0.2 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 7.0.

Alkaline sucrose sedimentation. Radioactive DNA was made 0.25 M NaOH in a final volume of 0.2 ml. After 15 min at room temperature, the sample was layered on a 4.8-ml 5 to 20% (wt/vol) sucrose and 0.25 to 0.5 M NaOH gradient in 1.0 M NaCl and 0.001 M EDTA at a final pH of > 12.5.

All equilibrium and sedimentation analyses were carried out in a Spinco SW50.1 rotor, under conditions outlined in the legend of each figure. Gradients were collected from the bottom of the tube directly on to 2.5-cm Whatman no. 1 filter paper squares, dried, washed twice in 10 ml of cold 5% trichloroacetic acid per filter and once in absolute ethanol, and counted as above.

Exonuclease I digestion. Exonuclease I from *E. coli* was assayed and purified according to Lehman and Nussbaum (15). Fractions comprising the peak of activity from a DEAE cellulose column were pooled, precipitated with ammonium sulfate, and taken up in 0.05 M Tris buffer, pH 7.4. Radioactive DNA was digested before or after denaturation by incubation with 0.5 or 5.0 μg of the enzyme in 0.3 ml of 0.067 M

glycine-NaOH, 0.0067 M MgCl_2 , 0.0017 M 2-mercaptoethanol, pH 9.5. The reaction was stopped by the addition of 50 μg of denatured salmon sperm DNA and 5 volumes of cold 10% trichloroacetic acid containing 10% each of saturated tetrasodium pyrophosphate and trisodium orthophosphate (acid/phosphate). The precipitate was collected on a presoaked GF/C glass-fiber filter and washed with 20 ml of acid/phosphate and counted as before. Less than 3% endonucleolytic conversion of SV40 component I DNA to component II DNA was observed during the incubation of 5 μg of this preparation of exonuclease I with 0.02 μg of viral DNA (5×10^8 counts per min per μg) for 30 min at 37 C, under the assay conditions described.

DNA samples were denatured in 0.01 M Tris, 0.001 M EDTA, pH 7.5, by heating to 100 C for 2 min and quench cooling in ice water. Alternatively, the DNA was denatured by exposure to 0.5 M NaOH for 10 min. The solution was neutralized with 1 M HCl and made 0.05 M in Tris buffer, pH 7.4.

Denaturation, reannealing. DNA in a volume of 100 μliters was heat denatured and quench cooled as before. After cooling, 5 M NaCl was added to a final concentration of 0.25 M and the solution was maintained at 65 C for 48 h.

Displacement hybridization. Samples of labeled DNA were heat denatured in the presence of known amounts of unlabeled DNA, and allowed to reanneal as before, for 48 h. After reannealing, the amount of labeled DNA in duplex form was measured by adding the constituents of the exonuclease I incubation mixture and 5 μg of enzyme and then incubating for 30 min at 37 C.

RESULTS

DNA synthesis in infected and control cells. The incorporation of ^3H -thymidine in control and MVM-infected A9 cell cultures is shown in Fig. 1a. A slight depression in incorporation in infected cells was observed in the latter half of the labeling period. This may be due to the fact that L cells do not divide after infection with MVM at a multiplicity of 5 PFU/cell (Tattersall and Shatkin, unpublished observation, D. C. Ward, personal communication). When the labeled DNA was fractionated by the selective extraction procedure of Hirt (10), however, differences were observed in the type of DNA synthesized in infected and control cells. This procedure is based on the selective pelleting of host DNA by SDS, in the cold, in the presence of 1.0 M NaCl. As shown in Fig. 1b, a fixed amount (0.8%) of the labeled DNA in control cells appears as supernatant DNA, whereas in infected cells, between 8 and 14 h, there is a considerable increase in the proportion of total label appearing in the supernatant fluid, reaching 25% of the total DNA synthesized by 20 h. The appearance of this class of DNA closely parallels the production of cell-associated viral hemagglutinin. This DNA was

characterized further to determine its relationship to viral DNA.

Hydroxyapatite chromatography of supernatant DNA. The secondary structures of the supernatant DNA from both infected and control cells were determined by fractionation on columns of hydroxyapatite (1, 2). Chromatography of supernatant DNA from control cells revealed only one component, eluting as double-stranded DNA (Fig. 2a). Supernatant DNA from infected cells, however, was resolved into two components, one eluting in the position of single-stranded DNA (σ) and one eluting in the position of double-stranded DNA (δ). The component eluting first comprised 20% of the total supernatant DNA at 14 and 20 h (Fig. 2b and c). Correcting for the differential loss of single-stranded DNA observed in reconstruction experiments, this would indicate that the supernatant fluid contains approximately equal amounts of single- and double-stranded DNA at each time examined.

Centrifugation of supernatant DNA. In

FIG. 1a. Incorporation of ^3H -thymidine into acid precipitable material in mock-infected (O) and MVM-infected (●) suspension cultures of A9 cells. Samples from cultures prepared and labeled as described in the text, were lysed by adding 5 volumes of cold 0.01 M EDTA and precipitated with an equal volume of 10% acid/phosphate. The precipitates were collected on GF/C filters, washed, dried, and counted as described. (b), Formation of viral hemagglutinin and labeled supernatant DNA. Samples containing 10^6 infected cells were pelleted, cell-associated virus was extracted, and the hemagglutinin titers (Δ) were determined as previously described (29). Samples containing 2×10^6 cells were lysed by the SDS-Pro-nase procedure described in the text. The percentage of the total radioactivity appearing in supernatant DNA is shown for samples from mock-infected (O) and infected (●) cultures.

FIG. 2. Labeled DNA preparations from SDS-Pronase supernatant fluids were chromatographed on hydroxyapatite as described in the text. The vertical arrows marked σ and δ show the elution positions of single-stranded MVM DNA and double-stranded polyoma component I DNAs, respectively. (a), Mock-infected culture labeled 0 to 20 h; (b), infected culture labeled 0 to 14 h, and (c), infected culture labeled 0 to 20 h. Samples bracketed and designated A, B, C, and D were pooled separately for further analysis.

order to examine the size and structure of the hydroxyapatite fractions further, the peaks were pooled and compared with MVM DNA and polyoma virus DNA on sucrose gradients.

In alkaline sucrose, DNA from purified virus sedimented as a sharp peak slightly slower than 14S polyoma DNA (Fig. 3b). The DNA from HAP pool C (Fig. 2c) sedimented in an identical manner (Fig. 3a), suggesting that it is progeny viral DNA, although confirmation of this requires hybridization or infectivity studies. The significance of the shoulder of material sedi-

menting slower than the peak in both preparations is not understood at present.

Figure 4a shows that 60% of the double-stranded DNA from infected cell supernatant fluids sediments as a distinct peak slightly behind polyoma component II DNA (16S) in neutral sucrose. DNA with similar sedimentation properties has been demonstrated by pulse labeling of MVM infected L cells (7). The remaining DNA sediments faster and appears to be heterogeneous. The possibility that this fast-sedimenting DNA may comprise com-

FIG. 3a. ^3H -thymidine labeled DNA from HAP pool C sedimented in alkaline sucrose at $120,000 \times g$ (average) for 5 h at 5 C. (b), ^3H -thymidine-labeled, purified MVM virus was lysed by exposure to 0.25 M NaOH for 15 min at room temperature and sedimented in alkaline sucrose as in (a). The vertical arrows marked I and II show the positions of ^{14}C -polyoma DNA components I and II, respectively, in the same gradients.

FIG. 4a. ^3H -thymidine labeled DNA from HAP pool D was sedimented in neutral sucrose at $120,000 \times g$ (average) for 4.5 h at 5 C. (b), ^3H -thymidine labeled DNA from HAP pool D was sedimented in an alkaline sucrose gradient at $120,000 \times g$ (average) for 5 h at 5 C. The vertical arrows marked I and II show the positions of ^{14}C -polyoma DNA components I and II, respectively, in the same gradients.

plexes of genome length viral DNA is suggested by the results shown in Fig. 8a and is discussed in a following section. In alkaline sucrose, essentially all the double-stranded supernatant DNA sediments as a peak with an average *S* value again slightly lower than that of polyoma DNA (Fig. 4b). No material was detected in the region where a closed circular double-stranded form of MVM DNA would be expected to sediment (50 to 55S).

In ethidium bromide-caesium chloride essentially all of the double-stranded supernatant DNA banded with polyoma component II DNA, ruling out the possible existence of alkali-sensitive closed circular molecules (Fig. 5). In neutral caesium chloride over 95% of the ³H-labeled DNA banded with A9 cell DNA (not shown), consistent with a G plus C content of approximately 40% and ruling out the possibility that the molecules were DNA-RNA hybrids. This is in agreement with the expected G plus C content of a double-stranded form of MVM calculated from the base analysis of the viral strand (6).

Exonuclease I digestion of supernatant DNA. The secondary structures of the various hydroxyapatite fractions of supernatant DNA

FIG. 5. ³H-thymidine labeled DNA from HAP pool D (●) and ¹⁴C-polyoma components I and II DNA (○) were centrifuged to equilibrium in a caesium chloride-ethidium bromide density gradient at 100,000 × *g* (average) for 60 h at 20 C.

were examined further by using exonuclease I from *E. coli*. This enzyme specifically hydrolyzes single-stranded DNA from the 3'OH end (15). Figure 6a shows the hydrolysis of control cell supernatant DNA (pool A, Fig. 2a) before and after denaturation. The initial rate of hydrolysis of denatured DNA was greater than 500 times that of native DNA. The final extent of hydrolysis at this concentration of enzyme for denatured DNA was 93%. The resistance of the remaining 7% may have been due to renaturation of repetitive sequences in the DNA during the incubation, for at a 10-fold higher enzyme concentration, where the reaction was complete within 2 min, 97% hydrolysis was observed (Table 1). DNA from pool C (Fig. 2c) was hydrolyzed at similar rates before and after heat denaturation, indicating that this DNA is indeed single stranded (Fig. 6b). The extents of hydrolysis were 79% for unheated and 89% for heated DNA. In contrast, unheated DNA from pool D (Fig. 2c) was resistant to hydrolysis (Fig. 6c). The initial rate of hydrolysis of this DNA after denaturation is equivalent to that of the other denatured DNAs, but the extent of hydrolysis achieved was only 64% (Table 1). At a 10-fold higher enzyme concentration, 69% hydrolysis was observed. At this enzyme level, the reaction was complete within 2 min and no further hydrolysis was obtained by adding more enzyme. The resistant fraction was also observed after alkali denaturation (Table 1). This anomalous behavior has been observed for other double-stranded DNA preparations from infected cells extracted both at 14 and 20 h after infection. In different experiments, the exonuclease I-resistant fraction was found to be between 20 and 40% of the total supernatant double-stranded DNA.

To determine whether the resistance was due to an interaction between two or more molecules, the effect of varying the DNA concentra-

TABLE 1. Limit digestion of denatured supernatant DNA fractions with *E. coli* exonuclease I^a

DNA source	Denaturation procedure	Amount of enzyme (μg)	Hydrolysis (%)
Pool D	Heat	0.5	64
Pool D	Heat	5.0	69
Pool D	Alkali	5.0	52
Pool B	Heat	5.0	64
Pool A	Heat	0.5	93
Pool A	Heat	5.0	97
Pool C	Heat	0.5	89

^a Equal amounts of labeled HAP fractionated supernatant DNA were denatured as described in the text and digested with 0.5 or 5.0 μg of exonuclease I for 30 min at 37 C.

FIG. 6. Exonuclease I digestion of (a) HAP pool A DNA, (b) HAP pool C DNA, and (c) HAP pool D DNA, both native (●) and after heat denaturation (○). Each DNA was incubated with 0.5 μ g of exonuclease I as described in the text.

tion during denaturation was investigated. If the residual resistance were due to the reannealing of separate strands, a 10-fold reduction in concentration should lead to a 100-fold reduction in rate of reannealing which would be reflected in the fraction resistant to exonuclease I after a short incubation period (<30 min under these conditions). As can be seen from Table 2, dilution had relatively little effect on the size of the resistant fraction. This evidence suggests either that a fraction of the single strands is resistant to exonuclease I because of some modification of the 3'-terminus (i.e., circularity, presence of a blocking group) or that digestion is not complete because of some intramolecular renaturation. To distinguish between these possibilities, the denaturation products were chromatographed on hydroxyapatite. After denaturation, DNA from pool D (Fig. 2c) was resolved into two components on hydroxyapatite (Fig. 7a): one, comprising 60 to 65% of the total, eluting as single-stranded DNA and the remainder eluting as double-stranded DNA. When the denaturation products of the original DNA were digested with exonuclease I prior to chromatography, more than 85% of the acid-precipitable label eluted as double-stranded DNA (Fig. 7b). These results indicate that the residual resistance to exonuclease I after denaturation is due to spontaneous, intramolecular renaturation. The denaturable fraction and the spontaneously renaturing fractions are denoted dN DNA and rN DNA, respectively, using the notation of Szybalski et al. (28).

Displacement hybridization. The extents of homology between viral DNA and supernatant double-stranded DNAs from infected and mock-infected cells were examined by displacement hybridization. This measures the ability

TABLE 2. Effect of DNA concentration on resistance to exonuclease I after denaturation^a

Relative dilution	Percentage resistant
1	38
5	33
10	29

^a Samples of HAP pool D DNA were prepared at three different concentrations and the overall DNA concentration was adjusted to 0.1 μ g/ml with salmon sperm DNA. These preparations were heat denatured as described in the text. After cooling, the standard exonuclease I incubation mixture and denatured salmon sperm DNA (0.1 μ g/ml) were added to make all preparations up to the same volume and DNA content. The denatured DNAs were then digested with 5 μ g of exonuclease I for 30 min at 37 C.

FIG. 7a. HAP pool D DNA was heat denatured, quench cooled in ice water and rechromatographed on hydroxyapatite as described previously. (b), HAP pool D DNA was heat denatured, quench cooled and digested with 5 μ g of exonuclease I for 30 min at 37 C. The remaining DNA was rechromatographed on hydroxyapatite as before. The vertical arrows marked σ and δ show the elution positions of single-stranded MVM DNA and double-stranded polyoma component I DNA, respectively.

of unlabeled viral DNA strands to compete during renaturation with labeled strands from denatured double-stranded DNA. A large excess concentration of unlabeled viral strands would effectively displace all labeled strands with viral sequences, rendering them sensitive to exonuclease I after reannealing has gone to completion. Table 3 shows the results of an experiment where unlabeled viral DNA was used to compete for viral sequences in double-stranded supernatant DNA from infected and mock-infected cells. The addition of a large amount of unlabeled viral DNA to reannealing host DNA did not increase the amount of host DNA sensitive to exonuclease I. However, the addition of viral DNA displaced up to 35% of the label from pool D DNA (Fig. 2c). Since the virus contains only one strand, this indicates that at least 70% of this DNA is virus specific. This may be an underestimate since it is unlikely that the spontaneously denaturing sequences in the rN structure can be competed for by added viral DNA. A larger excess of unlabeled viral DNA might be required to compete for sequences involved in intra-strand renaturation. Single-stranded DNA from bacteriophage ϕ x174 also competed for sequences in infected cell DNA, but with approximately one hundredth of the efficiency of MVM DNA. The results of these hybridization studies show that the majority (and possibly all) of this hydroxyapatite fraction from MVM infected cells contains viral sequences.

Essentially all of the labeled DNA, denatured and reannealed in the absence of added viral

TABLE 3. Displacement hybridization with supernatant DNA fractions^a

Sources of DNA		Amount of competing DNA (ng)	Label remaining in hybrid (%)	Maximum theoretical displacement (%)
Labeled DNA	Competing DNA			
Pool D	None	0	100	0
Pool D	MVM	10	79	42
Pool D	MVM	50	66	68
Pool D	MVM	100	69	62
Pool D	MVM	500	65	70
Pool D	None	0	100	0
Pool D	ϕ X 174 ^b	200	95	10
Pool D	ϕ X 174 ^b	1,000	80	40
Pool A	None	0	43 ^c	0
Pool A	MVM	500	49 ^c	0

^a Displacement of single-strands homologous to MVM and ϕ X 174 from uniformly labeled supernatant double-stranded DNA was performed as described in the text. Results are expressed as the percentage of the maximum label (50%) that would be exonuclease I-sensitive at saturation, if all the supernatant DNA were double-stranded viral DNA.

^b ϕ X 174 was extracted from infected cells according to Sinsheimer (27). Virus purification and DNA extraction were as described in the text for MVM.

^c The maximum re-annealing of host DNA under these conditions was between 40 and 50%.

DNA, sedimented as a single peak in neutral sucrose as shown in Fig. 8b. The homogeneity of this material suggests that complete hybrids of a molecular weight of $\sim 3 \times 10^6$ had been formed. DNA which had been incubated under reannealing conditions for 48 h, but without prior denaturation, showed the same sedimentation profile as the reannealed DNA (Fig. 8a). It differed from the DNA as isolated from hydroxyapatite in that the faster sedimenting DNA, comprising 40% of the total (Fig. 4a), was shifted to the position of the main peak. This suggests that the faster sedimenting DNA consists of complexes of the 3×10^6 MW duplex, that are able to rearrange under the conditions described.

Benzoylated DEAE-cellulose chromatography. A further characterization of the double-stranded MVM DNA found in infected cell supernatants was attempted by BDC chromatography. This technique separates single- and double-stranded nucleic acids in a manner complementary to that of hydroxyapatite. Double-stranded DNA is eluted with a gradient of increasing NaCl concentration and single-stranded DNA or DNA molecules containing single-stranded regions are eluted with a gradient of increasing caffeine concentration in 1.0 M NaCl. Iyer and Rupp (11) have shown, with benzoylated, naphthoylated DEAE-cellulose,

FIG. 8a. HAP pool D DNA was maintained at 65°C in 0.25 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 7.5, for 48 h and then sedimented in neutral sucrose at $120,000 \times g$ (average) for 2.5 h at 5°C. (b), HAP pool D DNA was heat denatured, and allowed to reanneal as in (a) for 48 h at 65°C and then sedimented in neutral sucrose as before. Symbols: ●, ^3H HAP pool D DNA; ○, ^{14}C SV40 component II DNA.

that there is a linear relationship between the fraction of single-stranded DNA in each molecule, and the concentration of caffeine at which it elutes. Figure 9 shows that the DNA from infected cell supernatants which elutes as double-stranded DNA from hydroxyapatite is resolved into two components on BD cellulose, one eluting as completely double-stranded DNA and one which, although partially double-stranded, presumably contains single-stranded regions. This property has been reported for the replicating DNA molecules of a number of viruses (12, 13, 16, 33). The fraction of rN DNA in both classes of DNA was approximately equal. That the DNA eluting in the caffeine gradient does contain replicating DNA is shown by the experimental results in Fig. 10. A short pulse (2 min) of ^3H -thymidine at 18.5 h after MVM infection preferentially labels supernatant double-stranded DNA as determined by hydroxyapatite chromatography (Fig. 10a). When this DNA was pooled and rechromatographed on BD cellulose, the pulse label was found almost exclusively in DNA which eluted in the caffeine gradient (Fig. 10b).

DISCUSSION

We have used the selective extraction technique of Hirt (10) to follow the synthesis and determine the structures of various intracellular forms of MVM DNA present during the first 20 h of infection. This procedure is reported to

precipitate high molecular weight host DNA, selectively extracting small viral molecules into the supernatant fluid. The work of Pearson and Hanawalt (18) suggests that, in the cold, SDS crystals bind to membrane fragments precipitating the fragments and replicating cell DNA attached to them. Under conditions of minimum shear, this would presumably precipitate the entire nuclear DNA of the host. Digestion of the lysate with Pronase before precipitation did not affect the result (18). It is possible, therefore, that viral DNA attached to cell membranes would be precipitated with host DNA by this procedure. In this report we have not examined the DNA in the pellet for viral DNA content. A considerable proportion of the total label in infected cell DNA (25% by 20 h after infection) was extracted into the supernatant fluid. It was found to comprise approximately equal amounts of single-stranded and double-stranded DNA. Sedimentation analysis suggests that the single-stranded DNA is progeny viral DNA. Most of the double-stranded DNA was shown to contain viral sequences by hybridization and sediments in the position expected of a duplex viral genome. The time course for the accumulation of newly synthesized single-stranded MVM DNA corresponds with that determined for the production of progeny viral DNA for the parvoviruses RV (22) and Lu III (26).

Although single-stranded viral DNA appears to be linear by electron microscopy (6), it is partially resistant to exonuclease I, even after heat denaturation. That this may be due to secondary structure in the molecule is supported by the finding that this DNA is also partially resistant to S1 nuclease, a single-strand-specific endonuclease (D. C. Ward, personal communication). The single strands of AAV DNA have been shown to contain self-com-

FIG. 9. HAP pool D DNA was chromatographed on BD cellulose as described in the text.

FIG. 10. A9 cells, infected with MVM as described in the text, were labeled for 18 h with $[-2-^{14}\text{C}]$ thymidine ($0.25 \mu\text{Ci/ml}$, $2.5 \times 10^{-6} \text{ M}$). The cells were then washed twice with warm DP medium and resuspended at 10^7 cells/ml in DP medium containing $6 \times 10^{-5} \text{ M}$ fluorodeoxyuridine. (Fluorodeoxyuridine was a kind gift of Roche Products Limited, Welwyn Garden City, England.) After 30 min at 37°C , $5 \mu\text{Ci/ml}$ of $[\text{methyl-}^3\text{H}]\text{-thymidine}$ (21 Ci/mmol) was added. Two minutes later, DNA synthesis was stopped by pipetting 1 ml of the cells into 10 ml of acetone at -20°C . The cells were then pelleted, washed twice, and selectively extracted as described in the text. The supernatant DNA was chromatographed on hydroxyapatite (a) and the double-stranded DNA (pool E) was rechromatographed on BD cellulose (b) as previously described. In this experiment, the NaCl gradient was followed by a 20 ml gradient of 0 to 2% caffeine in 1.0 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 7.5 (prewarmed at 37°C). Symbols: $\circ$, ^{14}C -thymidine DNA labeled 0 to 18 h; $\bullet$, ^3H -thymidine DNA pulse labeled for 2 min at 18.5 h after infection.

plementary regions (4), including complementary sequences at each end of the DNA. Consequently, AAV DNA can form a "panhandle" structure giving rise to hydrogen bonded single-stranded circles (14). A short duplex region at or near the 5' end of the viral single-stranded (v strand) of MVM would render the majority of the molecule sensitive to exonuclease I, but the complementary strand (c strand) would have a corresponding duplex region at or near the 3' end making the majority of the c strand resistant to exonuclease I.

If this were the explanation for the exonuclease I resistant fraction in denatured MVM double-stranded DNA, one would expect the v and c strands to chromatograph identically on hydroxyapatite, that is, as single-stranded DNA. However, 40% of the DNA chromatographs as double stranded after denaturation (Fig. 7a) suggesting that the rN structure in the double-stranded DNA is different from the exonuclease I resistant structure in the v strand. That this spontaneously renaturing DNA is identical with the exonuclease I resistant fraction is shown by the experiment described in Fig. 7b.

Further experiments designed to investigate the secondary structure of the viral DNA and the double-stranded forms are required to establish the nature of the rN structure and its role, if any, in the replication of the viral DNA. It is of interest to note that similar rN fractions have been reported in preparations of vaccinia virus DNA (3) and fowlpox virus DNA (28), where the presence of one interstrand link per genome was indicated. It is possible that such rN structures are formed by the use of the 3' end of one strand as a primer for the synthesis of the other strand (structure A)

giving rise to spontaneously renaturing, "hair-pin" intermediates (structure B).

This implies some previous replication mechanism which generates a terminally redundant loop (r) at the 3' end of the template strand, in

order to conserve the entire base sequence of the genome. A model structure for the viral DNA which allows for such a mechanism is currently being investigated.

Extensive studies on the replication of the single-stranded DNA of bacteriophage ϕ x174 have demonstrated three types of reaction: (i) conversion of the parental single-stranded to a double-stranded form; (ii) replication of this double-stranded form to give further double-stranded intermediates (which appears to occur at a specific membrane site), and (iii) replication of these intermediates yielding single-stranded progeny molecules. In a synchronous infection, these events are sequential, giving rise to three definite stages in the replication of the viral DNA. In MVM infected cultures examined by the techniques outlined above, single-stranded progeny molecules are detectable at the same time as double-stranded intermediates, in the Hirt supernatant fraction. A short pulse of ^3H -thymidine late in infection labeled both pellet and supernatant DNA. Further fractionation of the supernatant DNA on hydroxyapatite and BD cellulose showed that the pulse predominantly labeled double-stranded DNA containing single-stranded regions (Fig. 10a and b). The increase in the ratio of pulse label (^3H) to uniform label (^{14}C) through the caffeine gradient (Fig. 10b) suggests that this DNA contains replicating intermediates involved in the final stage of replication, giving rise to progeny single strands as in reaction (iii) above. The uniform label in native molecules is resistant to exonuclease I (Fig. 6c), indicating that the single-stranded portions of these molecules are either internal, or have 5' termini. The latter type of molecule could arise by displacement of one v strand by the synthesis, in the 5' to 3' direction of a second v strand using the same c strand as a template (structure C).

The difficulty of constructing an overall model for replication which gives rise to such a large proportion of intermediate (i.e., double-stranded) molecules, at any given time, suggests that a class of intermediate, perhaps involved in a reaction analogous to (ii) above, is lost into the pellet with this extraction technique. In addition, if the S phase dependence of this and other parvoviruses is due to the requirement for host programmed events in the replication of the

viral DNA, then one would expect the replication cycle to be asynchronous in asynchronous cell cultures, and individual stages in the replication to be masked. Such possibilities are currently being examined in synchronous cell cultures.

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