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DNA of Minute Virus of Mice: Self-Priming, Nonpermuted, Single-Stranded Genome with a 5'-Terminal Hairpin Duplex

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The genome of the nondefective parvovirus minute virus of mice (MVM) is a linear DNA molecule of molecular weight 1.48×10^6, which is single stranded for ~94% of its length. In contrast to the genomes from defective parvoviruses, MVM DNA does not contain a detectable inverted terminal redundancy. A combination of enzymatic and physical techniques has shown that the molecule contains a stable hairpin duplex of approximately 130 base pairs located at the 5' terminus of the genome. MVM DNA is efficiently utilized as a template-primer by a number of DNA polymerases, including reverse transcriptases. Polymerases lacking 5' to 3' exonuclease activity yield a duplex DNA product with a molecular weight 1.96 times that of the viral genome, in which the newly synthesized complementary strand is covalently attached to the template. This duplex contains an internal "nick" that can be sealed by DNA ligase to produce a self-complementary single-strand circle. The MVM DNA duplex is cleaved twice by *Eco*R·RI restriction endonuclease to yield three distinct fragments in molar amounts. These results suggest that the initiation of DNA synthesis in vitro occurs at a point within 100 bases of the 3' end of the genome, using the 3' terminus of viral DNA as a primer, and that the sequence of nucleotides in the genome is not permuted.

Parvoviruses are among the smallest known DNA viruses. The infectious virions are icosahedral particles, with diameters of 18 to 30 nm, that contain linear single-stranded genomes of 1.35×10^6 to 1.70×10^6 molecular weight (4, 32). The arrangement of nucleotide sequences in parvovirus genomes has been examined in some detail for a defective subgroup, the adenovirus-associated viruses (AAV) (4). These viruses package "plus" and "minus" DNA strands in separate virions that, on release from the particles, can anneal to form duplex molecules (6, 29, 33). Although the AAV genome is not randomly circularly permuted (4, 8, 17), it contains a limited number of terminal sequence permutations representing <6% of its length (4, 17). The majority of the single strands also contains an inverted terminal repetition, allowing them to form "panhandled" circles by self-annealing (5, 23).

In contrast to AAV, little is known about the secondary structure or sequence arrangements in the DNA of nondefective parvoviruses. This group of viruses packages a unique viral DNA strand (13, 27), equivalent in base composition to the strand of AAV DNA complementary to the single stable mRNA species transcribed in vivo (9-11).

We have examined the structure of the genome from minute virus of mice (MVM), a nondefective parvovirus, to compare its properties with that of AAV DNA and in an attempt to understand how certain intermediates in MVM DNA replication might be synthesized. Replication of MVM DNA in vivo involves the formation of double-stranded intermediates, some of which behave as though viral and complementary strands are covalently linked (43). Similar results have been reported recently for AAV (E. D. Sebring, S. E. Strauss, H. G. Ginsberg, and J. A. Rose, Fed. Proc. 34:639, 1975). Tattersall et al. (43) originally proposed that such intermediates might be formed by using the 3' end of one strand as a primer for synthesis of the other strand. This would necessitate a mechanism for replicating the terminal loop at the 3' end of the template strand to conserve the entire structure of the genome.

This report shows that the MVM genome is a nonpermuted DNA that contains a stable hairpin duplex at its 5' terminus and a 3'-terminal structure suitable for priming complementary

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strand synthesis in vitro. Whether such terminal nucleotide structures play an important role in MVM DNA replication in vivo is under current investigation.

MATERIALS AND METHODS

Virus and cell cultures. The plaque-purified strain T of MVM (41) was used in all studies. Virus labeled with [^{32}P]phosphate was prepared by infecting subconfluent RL5E rat cell monolayers (7) at a multiplicity of infection (MOI) of 5 PFU/cell. After a 90-min adsorption period the inoculum was removed, and 9 ml of D medium containing 2% of the normal inorganic phosphate and supplemented with 5% dialyzed fetal calf serum was added to each dish. At 10 h after infection a further 1 ml of the same medium containing 250 μCi of ^{32}P (as carrier-free phosphoric acid, New England Nuclear) was added to each dish. The infected cells were scraped into the medium and harvested by low-speed centrifugation at 48 h postinfection. Virus was extracted and purified as before (42) with the following modifications. Phenylmethyl sulfonyl fluoride was omitted from the cell homogenate (fraction 1), and the cytoplasmic supernatant (fraction 2) was made 25 mM in $CaCl_2$ and 5 μg/ml in micrococcal nuclease (Worthington) and digested for 30 min at 37°C. Trypsin (Merck) was then added to 100 μg/ml, and the incubation was continued for an additional 30 min. The precipitate of virus was collected by centrifugation and further purified as reported previously (42). Unlabeled virus and virus labeled with [^{3}H]- or [^{14}C]thymidine were produced from high-multiplicity or low-multiplicity infections of Ehrlich ascites and A-9 L cells as described before (42) and extracted as above.

Purification of viral DNAs. (i) MVM DNA. Purified "full" virus (0.1- to 0.2-ml suspension in TE 8.7 buffer [50 mM Tris-hydrochloride–0.5 mM EDTA, pH 8.7]) was sedimented into a 5-ml alkaline sucrose gradient (5 to 20% [wt/vol] sucrose-0.3 M NaOH-0.7 M NaCl-0.15% Sarkosyl) by centrifuging in a Beckman SW50.1 rotor at 48,000 rpm for 4 h at 5°C. The peak DNA-containing fractions (see Fig. 1) were pooled and either dialyzed exhaustively against TE 7.5 buffer (10 mM Tris-hydrochloride–1 mM EDTA, pH 7.5) or desalted on a 5-ml G-25 Sephadex column equilibrated with TE 7.5 buffer.

(ii) ^{3}H-labeled fd DNA. fd phage was grown in *Escherichia coli* KBL 828 (Hfr *thy*$^-$ *leu*$^-$ *lac*$^-$) in minimal medium 56 (26) plus 0.5% Casamino Acids, 2 μg of thiamine per ml, and 2.5 μg of [^{3}H]thymidine per ml (4 μCi/μg). The phage was purified by polyethylene glycol precipitation and CsCl density gradient centrifugation (48). The DNA was isolated by sedimentation of the purified phage into alkaline sucrose gradients using the same conditions as described for MVM DNA.

(iii) Other viral DNAs. ^{14}C-labeled simian virus 40 (SV40) DNA was prepared as described previously (14). Purified ^{32}P-labeled SV40 DNA was provided by K. Subramanian. ^{14}C-labeled λ phage DNA was obtained from J. Sklar, ϕX-176 RFII DNA was from N. Godson, and ^{3}H- and bromodeoxyuridine-labeled AAV DNA was provided by B. Carter.

Enzymes. *E. coli* DNA polymerase I holoenzyme, fraction 7 (specific activity, 16,000 U/mg of protein), was purified as described by Jovin et al. (22). The proteolytic fragment of *E. coli* DNA polymerase I, lacking 5′→3′ exonuclease activity (specific activity, 9,600 U/mg protein), was obtained from Boehringer Mannheim Corp. Both enzyme preparations were free of single- or double-stranded endonuclease. Bacteriophage T4 DNA polymerase (specific activity, 40,000 U/mg of protein) was the generous gift of P. Englund. Rauscher murine leukemia virus reverse transcriptase, prepared by the method of Abrell and Gallo (1), was obtained from Litton Bionetics, Inc., and avian myeloblastosis reverse transcriptase was kindly provided by D. Kacian. Exonuclease I from *E. coli* was purified by the procedure of Lehman and Nussbaum (25) and assayed as described previously (43). The exonuclease I preparation was essentially free of both single- and double-stranded endonuclease activity. Polynucleotide kinase from T4-infected *E. coli* B, purchased from P-L Biochemicals, Inc., had a specific activity of 16,400 U/mg of protein. Bacteriophage T4 DNA ligase (specific activity, 44,000 U/mg) and *E. coli* alkaline phosphatase (specific activity, 191 U/mg) were kindly provided by C. Richardson and J. Chlebowski, respectively. *Eco*R·RI restriction endonuclease, the gift of J. Skare, was purified, assayed and stored as described by Greene et al. (19). The single-strand-specific S1 endonuclease was prepared by the method of Vogt (45) and had a specific activity of 55,000 U/mg of protein. The enzyme was stored frozen in 50% glycerol at −70°C.

Enzyme assay conditions. (i) In vitro synthesis of double-stranded MVM DNA. Synthesis was generally carried out in the reaction mixture described by Richardson et al. (30) containing either 25 μCi of [^{3}H]TTP (0.8 μCi/nmol) or 10 to 30 μCi of α-[^{32}P]TTP (1 to 2 μCi/nmol) per ml. Typically, 0.5 μg of input MVM DNA was incubated with 4 U of *E. coli* DNA polymerase fragment (lacking the 5′ to 3′ exonuclease) in a total volume 0.2 ml at 37°C for 60 to 90 min. The reaction usually reached completion by 45 min of incubation. Portions of the reaction mixture were withdrawn at various times and added to 0.1 ml of 10% trichloroacetic acid-5% Na_3PO_4-5% $Na_4P_2O_7$, followed by the addition of 50 μg of carrier calf thymus DNA. All samples were left at 0°C for at least 10 min, filtered onto GF/C or GF/A glass fiber filters (Whatman), washed with 15 ml of 5% trichloroacetic acid and 5 ml of EtOH, dried, and counted in a toluene-based scintillation fluid.

The duplex MVM DNA product was isolated by sedimentation in alkaline sucrose as described for the isolation of single-stranded MVM DNA, except that the enzyme reaction mixture was made 25 mM in EDTA (to prevent aggregation) prior to layering on the gradient. When required, fractions containing duplex MVM DNA were concentrated ~10-fold either by dialysis against dry Sephadex G-200 or by blowing N_2 over the surface of the solution. The DNA was then dialyzed exhaustively against TE 7.5 buffer.

(ii) S1 nuclease. Standard reactions were carried out in 0.2 ml of 0.03 M sodium acetate-0.30 M NaCl-2 mM $ZnCl_2$, pH 4.5, containing 2 μg of unlabeled calf

thymus DNA (Sigma) and 10 U of S1 nuclease. After various times of incubation at 37°C, portions of the reaction mixture were withdrawn, and the amount of trichloroacetic acid-insoluble radioactivity was determined as described above.

Since the S1 nuclease preparation was found to have a low level of phosphatase activity (<20 pmol of PO_4 hydrolysis/h per 100 U), reaction mixtures used for digestion of MVM DNA bearing a 5'-terminal ^{32}P label also contained 1.0 mM dCTP. By providing a vast excess of an alternative phosphatase substrate, artifactual solubilization of the terminal [^{32}P]PO_4 could be avoided.

(iii) Exonuclease I. Samples were digested at 37°C in 0.3 ml of 66 mM glycine-NaOH-6.6 mM $MgCl_2$-1.7 mM mercaptoethanol, pH 9.5, containing 4 μg or less of DNA and approximately 2 μg of exonuclease I. Acid-insoluble radioactivity was determined as described above.

(iv) Alkaline phosphatase. A 0.2-ml solution of [^3H]MVM DNA (8 to 20 μg/ml) in 0.05 M Tris-hydrochloride-1.0 mM EDTA, pH 8.7, was treated with 0.1 to 0.2 U of *E. coli* alkaline phosphatase for 30 min at 65°C. After treatment, the viral DNA was purified by alkaline sucrose gradient centrifugation (as above) and dialyzed against 0.01 M Tris-hydrochloride-0.02 M NaCl-0.5 mM EDTA, pH 7.4.

(v) Polynucleotide kinase. Phosphatase-treated [^3H]MVM DNA was incubated at 37°C for 30 min with polynucleotide kinase as described by Weiss et al. (46). Reactions (0.25 ml) contained MVM DNA, 3.5 μg (~2.5 pmol of 5'-terminal nucleoside); Tris-hydrochloride buffer, pH 7.4, 20 mM; $MgCl_2$, 10 mM; mercaptoethanol, 10 mM; γ-[^{32}P]ATP, 3.4 μM (specific activity, 6×10^4 cpm/pmol); and polynucleotide kinase, 2.5 U. An additional 2.5 U of enzyme was added after 15 min of incubation. The reaction was terminated by the addition of 0.05 ml of 0.1 M EDTA, and the DNA was purified by alkaline sucrose centrifugation.

(vi) DNA ligase. Bacteriophage T4 DNA ligase reactions were done as described by Masamune and Richardson (28) using both the ligase alone or in combination with the subtilisin fragment of *E. coli* DNA polymerase I.

Gel electrophoresis. (i) Acrylamide slab gel. Electrophoresis was performed as described by Jeppesen (21) using a 3.5 to 7.5% acrylamide gradient and a 2.5% acrylamide stacking gel. Gels were run at room temperature for approximately 4.5 h with a current of 50 mA (150 V), dried under vacuum onto Whatman 1 paper, and exposed to Kodak RP/R54 X-ray film.

(ii) Agarose slab gel. Agarose slab gels (1.4%) were run as described by Sharp et al. (37), except that ethidium bromide was omitted in most experiments. Gels were run for about 3 h at room temperature with a current of 40 mA (120 V) and then dried and autoradiographed as above.

Hydroxyapatite chromatography. A 50-μl sample containing ^{32}P-labeled MVM DNA and ^3H-labeled fd DNA in 10 mM Tris-hydrochloride-1.0 mM EDTA, pH 7.5, was adsorbed to a 0.3-ml column of hydroxyapatite (Bio-Rad Laboratories, DNA grade) and then eluted with a 40-ml linear gradient of K_2PO_4 buffer, pH 6.8 (0.08 to 0.2 M), as described by Wilson and Thomas (47). Fractions (0.5 ml) were collected and mixed with 0.2 ml of water and 5.0 ml of Aquasol, and their relative ^{32}P-^3H content was determined in a liquid scintillation counter using double-label settings. Phosphate concentration was measured by refractive index as determined against a standard calibration curve.

Ethidium bromide fluorimetry. Fluorescence intensity measurements were made on the ultrasensitive spectrofluorimeter constructed by L. Stryer et al. as described by Yguerabide (49). The excitation wavelength was set at 520 nm, and the fluorescence emission was monitored through a Kodak 3-66 filter, which transmitted light of 580 nm or above. All measurements were taken on DNA samples at room temperature in pH 12 buffer (20 mM KPO_4-0.2 mM EDTA-0.5 μg of ethidium bromide per ml, pH 12) that Coulter et al. (12) found optimal for detection of stable duplex DNA. Total DNA concentrations were determined spectrophotometrically, using 49.2 μg/ml per optical density unit at 260 nm for double-stranded DNA and 35.7 μg/ml per optical density unit at 260 nm for single-stranded DNA (38).

Electron microscopy. All DNA samples were mounted by the formamide modification of the Kleinschmidt technique, stained with uranyl acetate, and rotary shadowed with platinum-palladium (15, 16).

RESULTS

Size of MVM DNA. MVM DNA was isolated by sedimenting full virus particles purified to fraction 5 (42) into an alkaline sucrose gradient. A single band of DNA with an average sedimentation coefficient of about 15S was usually obtained. In some virus preparations, however, the DNA band had a definite skew to its trailing edge, indicating the presence of smaller pieces of DNA. Although the size heterogeneity has been observed with viral preparations from both low- and high-MOI infections, the extent of heterogeneity is generally greater with high-MOI-derived virus. It is quite likely that the smaller pieces reflect the presence of defective virions, since serial passage of MVM at high MOI rapidly generate particles containing less than a complete genome equivalent of DNA (P. J. Tattersall, P. J. Cawte, and D. Ward, unpublished observations).

If the isolated 15S DNA is resedimented on a second alkaline sucrose gradient, it again sediments as one band at the 15S position (Fig. 1, left). On a neutral sucrose gradient the DNA shows an approximate sedimentation coefficient of 20S (Fig. 1, right). Using the equations determined by Studier (39), which relate the sedimentation coefficient of linear DNA to its molecular weight, we have estimated a molecular weight of approximately 1.5×10^6 for MVM DNA. This result agrees closely with the ear-

lier molecular weight determinations of Crawford et al. (13).

To obtain a more precise measurement of molecular weight and also examine more closely the homogeneity of the isolated MVM DNA, length measurements were taken on four different preparations using standard electron microscope procedures. A representative field of DNA molecules is shown in Fig. 2. Circular, single-stranded fd DNA was added to all samples as in internal length standard. The results of the length measurements are summarized in Table 1; a histogram of the complete length distribution of the DNA in preparation 1 is given in Fig. 3. No significant difference was found between the average lengths of the "full-length" molecules in the four different MVM samples when measured relative to the stand-

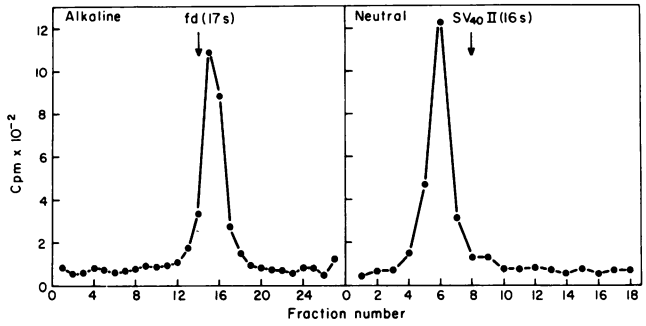

FIG. 1. *Sucrose gradient centrifugation of MVM DNA. The alkaline gradient (5 to 20% sucrose) contained 0.3 N NaOH, 0.7 M NaCl, and 0.15% Sarkosyl and was run for 4 h at 48,000 rpm (5°C) in a Beckman SW 50.1 rotor. The neutral gradient (5 to 20% sucrose) contained 1.0 M NaCl, 10 mM Tris-hydrochloride, and 1 mM EDTA, pH 7.5, and was run for 3.5 h at 48,000 rpm (5°C) in a Beckman SW50.1 rotor. fd and SV40 DNA markers were run on identical, parallel gradients.*

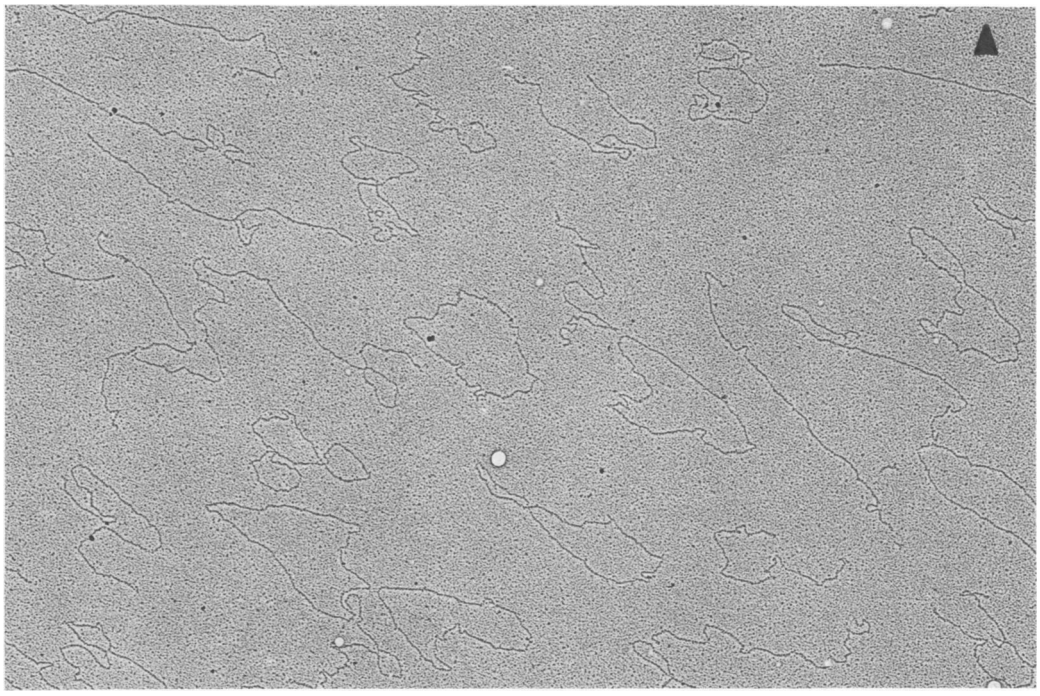

FIG. 2. *Electron micrograph of MVM DNA (linear molecules) with circular fd DNA added as a length standard. MVM DNA was isolated by alkaline sucrose gradient centrifugation. The entire band of DNA (see Fig. 1) was dialyzed into 10 mM Tris-hydrochloride-1 mM EDTA, pH 7.5, and mounted for electron microscopic analysis using the formamide spreading technique.*

TABLE 1. *Electron microscope measurements of MVM DNA*

Prep	Host cell line	No. of molecules measured	% Full-length molecules[a]	Avg length ($\pm\sigma$)[b]
I	A-9	92	70	0.77 ± 0.09
II	A-9	104	75	0.76 ± 0.05
III	A-9	56	82	0.76 ± 0.08
IV	EA	54	94	0.75 ± 0.09

[a] Full-length molecules were chosen as all molecules within ±15% of peak length. This is illustrated in Fig. 3 for preparation I DNA.

[b] Length is expressed as the fraction of the average length of the circular fd DNA molecules measured on the same grid. Only full-length MVM DNA molecules were included in these calculations.

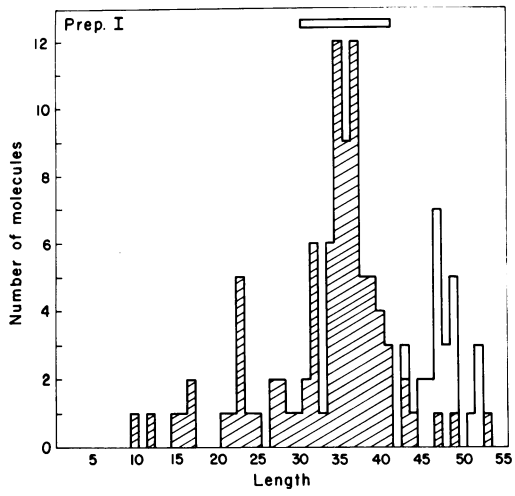

FIG. 3. *Histogram of the length distribution of MVM DNA. Preparation I (see Table 1) was used. The open bar above the main peak spans the range of lengths chosen to contain full-length molecules. The length distribution of circular fd DNA is shown without cross-hatching.*

ard fd DNA. Full-length molecules are defined as all molecules within ±15% of the peak length. Overall, full-length MVM DNA in the four preparations measured an average of 0.76 ± 0.08 fractional lengths of fd DNA. Based on the recent determination of 1.90×10^6 as the molecular weight of fd DNA (3), we have calculated an apparent molecular weight of $(1.44 \pm 0.15) \times 10^6$ for MVM DNA. Since we have determined that approximately 260 of the total nucleotides in MVM DNA exist in a double-stranded conformation (see below), an additional 0.04×10^6—the molecular weight of 130 nucleotides—should be added to the apparent molecular weight. Therefore, we estimate the molecular weight of MVM DNA to be 1.48×10^6.

Considerable size heterogeneity was observed in all MVM DNA preparations. Molecules smaller than those estimated to be full length constituted between 6 and 30% of the total molecules measured in the four different samples (Table 1). Furthermore, the standard deviation in the length measurements of the full-length molecules is about 11% of the average length of MVM DNA as compared to 5.5% for the fd DNA measurements. Although this implies that there is heterogeneity within the population of full-length molecules, the statistics are biased against MVM, since linear fragments of fd were not scored.

Furthermore, most of the viral DNA preparations examined in the electron microscope were found to contain a low percentage of linear, double-stranded molecules (approximately 1% of the total), which appeared to be somewhat shorter than single-stranded MVM DNA. It is not known if these full double-stranded molecules are viral or host in origin. However, they are unlikely to be randomly packaged, double-stranded host DNA, since the DNA isolation was performed under alkaline conditions, and the observed duplex DNA must be capable of fairly rapid reannealing. The presence of duplex viral DNA could arise as a result of a low frequency error in packaging the viral genome. Although AAVs are known to encapsidate in separate particles both plus and minus viral DNA strands with equal efficiency (6, 29, 33), MVM (and other nondefective parvoviruses) was thought to package only the plus DNA strand. However, virion DNA strand selection has never been convincingly demonstrated to the 1% level.

Configuration of MVM DNA. The defective AAVs contain a linear single-stranded DNA genome that is able to circularize due to an inverted terminal repetition of about 50 bases (5, 23). Consequently, it was of interest to determine whether MVM, as a representative of the nondefective subgroup, had a similar terminal nucleotide sequence arrangement. Separate samples of AAV and MVM DNA were simultaneously denatured by dialysis against the same solution of 95% formamide-5 mM EDTA, pH 8.2, at room temperature for 90 min and then allowed to reanneal by dialysis against 50% formamide-0.1 M Tris-0.01 M EDTA, pH 8.2, at room temperature for 2 to 48 h. Portions were

withdrawn from both DNA samples under the denaturing and renaturing conditions and immediately mounted for electron microscope examination as described above. Representative fields of the two DNAs under both conditions are shown in Fig. 4. As expected, the majority of the AAV DNA molecules formed panhandle-type circular structures when renatured (Fig. 4B). However, circular molecules were never observed with MVM DNA, even after 48 h of incubation in the 50% formamide solution. More than 500 MVM DNA molecules were scanned in these preparations.

Although both AAV and MVM DNA have about the same overall base composition (40% guanine plus cytosine) (27), it is possible that MVM DNA could have an inverted terminal redundancy which has a very low melting temperature. Therefore, the DNA could have remained linear under the renaturation or spreading conditions used. However, this is considered very unlikely, since virus disrupted by either mild heating (50°C) in 0.2% sodium dodecyl sulfate-0.1 M NaCl or incubation at room temperature in 30% formamide-0.1 M Tris, pH 8.7, releases DNA which, when free of capsid protein, always appears as linear molecules in the electron microscope when spread under the standard 30% formamide conditions (15). In addition, since the 5′ end of MVM DNA exists as a stable hairpin duplex (see below), base pairing between the 5′ and 3′ ends of the DNA would be extremely unlikely if the inverted terminal repetition was present within the hairpin.

Secondary structure of MVM DNA. (i) Evidence for a partially duplex genome. The existence of a small proportion of a stable, duplex DNA in the predominantly single-stranded MVM DNA has been established by three independent methods: (i) limit digestion with S1 endonuclease; (ii) hydroxyapatite chromatography; and (iii) ethidium bromide fluorimetry.

Limit S1 digestions were carried out on a number of different radioactive MVM DNA preparations. Two typical experiments are presented in Table 2 in comparison with limit S1 digests of single-stranded fd DNA and double-stranded λ DNA. The average S1 resistance for six different MVM DNA preparations is 6.5%, with a range of 4.6 to 8.7%. Two samples were also heat denatured and quenched to 0°C before digestion. In both cases, the S1 resistance dropped from about 8.5% before heat denaturation to about 6%, e.g., see MVM (II) in Table 2. This decrease in S1 resistance observed with some preparations after heat denaturation is most likely due to the presence of the small number of full double-stranded molecules in the preparation. It is also of interest that fd DNA, which we have found to be approximately 2.0% resistant to S1 digestion, is known to contain a "core" of double-stranded DNA estimated to represent 2.5% of the total fd nucleotides (35, 36).

Hydroxyapatite chromatography was the second method employed to demonstrate that a small portion of the MVM DNA molecule is in a double-stranded conformation. Completely single-stranded DNA elutes from hydroxyapatite below 0.12 M potassium phosphate buffer, pH 6.8, at 25°C, whereas double-stranded DNA as short as 17 base pairs requires at least 0.14 M KP_i for elution (47). Consequently, one would predict that MVM DNA should require at least 0.14 M KP_i for elution. As is shown in Fig. 5, this is, in fact, the case. A mixture of ^{32}P-labeled MVM DNA and ^{3}H-labeled fd DNA was applied to a small hydroxyapatite column and eluted with a shallow gradient of potassium phosphate buffer, pH 6.8 (0.08 to 0.20 M over 40 ml). The peaks of MVM DNA and fd DNA were found to elute at 0.146 M KP_i and 0.141 M KP_i, respectively. This small difference in elution position was reproducible with different preparations of MVM DNA. Therefore, both MVM and fd DNAs appear to contain a small amount of double-strand character. Since none of the MVM DNA eluted below 0.13 M KP_i, the results suggest that duplex structure is present in all molecules. However, it is not possible on the basis of these data to obtain an accurate estimate of the size of the duplex regions, since no data are available on the elution profiles of comparable DNAs (i.e., DNAs containing short double-strand regions of various sizes attached to relatively long single-strand regions). The fact that MVM DNA appears to contain a somewhat greater amount of double-stranded DNA than fd DNA is consistent with the previous S1 nuclease digestion results.

Finally, we have used ethidium bromide fluorimetry to obtain a direct, physical measurement of the percentage of double-strand DNA in several MVM preparations. This method relies on the fact that at pH 12, unstable, nonspecific, base-paired regions are eliminated, whereas stable, duplex DNA retains its double-stranded structure (12). A calibration curve for the increase in the fluorescence intensity of ethidium bromide ($I - I_0$) as a function of the concentration of double-stranded DNA is presented in Fig. 6. Single-stranded DNA does not produce an increase in the fluorescence, which requires the dye molecule to be intercalated into a double-helical region (12). The two arrows in Fig. 6 indicate the increases in fluorescence intensity obtained with one MVM

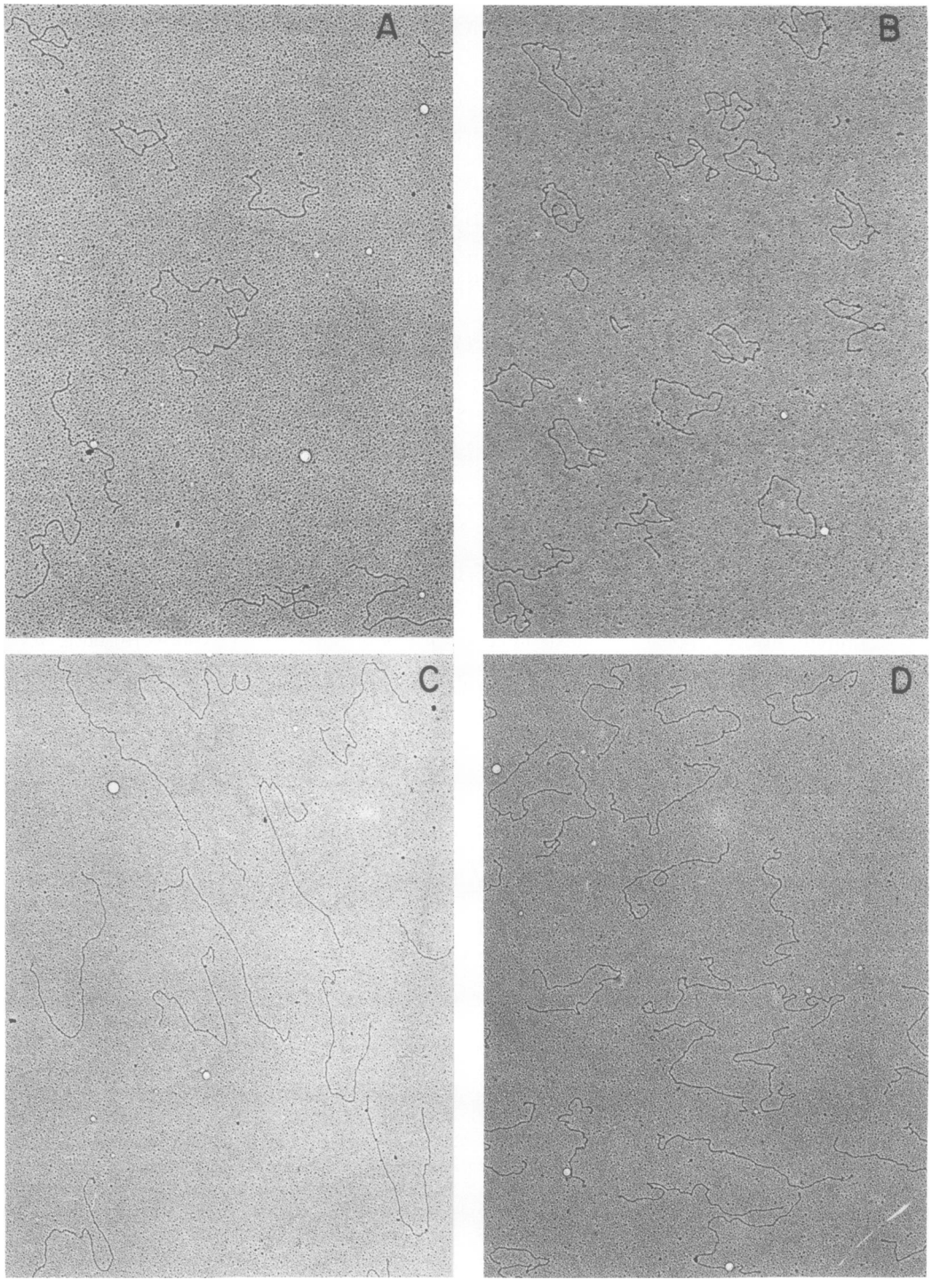

FIG. 4. *Electron micrographs of MVM and AAV DNA under denaturing and renaturing conditions. (A) Denatured AAV DNA; (B) renatured AAV DNA; (C) denatured MVM DNA; (D) renatured MVM DNA.*

TABLE 2. *Limit S1 nuclease digestion of MVM DNA*

DNA sample	Trichloroacetic acid-insoluble radioactivity (cpm) at:		% DNA S1 resistant
	0 min	120 min[a]	
^{32}P-labeled MVM (I)[b]	15,100	950	6.3
^{3}H-labeled fd	52,200	1,150	2.2
^{14}C-labeled λ	1,410	1,360	96.3
^{32}P-labeled MVM (II)	4,960	431	8.7
Denatured ^{32}P-labeled MVM (II)[c]	5,030	305	6.1

[a] These values represent limit digests as determined kinetically. Essentially the same degree of digestion was observed by 90 min of incubation.

[b] Roman numerals denote different DNA preparations.

[c] Denaturation was obtained by incubating the DNA in TE 7.5 at 100°C for 5 min followed by immediate quenching to 0°C.

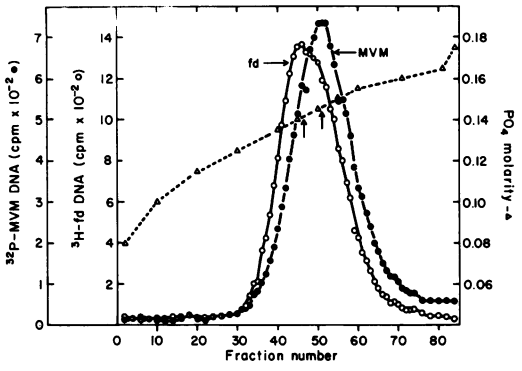

FIG. 5. *Hydroxyapatite chromatography of a mixture of ^{3}H-labeled fd DNA and ^{32}P-labeled MVM DNA. The two arrows indicate the phosphate molarity at the peak elution positions of the two samples.*

DNA sample and with fd DNA. A summary of the results obtained with three different MVM preparations is given in Table 3. From these data, we estimate that approximately 3% (±1%) of the nucleotides in MVM DNA exist in a duplex conformation at pH 12. MVM DNA (III) appears to be free of any heat-denaturable double-stranded DNA, in contrast to the two preparations tested by incubation with S1 nuclease (Table 2). Clearly, fd DNA produces no detectable increase in fluorescence intensity, indicating that its duplex regions are not stable in the pH 12 buffer. This probably is due to their small size and/or mismatched base pairs (35).

(ii) Location of the double-stranded region in MVM DNA. To determine whether the stable, double-stranded DNA is localized in only one region of the MVM genome, the size of the S1 nuclease-resistant material was measured by velocity sedimentation and by gel electrophoresis. On neutral sucrose gradients the limit S1 digestion product sedimented as one band with an average sedimentation coefficient of about 5S. This corresponds to a piece of DNA

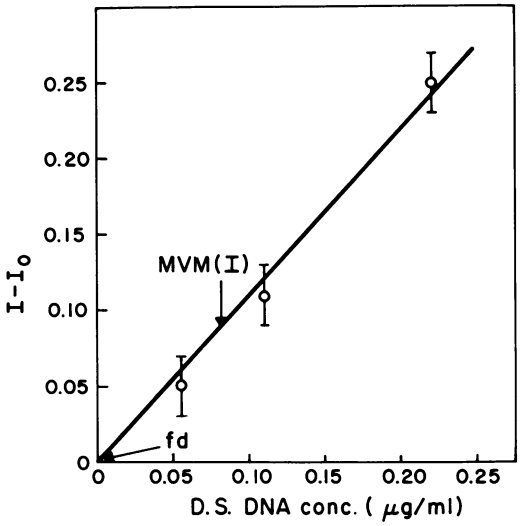

FIG. 6. *Ethidium bromide fluorimetry analysis of the amount of double strandedness in MVM DNA at pH 12. $I - I_0$ is the increase in fluorescence intensity given in arbitrary units. The straight line calibration plot was determined using known concentrations of pure double-stranded lambda DNA. MVM (I) refers to a particular MVM DNA sample (see Table 3). No double-stranded DNA was detected in fd DNA at pH 12.*

TABLE 3. *Measurements of the amounts of double strandedness in MVM DNA by ethidium bromide fluorimetry*

DNA sample	Total DNA concn[a] (μg/ml)	Double-stranded DNA concn[b] (μg/ml)	% Double-stranded DNA
MVM (I)[c]	2.27	0.08	3.5
MVM (II)	7.34	0.15	2.1
MVM (III)	2.02	0.07	3.4
Denatured MVM (III)[d]	2.02	0.07	3.4
fd	3.25	0.00	0.00

[a] DNA concentration was measured spectrophotometrically; 1.0 optical density unit at 260 nm/ml for single-stranded DNA was taken as 36 μg/ml (38).

[b] Taken from calibration curve shown in Fig. 8.

[c] Roman numerals denote different DNA preparations.

[d] Denatured as described in Table 2, footnote *c*.

containing approximately 100 base pairs. A more accurate estimate of the size of this material was obtained by comparing its electrophoretic mobility with that of fragments of SV40 DNA produced by treatment with *Hae* III restriction endonuclease. An autoradiogram of such an acrylamide gradient gel (Fig. 7) shows that the S1 nuclease-resistant DNA is somewhat smaller than the SV40 *Hae* H fragment, which contains about 165 base pairs (24, 40). From the straight line calibration plot presented in Fig. 8, it is estimated that the limit S1 digestion product is a piece of DNA with an average size of 130 ± 20 base pairs. This represents approximately 6.0% of the total number of bases (~4,400) in MVM DNA. Since the percentage of MVM DNA resistant to S1 digestion is also 6%, essentially all of the double-stranded DNA must occur in one segment of the MVM genome. We believe that the second band (see arrow in Fig. 7), which occurred only in the undigested sample and the nondenatured, S1-treated sample, corresponds to the fully double-stranded DNA molecules observed at low frequency in the electron microscope.

As a first step in localizing the duplex region within the MVM DNA molecule, we tested the viral DNA for its susceptibility to digestion by exonuclease I, an enzyme which attacks single-stranded DNA from the 3′ end of the polymer chain. Since previous studies (34, 43) had shown that Kilham rat virus DNA and MVM DNA were extensively degraded by exonuclease I, a large majority, if not all, of the double-stranded DNA should be near the 5′ end of the MVM genome. This expectation is confirmed by the results given in Table 4: for a typical MVM DNA preparation, approximately 4 to 5% of the input DNA remained trichloroacetic acid insoluble after a limit digestion with exonuclease I. This percentage resistance is clearly very close to that obtained by S1 nuclease digestion and to the estimate of duplex DNA obtained in the ethidium bromide experiments.

To determine whether the 5′-terminal nucleotide exists in a stable duplex conformation, the 5′ end of ^{3}H-labeled MVM DNA was labeled with $^{32}PO_4$ using the standard procedures of alkaline phosphatase treatment followed by polynucleotide kinase and γ-[^{32}P]ATP (see above). Approximately 61% of the input MVM DNA was terminally labeled with ^{32}P. After reisolation from alkaline sucrose gradients, the ^{32}P- and ^{3}H-labeled MVM DNA was digested with S1 nuclease. If the 5′ terminus is in a double-stranded conformation, then the ^{32}P counts should remain resistant to enzymatic digestion. The data in Fig. 9 shows that although 90% of the ^{3}H radiolabel becomes acid soluble in 60 min, the majority (80%) of the terminal ^{32}P label remains acid precipitable. The observed ^{32}P solubilization may be due to fragmented molecules lacking the duplex structure at their 5′ terminus. Digestion due to "breathing" of the terminal duplex appears unlikely, since all of the ^{32}P loss occurs within the first 15 min of the 1-h reaction. This result, indicating the presence of a hairpin duplex at the 5′ terminus of the MVM genome, is further supported by the DNA ligase experiments described below.

In vitro-synthesized double-stranded MVM DNA. The observation that some of the double-stranded DNA intermediates in the replication of MVM (43) and AAV (Sebring et al., Fed. Proc. 34:639, 1975) renatured spontaneously in a monomolecular fashion suggested the presence of a covalent linkage between viral and complementary strands. It was proposed by Tattersall et al. (43) that such intermediates might be formed in vivo by using the 3′ end of the viral strand as a primer for the synthesis of the complementary strand. In these studies we have determined that purified MVM DNA functions as an efficient template-primer in vitro using several different DNA polymerases: *E. coli* DNA polymerase I (both the holoenzyme and the proteolytic fragment lacking the 5′ to 3′ exonuclease activity), T4 bacteriophage DNA polymerase, and avian myeloblastosis and Rauscher murine leukemia virus reverse transcriptases. All of the polymerases tested synthesize almost one complete copy of the template MVM DNA, except for the *E. coli* DNA polymerase I (holoenzyme), which is able to carry out net synthesis, as expected.

In all of the experiments described below, the *E. coli* DNA polymerase I fragment lacking the 5′ to 3′ exonuclease activity was used to synthesize double-stranded MVM DNA. The time course and extent of synthesis for a typical reaction is shown in Fig. 10. The amount of complementary strand synthesized was normally between 92 and 100% of the input viral DNA, the average of six independent experiments being 95.6%.

The product of the polymerization reaction was first analyzed by sedimentation on alkaline sucrose gradients (Fig. 11). Since both the template DNA and the synthesized DNA co-sediment at the position of ~20*S*, it is clear that the complementary strand is covalently attached to the template DNA and that the product has about twice the molecular weight (~3×10^6) of the input MVM DNA. This double-stranded DNA was further examined by electron microscopy and found to be essentially all linear, duplex DNA molecules. No terminal loops or sin-

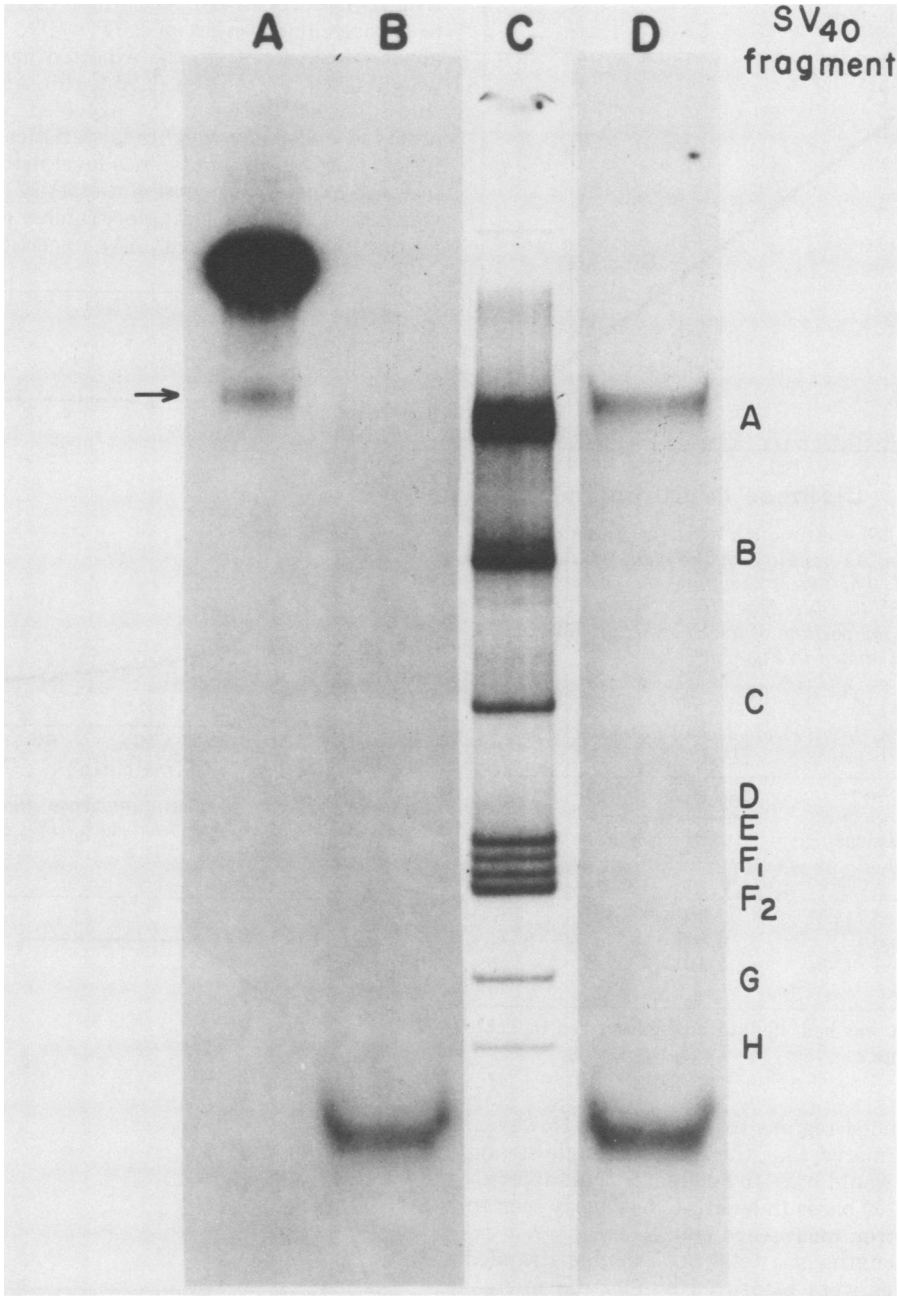

FIG. 7. *Polyacrylamide gradient gel electrophoresis of ^{32}P-labeled MVM DNA before and after digestion with S1 endonuclease. (A) MVM DNA before digestion; (B) MVM DNA after heat denaturation (100°C, 5 min), quick cooling, and then digestion with S1 endonuclease; (C) ^{32}P-labeled SV40 DNA digested with Hae III restriction endonuclease; (D) MVM DNA digested with S1 endonuclease without prior heat denaturation. The arrow indicates the gel position of double-stranded DNA (see text).*

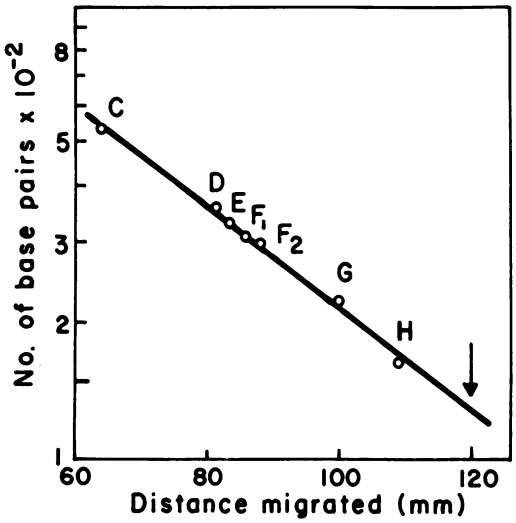

FIG. 8. *Size estimation of the S1-resistant MVM DNA from its mobility on the polyacrylamide gradient cell. The letters designate the positions of the various SV40 DNA Hae III fragments. The arrow indicates the position of the S1-resistant MVM DNA on the gel shown in Fig. 7.*

TABLE 4. *Limit exonuclease I digestion of MVM DNA*

DNA sample	Trichloroacetic acid-insoluble radioactivity (cpm) at:		% DNA exonuclease I resistant
	0 min	120 min	
^{32}P-labeled MVM	12,300	564	4.6
^{14}C-labeled λ	4,480	4,370	97.5
Denatured ^{14}C-labeled λ[a]	2,510	29	1.2

[a] DNA was heat denatured as described in Table 2, footnote *c*.

gle-stranded regions were apparent. However, it is estimated that the loops or single-strand regions would have to be greater than approximately 100 bases in length to be clearly seen in the electron microscope (15).

The lengths of a total of 77 double-stranded molecules were measured by electron microscopy using φX174 RFII DNA (molecular weight, 3.17 × 10^6) (3) as an internal standard. The standard deviation in the length measurements of the double-stranded MVM DNA molecules was somewhat greater than that found with the φX174 DNA molecules (8% versus 5.5%). This indication of heterogeneity was expected, since it was also observed in all single-stranded MVM DNA preparations. The average length was found to be 0.86 times the length of φX174 RFII DNA, which corresponds to a molecular weight of 2.74 × 10^6. This is about 4% shorter than the expected molecular weight of 2× MVM DNA (2.96 × 10^6). Although the difference between the expected and the observed molecular weights is statistically significant at the 99% confidence level using the *t* test, the molecular weight of the MVM genome was determined using single-stranded fd DNA as the internal standard. Any uncertainty in

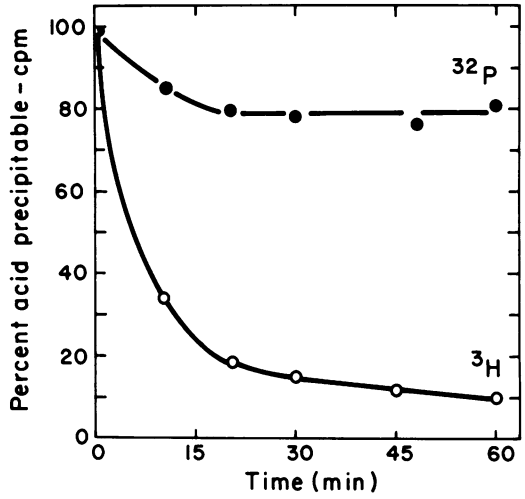

FIG. 9. *Kinetics of S1 endonuclease digestion of ^{3}H-labeled MVM DNA labeled with $^{32}PO_4$ at the 5'-terminal nucleotide.*

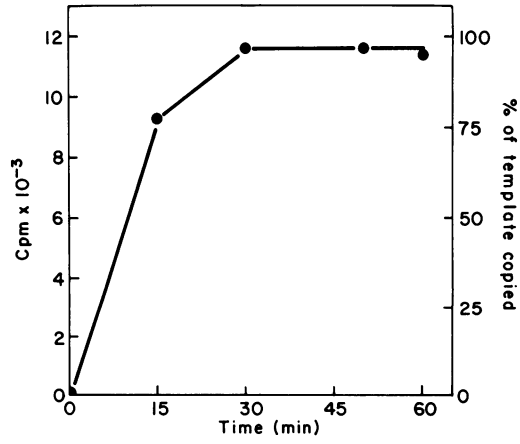

FIG. 10. *In vitro synthesis of double-stranded MVM DNA. E. coli DNA polymerase I fragment lacking the 5' to 3' exonuclease was used with purified MVM DNA as the template-primer. The percentage of template copied was calculated from the amount of input MVM DNA, the known base composition of the viral genome (13), and the specific activity of [^{3}H]TTP used in the polymerization reaction (see Materials and Methods).*

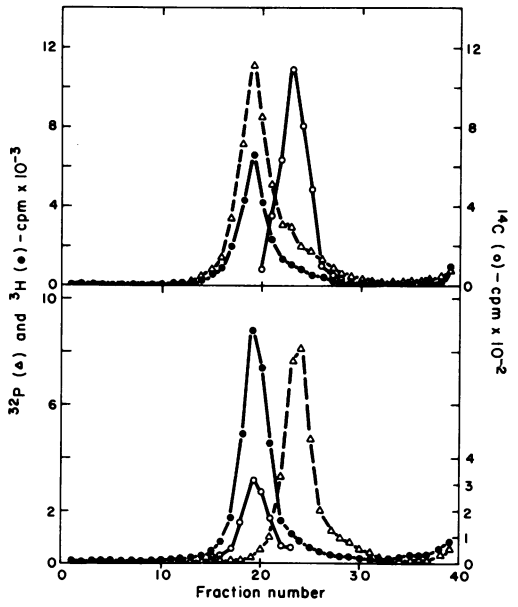

FIG. 11. *Alkaline sucrose sedimentation analysis of the product of the in vitro DNA polymerase I reaction. (A) ^{32}P-labeled MVM DNA was used as the template-primer, and the DNA polymerase reaction was done with [^{3}H]TTP as the radiolabeled substrate. The reaction was terminated after 90 min by the addition of EDTA (to 0.02 M), and ^{14}C-labeled MVM DNA marker was added. The entire reaction mixture was centrifuged on an alkaline sucrose gradient, and the amount of trichloroacetic acid-insoluble radioactivity in each fraction was determined (see Materials and Methods). (B) Reactions were run as described for (A) except that the MVM DNAs used were reversed; ^{14}C-labeled MVM DNA was used as the template-primer and ^{32}P-labeled MVM DNA was used as the marker.*

the molecular weight relationship between ϕX174 RFII DNA and fd DNA could, therefore, influence such a statistical analysis. In addition, 4% shortening due to looping back of the 3′ end to form a template-primer complex with the DNA polymerase would give a single-strand terminal loop of approximately 160 bases, which should be visible in the electron microscope.

Even though MVM DNA serves as a template-primer for several DNA polymerases, the 3′ end of the genome most likely does not exist in a stable duplex conformation, since the viral DNA is almost completely degraded by exonuclease I. Therefore, the 3′ terminus must form part of an unstable or transiently base-paired structure that is stabilized by the binding of the polymerase and thereby is able to function as a primer for DNA synthesis. In this regard, Goulian et al. (18) have observed that linear, single-strand M13 DNA will serve as a template-primer for T4 DNA polymerase and proposed that any single-stranded DNAs could, by looping back their 3′ end, form an unstable duplex region suitable for priming of DNA synthesis. We have also found that linear fragments of MVM DNA or fd DNA, produced by brief treatment with S1 nuclease, also function as template-primers (data not shown). However, examination of these in vitro-synthesized double-stranded molecules in the electron microscope showed that some molecules contained terminal single-strand loops. This is in contrast to the molecules synthesized using full-length MVM DNA as a template-primer, which never showed such loops. Consequently, we propose that the 3′ end of MVM DNA contains a specific hairpin duplex that is relatively unstable in vitro but that could provide the priming activity necessary to generate the spontaneously renaturing duplex MVM DNA molecules observed in vivo.

Restriction nuclease cleavage of double-stranded MVM DNA. The double-stranded MVM DNA synthesized in vitro was treated with *Eco*R·RI restriction endonuclease, and the products were analyzed on a 1.4% agarose gel (Fig. 12). Only three distinct bands were found. Analysis of the sample on a 3.5 to 7.5% acrylamide gradient gel revealed no other DNA fragments (data not shown). The smallest piece of DNA detected on this gel would be about 100 base pairs. Considering the size of the three fragments (relative to the SV40 *Hae* III A, B, and C fragments) and their relative radioactivity content, it is clear that they exist in molar amounts (Table 5). Therefore, we conclude that MVM DNA must contain a unique, nonpermuted sequence of nucleotides that is cleaved twice by the *Eco*R·RI enzyme. The possibility of a limited terminal permutation such as has been seen for AAV DNA cannot, however, be completely ruled out. Preliminary experiments with the *Hae* III restriction nuclease indicate that it cleaves double-stranded MVM DNA five times, producing six fragments ranging in size from 0.1×10^6 to 1.4×10^6 daltons. A more complete analysis of restriction enzyme cleavage patterns will be presented elsewhere.

Ligation of double-stranded MVM DNA. The double-stranded MVM DNA made in vitro, because of the hairpin duplex at the 5′ terminus of the viral genome, should contain an internal nick suitable for sealing by DNA ligase. The ligated product, under denaturing conditions a single-stranded circular DNA, will sediment approximately 10% faster in alkaline sucrose

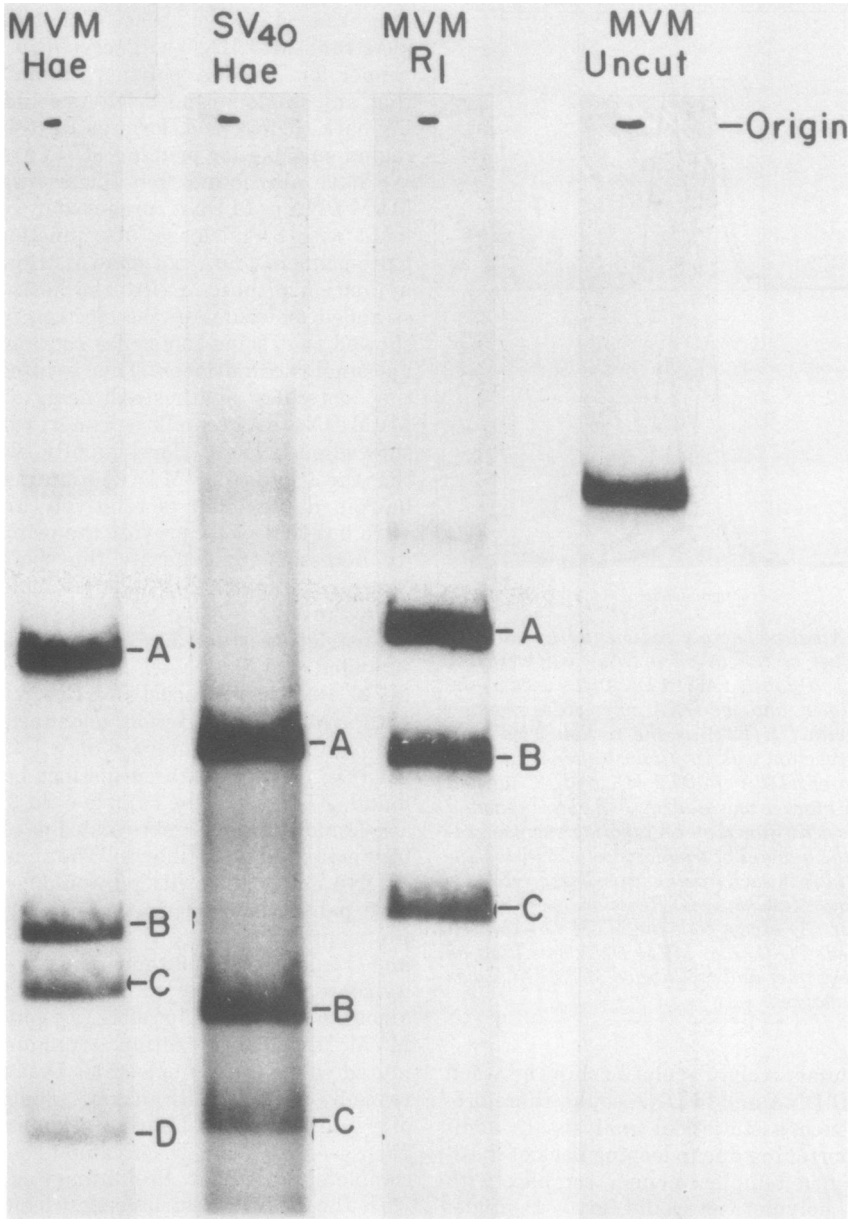

FIG. 12. *Agarose gel electrophoresis of duplex MVM DNA fragments generated by cleavage with EcoR·RI and Hae III restriction endonucleases. Unlabeled MVM DNA was used as the template-primer, and [^{32}P]TTP was the radioactively labeled substrate in the synthesis of duplex MVM DNA.*

than nonligated, linear DNA (44). As shown in Fig. 13, when duplex MVM DNA is prepared in the presence of DNA ligase, a significant proportion of the sample (about 36%) sediments with an *S* value (22S) 10% greater than that of duplex DNA prepared in the absence of DNA ligase (20*S*). However, the extent of ligation observed with different preparations of MVM DNA varied considerably and was never quantitative. Possible explanations for this variability are: (i) strand displacement of the 5′ hairpin by the polymerase before ligase seals the nick; and (ii) dephosphorylation or "blocking" of the 5′-terminal nucleotide in some of the DNA molecules, making them nonligatable.

Additional experimental evidence demon-

strating that duplex MVM DNA can be ligated was obtained using MVM DNA labeled in the 5'-terminal nucleotide with $^{32}PO_4$ (see above) as a template-primer for DNA polymerase both in the presence and absence of DNA ligase. Although over 98% of the ^{32}P was removed from the ligase-free polymerase product when subjected to alkaline phosphatase treatment at 65°C for 30 min, 41, 57, and 72% of the ^{32}P remained acid precipitable (three separate experiments) in the ligase-treated DNA.

DISCUSSION

Electron microscope analysis of several different preparations of MVM DNA has shown that the majority of the molecules have a molecular weight of 1.48×10^6. This is in excellent agreement with earlier estimates of the size on MVM DNA (13) and is similar to that of other parvoviruses, which all appear to contain genomes between 1.4×10^6 and 1.7×10^6 daltons (32). MVM DNA, free of capsid protein, is always found as a linear molecule even when isolated under mild, nondenaturing conditions. It does not form the circular panhandle structures as does AAV DNA under renaturing conditions. Consequently, there is clearly a difference in the types of terminal sequences which MVM and AAV DNA contain.

TABLE 5. *EcoR·RI fragments of double-stranded MVM DNA*

Fragment[a]	No. of base pairs[b]	% Total base pairs	cpm[c]	% Total cpm
A	2,300	48.1	1,750	51.4
B	1,500	31.4	980	28.8
C	980	20.5	670	19.8

[a] Fragments are labeled as shown in Fig. 12.

[b] Number of base pairs was determined from the position of bands relative to the three largest simian virus 40 *Hae* III fragments (A-C) on the same gel.

[c] The dried gel was cut up into 0.5-cm segments and counted directly in a toluene-based scintillation fluid. The values given are totals for each band.

Although MVM DNA is predominantly single stranded, a small proportion of the genome has been shown, by hydroxyapatite chromatography and ethidium bromide fluorescence, to be double stranded. Approximately 6% of the DNA (~260 nucleotides) is resistant to digestion by either S1 endonuclease or exonuclease I. Analysis of the product of the S1 limit digest on polyacrylamide gradient gels yields a single band of 130 ± 20 bases pairs, indicating that essentially all of the duplex structure is located in one region of the genome. The fact that exonuclease I digests MVM DNA to the same extent as S1 endonuclease (94 to 95%) implies that the duplex region is located at the 5' terminus. Direct proof for this location was obtained by digesting MVM DNA, which was terminally labeled at the 5' end with $^{32}PO_4$, with S1 endonuclease. Since a large majority (80%) of the ^{32}P radioactivity is not rendered acid soluble, the 5' terminus must be in a duplex conformation and resistant to S1. Further confirmation of a duplex structure at the 5' terminus was provided by the ability to join both ends of the in vitro-synthesized double-stranded MVM molecule by DNA ligase. Preliminary results with DNA isolated from other nondefective parvoviruses (H-1, H-3, and Kilham rat virus) indicate that they also contain a stable hairpin duplex of about 130 base pairs at their 5' terminus (M. B. Chow and D. C. Ward, unpublished observations).

Since subtilisin-fragmented *E. coli* DNA polymerase I is, unlike T4 DNA polymerase, capable of undergoing nick translation or strand displacement synthesis (28), the observed vari-

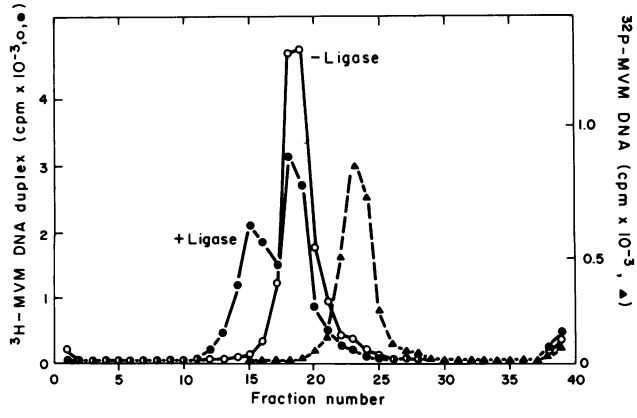

FIG. 13. *Alkaline sucrose sedimentation analysis of the double-stranded MVM DNA made in the presence of DNA polymerase I (lacking the 5' to 3' exonuclease) with and without added T_4 DNA ligase.*

ability in the ligation of MVM DNA duplexes may merely reflect the relative efficiency of these two competing processes under the various incubation conditions studied. Alternative possibilities, which cannot be excluded, are that the 5′ end of the MVM genome is either dephosphorylated or "blocked." The 5′ termini of adenovirus DNA is known to be linked to a viral protein (31). Although the 5′ terminal nucleotide of MVM DNA can be labeled with $^{32}PO_4$ by polynucleotide kinase after phosphatase treatment, unlike the 5′ end of adenovirus DNA (R. Roberts, personal communication), the extent of phosphorylation has always been considerably less than quantitative. Since our standard isolation procedure for MVM DNA employs sedimentation through alkaline sucrose gradients, and since the alkaline stability of the adenovirus DNA-protein complex has not been established (R. Roberts, personal communication), it is quite possible that the ability to ligate (or phosphorylate) MVM DNA may depend upon the length of time that the DNA preparation is exposed to alkaline conditions. Although the effect of alkali on the efficiency of ligation has not been examined, it is of interest to note that when MVM virions are disrupted at room temperature in 30% formamide–0.1 M Tris-hydrochloride, pH 8.7, and examined directly by electron microscopy, a significant number of the capsids remain associated with the released DNA. As shown in Fig. 14, these capsids are preferentially bound to terminal regions of the DNA molecules, in most cases one capsid per genome. Should an individual viral polypeptide(s) behave similarly, such an interaction could account for the observed variability in the ligase and kinase experiments.

Because MVM DNA is able to function as a primer-template for a number of DNA polymerases, yet is also extensively degraded by exonuclease I, the 3′ terminus is most likely part of

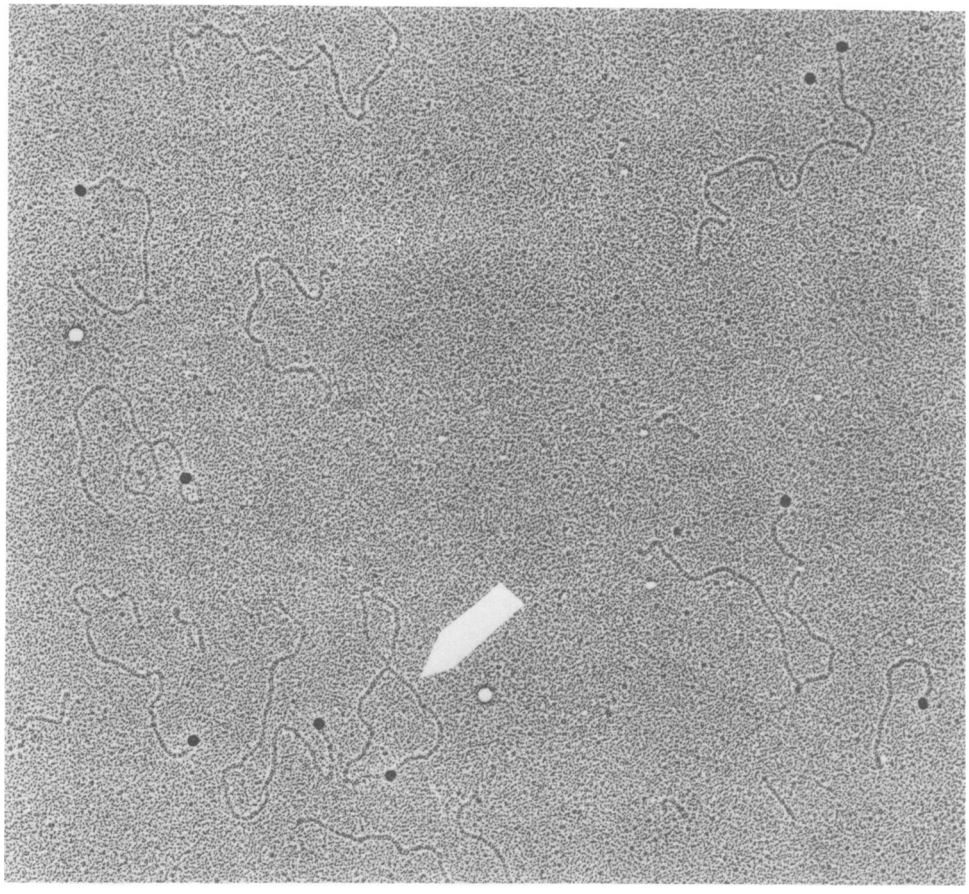

FIG. 14. *Electron micrograph of MVM virions after treatment at room temperature in 30% formamide–0.1 M Tris-hydrochloride buffer, pH 8.7. The arrow indicates one molecule of MVM DNA which has both its termini associated with the capsid.*

an unstable transiently base-paired structure. Although Goulian et al. (18) have proposed that any single-stranded DNA can prime in vitro DNA synthesis by looping back of the 3′ terminus, several observations suggest that the priming activity of MVM DNA may not be just a random phenomenon. The duplex DNA product shows no evidence of terminal single-stranded loops, unlike duplexes prepared from fragmented MVM DNA. Cleavage of the double-stranded MVM DNA molecule by *Eco*R·RI restriction endonuclease yields three discrete fragments in molar amounts, indicating that the MVM DNA contains a unique, rather than a permuted, sequence of nucleotides. In addition, viral reverse transcriptases, which do not exhibit either 3′ → 5′ or 5′ → 3′ exonucleolytic activity against DNA (2, 20), give duplex products that are indistinguishable from those prepared using DNA polymerases (*E. coli* and T4), which contain a 3′ → 5′ exonuclease. This suggests that the 3′ terminus is hydrogen bonded under the polymerase reaction conditions and does not contain a non-hydrogen-bonded "tail" that must be digested by the 3′ → 5′ exonuclease prior to initiating DNA synthesis.

It is of obvious interest to determine whether the first replicative intermediate in vivo has the same structure as the double-stranded molecule synthesized in vitro, and studies along this line are in progress. The roles that the 5′ terminal hairpin duplex and the self-priming ability of the 3′ terminus play in MVM DNA replication will require further examination of the structure of the replicative intermediates formed throughout the virus growth cycle.

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