Physicochemical Characterization of Adeno-associated Satellite Virus Type 4 and Its Nucleic Acid

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Based on protein, deoxyribonucleic acid (DNA), and phosphorus analyses, adeno-associated satellite virus type 4 has a DNA content of 26.5%, significantly larger than type 1 which contains 18.9% DNA. Type 4 DNA contains 58 to 62% guanine + cytosine, as calculated from thermal denaturation temperature and buoyant density measurements. Simian adenovirus SV15, which serves as a helper virus for type 4 satellite, contains 13.7% DNA with a guanine + cytosine content of 55 to 57%. The DNA extracted from type 4 satellite virus is a linear, double-stranded molecule, as shown by thermal denaturation temperature profile measurements and by electron microscopy. The contour length and sedimentation coefficient of type 4 DNA indicate a molecular weight of 3.0×10^6 daltons.

The similarity between the defective adeno-associated satellite viruses and the defective ribonucleic acid (RNA)-containing tobacco necrosis satellite virus (10) has been recognized (16). The plant satellite virus is defective presumably because of a paucity of nucleic acid (21) and the adeno-associated satellite viruses may possibly have a similar mechanism for their defectiveness. Rose et al. (22), however, recently reported that type 1 satellite virus deoxyribonucleic acid (DNA) has a molecular weight of 3.6×10^6 daltons, in the same size range as papovavirus DNA. They also demonstrated that the DNA was double-stranded with a guanine plus cytosine (G + C) content of 54.2%.

Type 4 satellite virus differs from the other currently known serotypes in having a higher buoyant density (1.43 g/cc compared to 1.38 g/cc) in cesium chloride (19). Type 4 also behaves as though it contains a single-stranded DNA by acridine-orange staining of purified whole virion (16). Characterization of type 4 satellite virus nucleic acid may help to clarify the relationship between these defective satellite virus serotypes and to establish the strandedness of the nucleic acid of a denser member of the satellite group. We report here the results of studies on the chemical composition of purified type 4 adeno-associated satellite virus and the base composition, molecular weight, and conformation of its nucleic acid.

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MATERIALS AND METHODS

Viruses and cells. The origins and adenovirus helpers of satellite virus serotypes 1 and 4 have been described (19). Virus stocks were prepared in primary African green monkey kidney cells growing in 16-oz (0.045-liter) prescription bottles and maintained in M-E medium (18). When viral cytopathic effect involved the entire cell monolayer, the virus was harvested by freezing at -35°C and rapid-thawing at 37°C . A preliminary purification was accomplished by mixing equal volumes of a fluorocarbon (Genetron 113 or Freon 113, trifluorotrichloroethane) and the virus stock in a Sorvall high-speed omnimixer at 4°C . Concentration of the satellite virus was then carried out by centrifugation in a Spinco 30 rotor for at least 3 hr at $78,000 \times g$. The virus was then purified by repeated equilibrium density gradient centrifugation in CsCl as previously described (19). Virions purified in this manner had a particle-to-infectivity ratio of less than 10, using methods already described (8, 19). Concentration and purification of simian adenovirus SV15(0) was performed as described by Green and Piña (4) for human adenoviruses.

Isolation of nucleic acids. Virus stocks which had been twice-banded to equilibrium in cesium chloride were used for nucleic acid isolation. The procedure described by Green and Piña (4) for adenovirus was used for SV15 and for satellite virus. As previously reported, high yields of adenovirus DNA were obtained by using this procedure; however, satellite virus DNA yields were only 30 to 50%, significantly less than the yields from adenovirus (80 to 90%). The 260:280 absorbancy ratio for these DNA preparations ranged from 1.6 to 1.9.

Chemical determinations. The DNA and protein content of purified virus preparations was determined by using micromodifications of the procedures of

Burton (1) and Lowry et al. (13). Calf thymus DNA and bovine serum albumin were employed as standards. A microdetermination of phosphorus (20) was also used to measure DNA content.

Determination of base composition. Procedures for thermal denaturation temperature and buoyant density measurements on viral DNA have been previously described (4, 20).

Molecular weight determinations of viral DNA. Protein film electron microscopy and analytical zone centrifugation were used to determine the molecular weight of satellite virus DNA. Electron microscopy of DNA molecules was performed by the Kleinschmidt technique (11). An instrumental magnification of 10,000 $\times$ was employed. Lengths of DNA molecules were measured on photographic enlargements with a map measure, and total magnification was determined from a replica grating.

$S_{20,w}$ values of native and alkali-denatured DNA were obtained by analytical zone centrifugation (26), as recently described (5), and these values were used to calculate the molecular weight of type 4 DNA (25).

RESULTS

Chemical composition of the virion. Table 1 shows the results of protein, DNA, and phosphorus determinations of purified types 1 and 4 satellite virus and of simian adenovirus SV15. Type 1 was found to contain 18.9% DNA. Multiple analyses of type 4 virus gave an average value of 26.5% DNA. SV15, which has a buoyant density typical of adenoviruses (1.345 g/cc), has a value of 13.7% DNA.

The purity of virus preparations employed for

these determinations was established by the following criteria: (i) each preparation was found to contain either satellite virions or adenovirions, but not both, when examined by electron microscopy; (ii) each preparation reacted only with homologous virus antiserum and not with anti-host cell material as determined by complement-fixation; (iii) each preparation sedimented as a single ultraviolet light-absorbing component in the analytical ultracentrifuge; and (iv) each preparation had a phosphorus (P) content in close agreement with the DNA content (DNA contains close to 10% P), thus indicating that RNA and phospholipid were absent. The DNA content for type 1 satellite virus and SV15, as determined by these methods, agreed with the estimates from the buoyant density of the virions in cesium chloride (28, 31). Type 4, however, has a DNA content of 26.5% as determined in the present study in contrast to an estimated value of about 37% from its buoyant density.

Base composition of viral nuclei acid. The base composition of type 4 satellite virus DNA was determined both by buoyant density measurements in cesium chloride and by thermal denaturation temperature measurements. Repeated determinations for type 4 DNA gave a value of 1.721 g/cc, which corresponds to guanine + cytosine (G + C) content of 62% (23). These values were reproducible to within 0.001 g/cc.

TABLE 1. Chemical analyses of purified satellite viruses types 1 and 4 and simian adenovirus, SV15

Prepn	Protein (μ g/ml)	DNA (μ g/ml)	Phosphorus (μ g/ml)	Per cent DNA ^a
Satellite virus type 1				
1	140.4	38.7	3.75	20.4
2	31.8	6.1	0.54	16.1
3	290.8	73.6	8.1	20.2
Avg				18.9 (± 1.4)
Satellite virus type 4				
1	295	101	10.1	25.6
2	114	45	4.5	28.2
3	207	62.4	5.2	23.2
4	482	155	15.5	24.3
5	564	179	15.1	24.0
6	360	121	13.3	25.2
Avg				26.5 (± 1.0) ^b
Simian adenovirus SV15				
1	56.7	9.4	—	14.1
2	1048	161	—	13.3
Avg				13.7 (± 0.4)

^a Percentage of DNA based on DNA measurements.

^b Average of 17 separate preparations.

Only one narrow band of viral DNA, a further indication of purity, was found.

The buoyant density of SV15 DNA was found to be 1.717 g/cc, a value significantly lower than that of its satellite type 4 DNA. This value corresponds to a G + C content of 58%. Type 1 satellite virus DNA and helper adenovirus type 7 were reported to have considerably lower G + C contents, 54.2% by direct analysis and 49 to 50%, respectively (22).

A further measurement of base composition (15), as well as strandedness of DNA, is the thermal denaturation temperature (T_m). The melting profile of type 4 satellite virus DNA is shown in Fig. 1. T_m values ranged from 92 to 94.5 C, with an average of 93.0 C, corresponding to a G + C content of 58%. The wide range of T_m values for different DNA preparations is not understood.

The thermal dissociation profiles were characteristic of double-stranded DNA. The discrepancy in the G + C content as measured by buoyant density (62%) and by T_m (58%) is similar to that reported for type 1 satellite virus (22) and the weakly oncogenic human adenoviruses (4), but the significance of this is not known. The T_m values for adenovirus SV15 DNA averaged 92.0 C (± 1.0), which indicated a G + C content of 55.4%. This value is in fair agreement with the 57.7% G + C content determined by buoyant density measurements.

FIG. 1. Melting profile of type 4 adeno-associated satellite virus DNA (15 μ g per ml) in standard sodium citrate (0.15 M sodium chloride plus 0.015 M sodium citrate, pH 7.4). Readings were made with a thermostatically controlled, continuously recording Gilford spectrophotometer set for reading absorbancy at 260 mμ. Adenovirus type 2 or 4 DNA was always included as a control DNA for T_m determinations.

FIG. 2. Histogram of molecular lengths of type 4 satellite virus DNA as measured by electron microscopy. The lengths of all DNA structures (total of 114 molecules) were measured and are shown. Five other preparations examined gave very similar results. As described in the text, the very short fragments 0.09 to 0.55 μ appear to be artifacts of preparation.

Molecular length of type 4 satellite DNA by electron microscopy. Extracted type 4 satellite DNA was examined by the Kleinschmidt technique (11) in the electron microscope. A histogram for one of several preparations examined, comparing the relative incidence of DNA molecules of various contour lengths, is shown in Fig. 2. Two main distributions of DNA strands were found, 1.4 to 1.6 μ and 0.1 to 0.3 μ . There were few strands of intermediate length. We assume that the long strands are unbroken DNA molecules and that the short strands resulted from breakage during preparation for electron microscopy (5).

The length of the longer strands was relatively constant in numerous preparations and averaged $1.5 \pm 0.2 \mu$. Using a value of 192 daltons per A, a value characteristic of the B-form of DNA (14, 30), a value of $2.9 (\pm 0.4) \times 10^6$ daltons was obtained for the molecular weight of type 4 satellite DNA. Recent reports have emphasized the influence of ionic concentrations on the contour lengths of DNA molecules (7, 12). However, studies with adenovirus DNA preparations, using the same conditions employed in the present work, gave molecular weights in excellent agreement with values derived from sedimentation coefficients (5).

Examples of the unfragmented type 4 satellite DNA molecules are shown in Fig. 3. Although some rosettes were noted (not shown), all extended strands were linear, and no circular forms were noted. The folds and turns of the molecules are not sharp and the molecules are not hypercoiled. Denatured (single-stranded)

FIG. 3. Electron micrographs of type 4 satellite DNA prepared by the Kleinschmidt technique. Fragments are present in the upper and middle right micrograph. No circular forms were noted. $\times 93,500$.

TABLE 2. Sedimentation coefficient and molecular weight estimation of type 4 satellite DNA by zone sedimentation of native DNA, alkali-denatured DNA, and DNA derived from virus by alkali treatment

Prepn no.	Neutral (DNA) ^a		Alkaline (DNA)		Alkaline (whole virus)	
	$S_{20,w}$	MW of duplex ^b	$S_{20,w}$	MW of duplex ^c	$S_{20,w}$	MW of duplex ^c
1	—	—	—	—	14.8	2.6×10^6
2	—	—	—	—	17.0	3.7×10^6
3	15.7	3.2×10^6	15.7	3.0×10^6	15.7	3.0×10^6
4	—	—	—	—	15.5	2.9×10^6
5	14.4	2.5×10^6	—	—	16.0	3.2×10^6
6	16.6	3.7×10^6	15.4	2.9×10^6	15.3	2.8×10^6
7	—	—	—	—	15.3	2.8×10^6
8	16.0	3.4×10^6	16.5	3.4×10^6	16.8	3.6×10^6
Avg	15.7	3.2×10^6	15.9	3.1×10^6	15.7	3.0×10^6

^a Preparations examined contained 10 to 30 $\mu\text{g/ml}$ of DNA.

^b Calculated from Studier (25). $S_{20,w} = 0.0882 \text{ M}^{0.346}$.

^c Calculated from Studier (25) $S_{20,w} = 0.0528 \text{ M}^{0.400}$. In alkali, native DNA undergoes strand separation; therefore the sedimentation coefficient actually corresponds to a molecular weight for the single strand (avg 1.5×10^6 daltons). The value shown above is for the duplex molecule.

DNA's, when examined under the same conditions as employed in the present study, show marked hypercoiling and pooling (3, 6). This indicates that the structural restraints present in type 4 satellite DNA molecules are characteristic of double-stranded DNA.

Zone centrifugation of type 4 satellite DNA.

FIG. 4. Band centrifugation pattern of type 4 satellite virus DNA prepared from purified whole virus in alkaline NaCl (0.9 M NaCl, 0.1 M NaOH). The heavy bands moving from left to right in successive pictures (photographed every 8 min at 52,000 rev/min) represent the single strands of DNA. The faint band at the left of each picture presumably represents viral protein. The sedimentation coefficient calculated from these pictures was 15.6 ($S_{20,w}$).

Zone centrifugation of satellite viral DNA at neutral pH (1 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane, pH 7.4) gave an average $S_{20,w}$ value of 15.7, which corresponds to a molecular weight of 3.2×10^6 daltons (Table 2).

Zone centrifugation of alkali-denatured DNA gave an average sedimentation coefficient of 15.9, which corresponds to a duplex molecular weight of 3.1×10^6 daltons, a figure in good agreement with the value obtained by band sedimentation of native DNA.

Zone centrifugation of DNA released from purified virus by alkali treatment in the analytical centrifuge yielded the sedimentation pattern shown in Fig. 4. The DNA component (fast moving) has a $S_{20,w}$ of 15.6; the slow moving band near the origin is probably viral capsid protein. Only a single DNA component was noted in numerous specimens examined. Alkaline-denaturation did not produce the very rapidly sedimenting forms characteristic of uninterrupted circular DNA molecules (29).

Microdensitometer tracings of the sedimentation patterns are presented in Fig. 5. A single sedimenting component was noted in the alkaline and neutral zone centrifugation of the type 4 DNA, thus suggesting the absence of significant amounts of either circular forms or fragmented nucleic acid components. The latter point was further evidence that the short molecules of DNA noted in electron microscopic studies were artifacts of preparation for electron microscopy.

$S_{20,w}$ values obtained by alkaline zone sedimentation of whole virus were in excellent agreement with $S_{20,w}$ values of alkali-treated extracted DNA (Table 2). In alkali the DNA is denatured and strands sediment presumably as single polynucleotide chains. The $S_{20,w}$ value for the single strands in alkaline zone sedimentation (of both DNA released from whole virus and extracted DNA) corresponded to a molecular weight of 1.5×10^6 , exactly half the molecular weight value determined by electron microscopy and from sedimentation coefficients of native DNA. We therefore conclude that type 4 DNA is a linear, double-stranded molecule of molecular weight 3×10^6 daltons. Preliminary analysis of type 1 DNA by zone centrifugation methods gave values similar to those obtained for type 4 DNA.

DISCUSSION

The secondary structure of the DNA obtained from type 4 satellite virus is clearly that of a linear, double-stranded molecule, as determined by thermal denaturation profile measurements, by electron microscopy, and by analytical zone

FIG. 5. Microdensitometer tracings of sedimentation patterns studied by three methods of analytical zone sedimentation of type 4 satellite DNA: whole virus in alkali, extracted DNA in alkali, and extracted DNA in neutral NaCl. Intervals between patterns are 8 min and sedimentation is from left to right at 52,000 rev/min at 20 C.

centrifugation. These findings on type 4 confirm the recent report of Rose et al. (22) for type 1 and are of interest in view of reports by Jamison and Mayor (9) and by Crawford (2) that two other "picodnaviruses" (16, 17), X-14 of hamsters and the minute virus of mice (MVM), have characteristics typical of single-stranded DNA molecules. Both of these rodent viruses have been reported to replicate autonomously and thus differ from the defective primate satellite viruses.

Crawford presented two types of evidence that the MVM DNA is single-stranded (2). First, he observed that the buoyant density of extracted MVM DNA at pH 8.5 was 1.722 g/cc (a value similar to that of type 4 satellite), and he noted that this could correspond to either a double-stranded DNA molecule with 63% G + C or a single-stranded molecule with 48% G + C. At pH 12.5, where native DNA is denatured (27), MVM DNA was stated to band at a position characteristic of a molecule with 48% G + C, therefore suggesting that the native DNA is a single-stranded molecule. Secondly, he showed that incubation of purified virus with formaldehyde resulted in hyperchromicity of the absorbancy pattern similar to that observed with ϕ X-174 bacteriophage (24).

Three independent measurements of molecular weight and conformation of type 4 satellite virus DNA were in good agreement. Electron-microscopic length measurements of extracted DNA revealed a linear, double-stranded molecule with a molecular weight of 2.9×10^6 daltons. Analytical zone centrifugation of extracted type 4 DNA at neutral pH indicated a molecular weight of 3.2×10^6 daltons for type 4, assuming a linear double-stranded molecule. Analytical zone centrifugation of DNA released from purified whole virus by alkali or extracted DNA treated with alkali, procedures which denature DNA to single polynucleotide chains, revealed a single component with a molecular weight of 1.5×10^6 daltons. This corresponds to a duplex molecule 3.0×10^6 daltons. The close agreement of these three independent determinations is strong evidence that the DNA molecule of type 4 satellite virus is a linear, double-stranded molecule containing two uninterrupted polynucleotide chains. However, the configuration of the DNA within the virion cannot be determined from the present study.

The values obtained for type 4 satellite virus in the present study are in good agreement with those of Rose et al. (22). The G + C content of type 4 satellite virus was determined by thermal

denaturation and buoyant density which gave values of 58 and 62%, respectively, significantly greater than the values of 54 and 58% for type 1 satellite determined with the same respective techniques (22). Type 1 was also studied by direct base analysis and found to have a 54.2% G + C content. The molecular weight of type 4 is 3.1×10^6 daltons, also slightly less than the value of 3.6×10^6 daltons reported for type 1 (22). Nevertheless, the similarity is enough to allow us to generalize that the defectiveness of the primate satellite viruses appears to be a function of the information content of the DNA, not its quantity, since other DNA-containing viruses of the papovavirus group contain DNA of the same size, but are not defective.

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