Topological Forms of Cauliflower Mosaic Virus Nucleic Acid

E. L. Civerolo and R. H. Lawson

United States Department of Agriculture, Agriculture Research Service, Beltsville, MD 20705.

ABSTRACT

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The nature of the topological forms of the genome of cauliflower mosaic virus (CauMV) in infectious nucleic acid (NA) preparations was examined by velocity sedimentation in sucrose density gradients, by equilibrium sedimentation in CsCl and CsCl-ethidium bromide (EthBr) density gradients, by electrophoresis in composite polyacrylamide-agaose gels, and by electron microscopy. The distribution of CauMV-NA in centrifuged sucrose density gradients is heterogeneous and CauMV-NA is susceptible to degradation by alkali (0.03 N and 0.3 N NaOH). The alkali-sensitive (0.3 N NaOH) sites may be located at specific, widely separated sites in the genome. Molecules of CauMV-NA are linear and circular. The circular forms of CauMV-NA are twisted and non-twisted. The twisted circular structures are the predominant forms in rapidly sedimenting fractions from centrifuged sucrose density gradients and in the slowest of three distinct electrophoretic components in composite polyacrylamide-agaose gels. The untwisted circles may be nicked or relaxed forms of an otherwise closed duplex. No covalently closed circular NA was detected by centrifugation in alkaline sucrose or in CsCl-EthBr density gradients. Topological constraints on the NA other than covalent closure of the strands might be responsible for these forms. The twisted circles appear to be stable and distinct form the untwisted, open circles. No linear or circular oligomeric forms of the monomeric unit length (2.3 - 2.5 μm) circles were observed. Infectivity was associated only with fractions containing the twisted circular forms of CauMV-NA from centrifuged sucrose density gradients.

Additional key words: circular DNA.

The caulimoviruses (5, 2) are a group of plant-infecting, icosahedral, cytoplasmic deoxyriboviruses (ICDV's) (12). The higher plant ICDV's are distinguished from other ICDV's by the smaller size of the virions and by the structure and properties of their nucleic acid (12). Cauliflower mosaic virus (CauMV) is the most extensively studied and characterized higher-plant ICDV (22, 23). The DNA of CauMV is double-stranded, consists of circular and linear forms, and has a molecular weight of 4 to 5 × 10^6 daltons (20, 22, 23, 24, 25, 26). However, the precise nature of the circular structure has not been clearly established. The results of studies designed to more fully understand the structure of the CauMV genome are presented in this communication.

MATERIALS AND METHODS

Virus purification. — The isolate of CauMV used in this work initially was obtained from the American Type Culture Collection (PV 45). The virus was maintained in and purified from mustard-spinach (*Brassica perviridis* Bailey 'Tendergreen'). For virus purification, infected leaves were harvested 4 to 6 wk after inoculation and frozen at –20 C. The frozen tissue was homogenized in 2.5 to 3.0 volumes of 0.1 M Na_2HPO_4-KH_2PO_4, pH 7.2; (phosphate buffer). The extract was clarified by adding *n*-butanol to a final concentration of 8 to 8.5% and stirring

for 1 to 3 hr at 3 C. Urea (6%, w/v) and Triton X-100 (2.5%, v/v) were added and the mixture was stirred overnight at 3 C (10). Further concentration and purification were achieved by two cycles of differential centrifugation and sucrose density gradient centrifugation. High-speed centrifugation pellets were resuspended in 0.01 M phosphate buffer.

Infectivity assay. — For infectivity tests, generally five to 10 young mustard-spinach seedlings were inoculated for each preparation tested. Since no satisfactory local lesion host is available for CauMV, a quantitative measure of the relative infectivity of virus and nucleic acid preparations on the systemic mustard-spinach host was based on the bioassay method described by Raymer and Diener (18).

Nucleic acid extraction. — The CauMV from centrifuged sucrose density gradients was reconcentrated by ultracentrifugation and resuspended on 0.01 M phosphate buffer. Virus concentrations were based on a specific extinction coefficient (uncorrected for light scattering) of 7 cm^2mg^{-1} at 260 nm and a 1-cm light path (22). Nucleic acid (NA) was extractd by a pronase-sodium dodecyl sulfate (SDS)-phenol method (20, 22, 24). The nucleic acid was maintained in 0.01 M phosphate buffer or in 0.01 M Tris-HCl, 0.001 M Na_2EDTA_2, pH 7.5 (TE buffer). Nucleic acid concentrations were determined spectrophotometrically using an extinction coefficient of 20 cm^2mg^{-1} at A_{260}nm and a 1-cm light path (22).

Sucrose density gradient centrifugation. — Usually 1 to 2 ml containing virus extracted from the equivalent of

100 to 200 gm of infected tissue was centrifuged through preformed 10 to 40% (w/v) sucrose density gradients [4, 6, 6, and 7 ml of 10, 20, 30, and 40% sucrose, respectively, in 0.01 M phosphate buffer for 2.5 hr at 24,000 rpm in a Beckman SW 25.1 rotor at 5 C (3)]. Successive 0.5- or 1.0-ml fractions were collected from the top of the gradient. Fractions of 0.5 ml were collected from the major ultraviolet (UV)-absorbing peak region in centrifuged density gradients. Otherwise, 1.0-ml fractions were collected. Rate-zonal sedimentation of extracted nucleic acid (approximately 50 to 200 μg in 0.5 to 1 ml of sample) was done in preformed 5 to 20% (w/v) sucrose density gradients (4, 7, 7, and 7 ml of 5, 10, 15, and 20% sucrose, respectively) in 0.01 M phosphate or TNES (0.01 M Tris-HCl, 1.0 M NaCl, 0.001 M Na_2EDTA_2, 0.1% Sarkosyl, pH 7.5) buffer at 24,000 rpm in a Beckman SW 25.1 rotor for 16 to 18 hr at 5 C. All preformed sucrose density gradients were stored at 3 C for 20 to 24 hr before use. Centrifuged gradients were analyzed and fractionated with an ISCO Model D density gradient fractionator and UA-2 UV-analyzer.

Equilibrium density gradient centrifugation—The CauMV-NA (57 to 77 μg) was centrifuged to equilibrium in 55 to 57% (w/w) CsCl dissolved in TNES, 1.0 × SSC (0.15 M NaCl+0.015 M sodium citrate) or 0.1× SSC in a Beckman SW 50.1 rotor for 24 hr at 20 C. After centrifugation, the tubes were punctured from the bottom and the contents were fractionated into 45 to 50 10-drop fractions (approximately 0.1 ml per fraction). The density of selected fractions was determined with an Abbé refractometer and using the formula $p(25 \text{ C}) = 10.8601 \, n_D^{25} - 13.4974 \, \text{gm/cm}^3$ (11). Each fraction was diluted with 1 ml of appropriate buffer and the A_{260} of each fraction was determined. Before centrifugation, 0.3 ml of CauMV-NA was dialyzed in 1 liter of the appropriate buffer overnight. CauMV-NA (50 to 200 μg) also was centrifuged in 56% (w/w) CsCl in the presence of 100 g of ethidium bromide (EthBr)/ml to determine if any superhelical forms of the DNA could be detected (7).

Alkali treatment. — Treatment of CauMV-NA with alkali was done to determine whether the genome is a covalently closed circular duplex DNA. In strand-separating alkaline conditions, a closed circular duplex is expected to sediment much more rapidly than at neutral pH due to the topological constraint imposed on the strands (7). Concentrated (10 N) NaOH was added to solutions of CauMV-NA in TE buffer to final concentrations of 0.003, 0.03, and 0.3 N. The solutions were incubated at room temperature for 30 min, layered on 5 to 20% (w/v) alkaline sucrose density gradients and centrifuged at 24,000 rpm for 16-17 hr in a Beckman SW 25.1 rotor at 5 C. The gradients were made in TES buffer with 0.003 N NaOH-0.997 M NaCl, 0.03 N NaOH-0.97 M NaCl or 0.3 N NaOH-0.7 M NaCl. The formulation of TES buffer is 0.01 M Tris-HCl, 0.001 M Na_2EDTA_2 (pH 7.5) with 0.1% Sarkosyl.

Gel electrophoresis. — Composite gels of 1.7 to 2.2% polyacrylamide-0.5% agarose (PAAGE) were prepared as described previously (6, 17). Electrophoresis buffer was 0.05 M Tris, 0.02 M sodium acetate, 0.002 M Na_2EDTA_2, 0.018 M NaCl (TEAN) adjusted to pH 8.2 with glacial acetic acid originally in 10 ×concentrated buffer (4, 8). Cylindrical gels (6 mm in diameter ×10 or 25 cm long)

were pre-electrophoresed for at least 30 min at 100 V at room temperature. After pre-electrophoresis, the buffer in the lower reservoir was replaced with fresh TEAN buffer. Samples of 0.1 to 0.2 ml contained approximately 1 to 3 μg of CauMV-NA in TE buffer with 20% sucrose. Samples were run into the gels at 50 or 100V for 5 min and then 2.5 to 3.5 V/cm for 10 to 13 hr at room temperature (4, 8). After electrophoresis, gels were extruded into 1 μg EthBr/ml TEAN (1, 8, 21). After 0.5 to 5 hr, the flourescent bands were visualized and photographed under long wavelength UV light. Gels were photographed using a yellow filter (Kodak No. 9 Wratten gelatin filter) and Kodak Tri-X Pan film (ASA 400).

The electrophoretic components of CauMV-NA represented by the flourescent bands in composite poly-acrylamide-agarose gels after staining in EthBr were recovered for examination by electron microscopy and infectivity tests. Material in gel slices containing the flourescent bands was eluted electrophoretically into dialysis bags (17) or manually squeezed out of frozen gel slices. Alternatively, frozen-thawed gel slices were triturated in a sterile mortar with a sterile pestle.

Electron microscopy.—CauMV-NA was prepared for observation by the protein monolayer procedure (13, 14, 15). Preparations with 0.15 to 0.28 A_{260}/ml contained a sufficient number of non-overlapping molecules when 0.1 ml of sample was diluted in 0.9 ml of 1.0 M ammonium acetate, pH 7.2, and 0.01% cytochrome C (Sigma). The mixture was spread on a hypophase of 0.15 M ammonium acetate, pH 7.2. Portions of the monolayer were picked up on carbon-coated grids and dried by touching the grid surface on ethanol for 20 sec and drying down with a filter paper strip. The grids were rotary shadowed at an angle of 8 degrees with 80-20% Pt-Pd. Length of the molecules was determined with a map measurer and compared with the latex size standards.

The CauMV-NA was collected in three fraction from centrifuged sucrose density gradients in two experiments and in four fractions in a third experiment. Each fraction was spread as previously described and the molecules were measured. Differences in conformation of the molecules were noted as well as the number of crossovers in the molecules.

RESULTS

Virus purification. — Rate-zonal sedimentation and distribution of infectivity of CauMV in a centrifuged 10 to 40% sucrose density gradient is shown in Fig. 1. The fractions with the greatest relative infectivity are directly associated with a single, major UV-absorbing component. In the UV-absorbance and relative infectivity profiles, the major peak is also skewed towards the fast-sedimenting side. The relative infectivity of fractions in the denser regions of the gradient was approximately 1/20 to 1/6 that of the relative infectivity of the peak fractions. A small peak of UV-absorbing material, sedimenting faster than the major peak, was present in all preparations of CauMV. Following an additional cycle of differential and sucrose density gradient centrifugation of the pooled fractions assocaited with the major peak, the minor, rapidly sedimenting peak still was present. The absorbance profiles of preparations of CauMV always
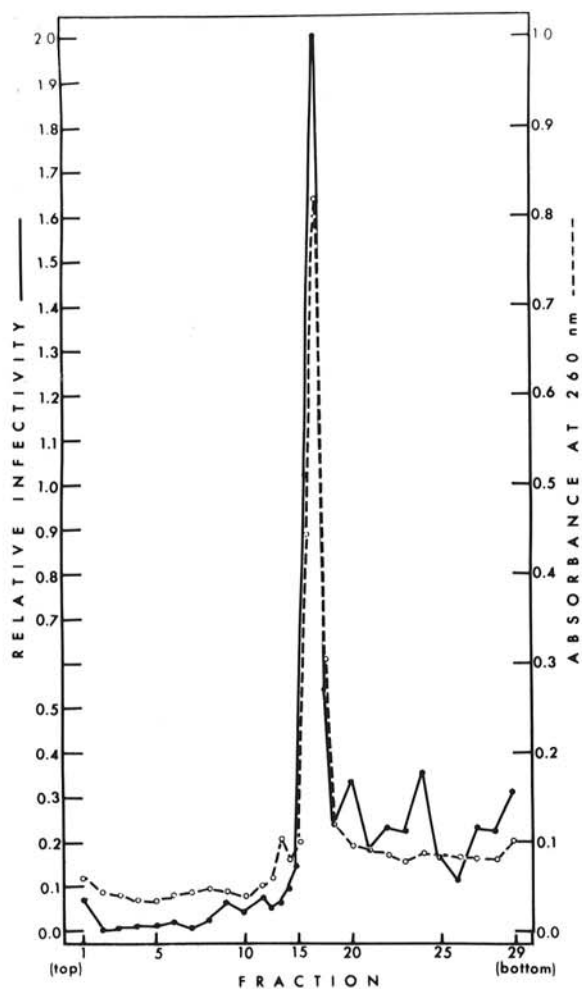
Fig. 1. Ultraviolet-absorbance profile and distribution of infectivity in a linear 10-40% (w/v) sucrose density gradient containing cauliflower mosaic virus and centrifuged for 2.5 hr at 24,000 rpm in a Beckman SW 25.1 rotor at 5 C. Sedimentation is from left to right.
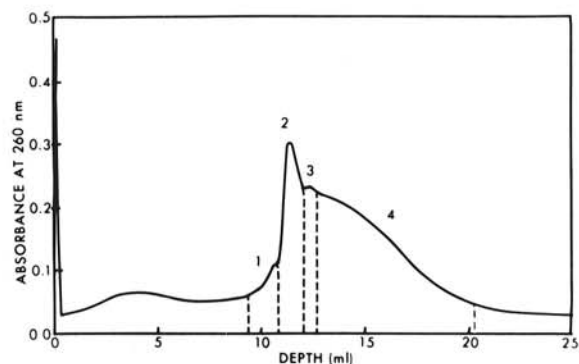

Fig. 2. Ultraviolet-absorbance profile of a linear 5-20% (w/v) sucrose density gradient in TNES (0.01 M Tris-HCl, 1.0 M NaCl, 0.01 M Na_2EDTA_2, 0.1% Sarkosyl, pH 7.5) containing cauliflower mosaic virus nucleic acid and centrifuged for 16 hr at 24,000 rpm in a Beckman SW 25.1 rotor at 5 C. Sedimentation is from left to right.

were characterized by a slight skewing of the major peak toward the denser portions of the gradient. Only the pooled fractions of the material associated with the major UV-absorbing, infectious peak were used for nucleic acid extraction.

Nucleic acid characterization.—Nucleic acid extracted from different preparations of CauMV consists of at least two distinct sedimenting components (1 and 2) and a relatively large amount of very heterogeneously distributed material sedimenting in the denser portions of sucrose density gradients (Fig. 2). The distribution of UV-absorbing material in sucrose gradients containing TNES or 0.01 M phosphate were generally the same qualitatively and quantitatively (data not shown). However, the resolution of components 1 and 2 was generally better when the density gradients were made with TNES.

Although unfractionated preparations of CauMV-NA were highly infectious, infectivity was not associated with any specific UV-absorbing component or fraction from sucrose gradients. In three trials, successive 1-ml fractions were collected from the top of the gradients. However, only a few fractions from the lower portions of the gradients containing the heterogeneously distributed UV-absorbing material (component 4 in Fig. 2) were infectious. The total infectivity in the few fractions from this region of sucrose gradients was less than that of the original preparation.

With increasing total amount of nucleic acid centrifuged in a sucrose density gradient, component 1 appears as a slight shoulder associated with the component 2 peak or merely as a skewing of the component 2 peak toward the less-dense side. The relative proportion of components 1 and 2 was variable in different preparations. However, in all preparations, the proportion of component 2 was greater than component 1.

Equilibrium density gradient centrifugation.—When approximately 57 to 77 μg of CauMV-NA was centrifuged to equilibrium in 56% (w/w) CsCl in TNES, 30 to 35% of the material in fractions 14-31 sedimented between 1.695 and 1.708 gm/cm³ (Fig. 3). In two trials, the density of the fraction containing the peak of A_{260} absorbance was 1.700 and 1.701 gm/cm³. This value is similar to the values of 1.6996 to 1.704 gm/cm³ reported earlier (20, 24). The CauMV-NA preparations also contain less dense material (amounting to approximately 24% of the total A_{260}-absorbing material of the preparation presented in Fig. 3) which is represented as a slight shoulder of A_{260} absorbance in the less-dense fractions (P = 1.637 to 1.687 gm/cm³).

When 50-200 μg of CauMV-NA was centrifuged in 56% (w/w) CsCl in the presence of 100 g EthBr/ml, only a single flourescent band was present in the upper portion of the gradient. In similar gradients without EthBr, the densities in this region of the gradient were 1.55 to 1.65 gm/cm³. These results are similar to those described previously (24).

Alkali treatment. — CauMV-NA is readily fragmented in the presence of 0.03 and 0.3 N NaOH (Fig. 4). After treatment with 0.3 N NaOH, CauMV-NA sediments as three distinct components (Fig. 4). All of these components sediment more slowly than component 1 (Fig. 4, control) of CauMV-NA in nontreated preparations. The proportion of the fastest sedimenting component in preparations treated with 0.3 N NaOH was higher than the

two slower-sedimenting components. The relative amount of CauMV-NA fragmented in 0.03 N NaOH appears to be less than that fragmented in 0.3 N NaOH. After exposure to 0.03 and 0.3 N NaOH, CauMV-NA also contained very heterogeneous material sedimenting just below the meniscus (Fig. 4).

Gel electrophoresis. — Preparations of CauMV-NA contain three distinct components when electrophoresed in composite 1.7 to 2.2% polyacrylamide -0.5% agarose gels (A, B, and C in order of increasing electrophoretic mobility, respectively) (Fig. 5). From electron microscopic examination, PAAGE component C consists primarily of linear forms. The PAAGE components A and B consist of circular forms (either untwisted circles or circles with varying numbers of twists or crossovers). Based on the widths and the intensities of the fluorescence of the bands in the gels, the relative amounts of the PAAGE components A and B in these CauMV-NA preparations were greater than PAAGE component C. Component A of PAAGE may not be electrophoretically homogeneous. A multiplicity of components can be resolved when CauMV-NA is electrophoresed in 0.7 - 1.0% agarose gels (Civerolo and Lawson, *unpublished*).

Only CauMV-NA PAAGE components A and B are infectious. However, these components were not adequately separated to preclude the possibility that only one component is infectious. There was no detectable en-hancement of infectivity when the PAAGE components are mixed in all possible combinations.

Electron microscopy of nucleic acid from sucrose density gradients. — Sucrose density gradient fraction 1 contains more linear forms than any other fraction (Table 1, Fig. 6-A). In one experiment only one circular molecule was present in this fraction and 42 linear forms varied in length from about 0.5 to 2.5 μm. In two other experiments, more circular forms were present in this fraction. (Table 1). Presumably the separation of this fraction from the adjacent peak was not as complete in the second and third experiments. In all three experiments, 35% of the linear molecules from all the sucrose density gradient fractions were 2.15 to 2.52 μm long (Fig. 7).

In fraction 2, 81 to 95% of the molecules in three experiments were circular (Fig. 6-B) and 4 to 19% were linear. In two experiments, most of the circular molecules were 2.29 μm long (Fig. 7). Assuming a molecular weight of $1.96 \times 10^6 / \mu$m (13), the mean molecular weight
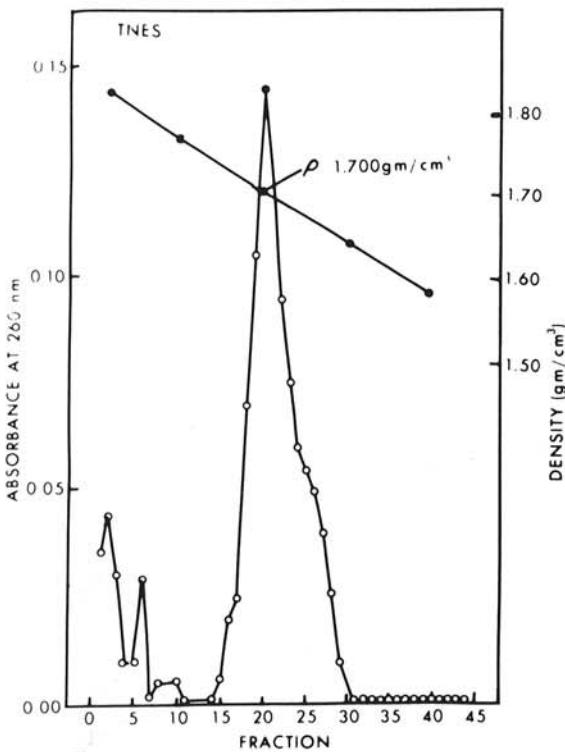

Fig. 3. Equilibrium density gradient centrifugation of cauliflower mosaic virus nucleic acid 56% (w/w) CsCl in TNES (0.01 M Tris-HCl, 1.0 M NaCl, 0.01 M Na$_2$EDTA$_2$, 0.1% Sarkosyl, pH 7.5) at 45,000 rpm in a Beckman SW 50.1 rotor for 24 hr at 20 C. Sedimentation is from right to left.
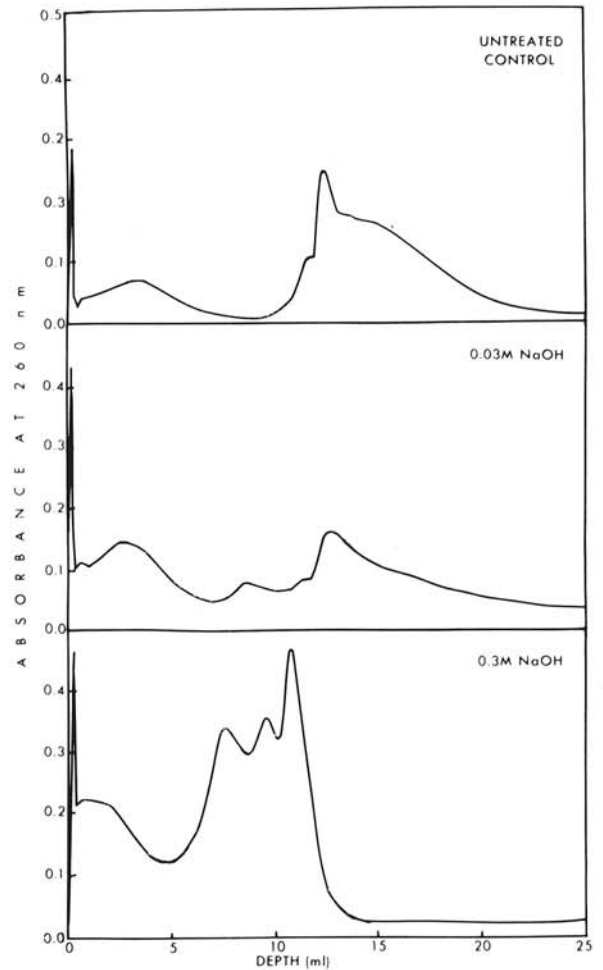

Fig. 4. Ultraviolet-absorbance profiles of linear 5-20% (w/v) sucrose density gradients containing cauliflower mosaic virus nucleic acid treated with 0.03 and 0.3 N NaOH and centrifuged for 16 hr at 24,000 rpm in Beckman SW 25.1 rotor at 5 C. Sedimentation is from left to right.

of CauMV-NA is 4.48×10^6 daltons. In the third experiment, the majority of molecules in this fraction were 2.46 μm long, equivalent to 4.70×10^6 daltons. The CauMV-NA prepared by the Kleinschmidt method (14, 25) measured 2.15 μm and that prepared by the method of Koller et al. (15, 25) ranged from 2.31 to 2.50 μm. Russell et al. (20) reported a contour length of 2.47 μm for the circular form of CauMV.

The length distribution of the circular molecules in fraction 3 was similar to those in fraction 2 and the peak length of the circular forms in fractions 2 and 3 was similar to all three experiments. However, there was a significant difference in the conformation of the molecules in fractions 2 and 3. Molecules in fraction 2 were generally untwisted while those in fraction 3 had several crossover points or twists (Fig. 6-B and 6-C).

The range in length of the few circular molecules measured in fraction 4 from one experiment was the same as in the other fractions with a lower proportion of molecules in the 2.27-μm length group. Only 29 molecules could be accurately measured in this fraction because of the extensive twisting or crossing over (Fig. 6-D).

Electron Microscopy of gel electrophoresis components.—Molecules in PAAGE component A were highly twisted and showed many crossovers (Table 2). No circular forms without crossovers were present in this gel fraction. The morphology of the molecules in sucrose gradient fraction 4 is similar to those in the PAAGE component A. This is consistent with the observation of forms with many crossovers which are also present in sucrose density gradient fraction 4.

Component B of PAAGE consisted of some circular forms with several crossovers but seven out of 22 circular molecules in this fraction showed no crossovers (Table 2). Fifteen of the circular molecules could be measured and the length distribution was similar to the circular molecules in sucrose density gradient fraction 2 with four of the 15 circular molecules from the gel measuring 2.39 μm. Samples from sucrose gradient fraction 3 contained molecular forms that are similar to those in the PAAGE component B. The high proportion of circular forms without crossovers in the PAAGE B component also were present in sucrose gradient fraction 2.

The PAAGE component C contained only linear forms of the molecule. The length of the linear forms ranged from 1.89 to 2.37 μm with most of the molecules measuring 2.21 μm. The linear forms in this gel component are similar to the linear forms present in sucrose gradient fraction 1.

DISCUSSION

As presently understood, the genome of CauMV consists of double-stranded DNA occurring as nicked circular and linear forms with a molecular weight of 4 to 5×10^6 daltons (20, 22, 23, 25). Buoyant density in CsCl, molecular weight as determined by electron microscopy, DNase-susceptibility, and RNase-resistance of the nucleic acid (data not shown) studied here, are consistent with the results presented in previous studies (20, 24).
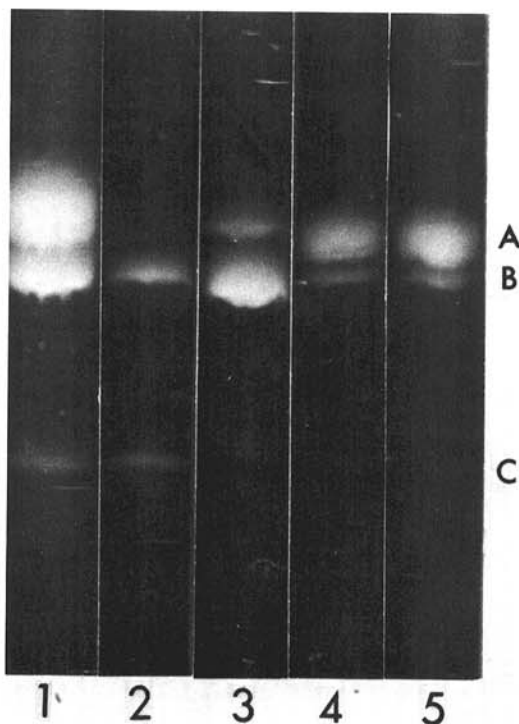

Fig. 5. Electrophoresis of cauliflower mosaic virus nucleic acid in composite 2.2% polyacrylamide-0.5% agarose gels. Nonfractionated (1) and fractionated (2-5) sucrose density gradient fractions 1, 2, 3, and 4, respectively. Electrophoresis was at 2.5 V/cm for 10 hr at room temperature. Electrophoresis buffer was 0.05 M Tris, 0.02 M sodium acetate, 0.002 M Na_2EDTA_2, 0.18 M NaCl, pH 8.2. Migration is from top (–) to bottom (+).

TABLE 1. Distribution of topological forms of cauliflower mosaic virus nucleic acid molecules in selected fractions from centrifuged sucrose density gradients (SDG) in TNES[a] (0.01 M Tris-HCl, 1.0 M NaCl, 0.01 M NA_2EDTA_2, 0.1% Sarkosyl, pH 7.5)

Experiment no.	SDG Fraction	Topological form			
		Linear		Circular[b]	
		(no.)	(%)	(no.)	%
1	1	17	45	21	55
	2	2	4	47	96
	3+4	6	8	72	92
2	1	42	98	1	2
	2	13	19	55	81
	3	35	34	69	66
3	1	11	69	5	31
	2	12	18	56	82
	3	9	16	47	84

[a]TNES is 0.01 M Tris-HCl, 1.0 M NaCl, 0.01 M Na_2 $EDTA_2$, 0.1% Sarkosyl, pH 7.5.

[b]Circular forms include open circles and twisted circles without visible ends.

However, additional information about the structural nature of the CauMV genome is provided by the present studies.

The centrifugal heterogeneity of CauMV-NA as determined by velocity sedimentation in sucrose density gradients is not due to the presence of distinct fragments
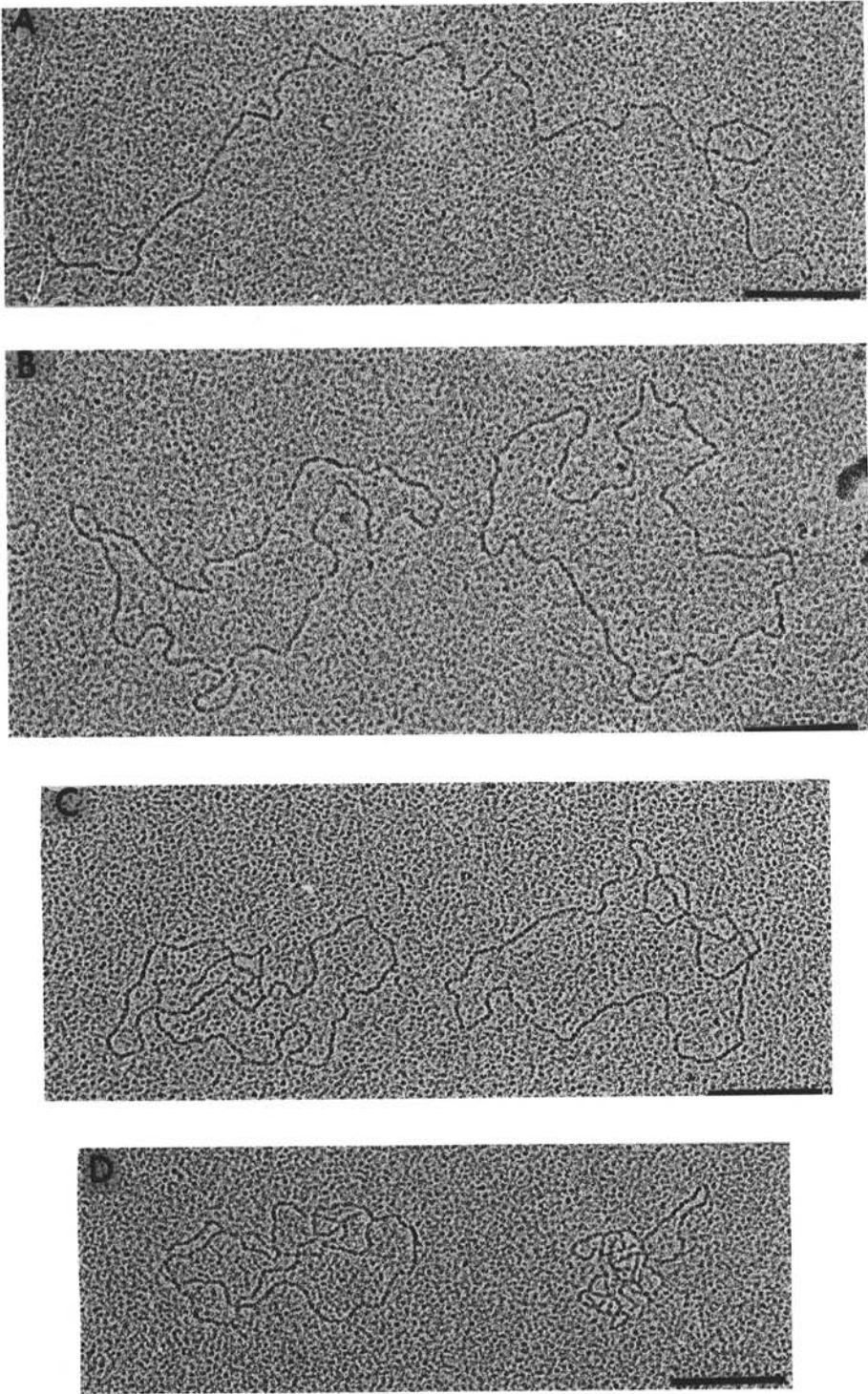

Fig. 6-(A to D). Electron micrographs of **A)** linear, **B)** untwisted circular and **C and D)** twisted circular forms of cauliflower mosaic virus nucleic acid in sucrose density gradient fractions 1(A), 2(B), 3(C), and 4(D). The bar equals 200 nm.

or oligomeric forms of varying size. The heterogenity of CauMV-NA in the denser regions of centrifuged sucrose density gradients could be due to different degrees of twisting or crossing over. Oligomeric forms of the monomeric unit or catenated molecules consisting of interlocked monomeric length molecules were not observed. The absence of multiple length linear and/or circular structures in unfractionated preparations of CauMV nucleic acid was clearly established by electron microscopy. There is no evidence that there is a significant difference in length of the circular molecules in fractions 2, 3, and 4. However, there is a marked increase in the number of crossovers on molecules in fractions 3 and 4 compared to those in fraction 2. Increased crossing over in the molecules in fractions 3 and 4 produces a more compact and more rapidly sedimenting structure. We do not know if crossing over is an artifact introduced into the molecules as a result of manipulation during extraction and purification. It is apparent, however, that fractions that contain molecules with extensive crossing over are also infectious and is consistent with the conclusion that these are not merely artifacts. When centrifuged to

equilibrium in CsCl, a single component with a slight shoulder is detected. These presumably represent the 20S and 18S components, respectively, and are circular and linear forms of the same molecule (20).

The alkaline lability of CauMV-NA is consistent with, but does not prove, the conclusion that the DNA genome contains covalently linked ribonucleotides as demonstrated for some other viruses (2, 9, 19, 27). Alternatively, the fragmentation under alkaline conditions could be a consequence of pre-existing single-strand interruptions or nicks induced during isolation. The possibility that the alkaline lability is due to the presence of unusual or modified bases (16, 28) is unlikely (22, 23, 24, 25).

The characteristic sedimentation behavior of CauMV-NA following treatment with 0.3 N NaOH presumably could be due to the presence of the alkaline labile regions located at widely separated specific sites in the genome. The 20S circular form of CauMV-NA was converted to a heterogeneous 17S component with an average molecular weight of 1.93×10^6 daltons at pH 12 to 13 (20).

The nature of the alkali-labile sites in CauMV nucleic acid cannot be conculsively established from these
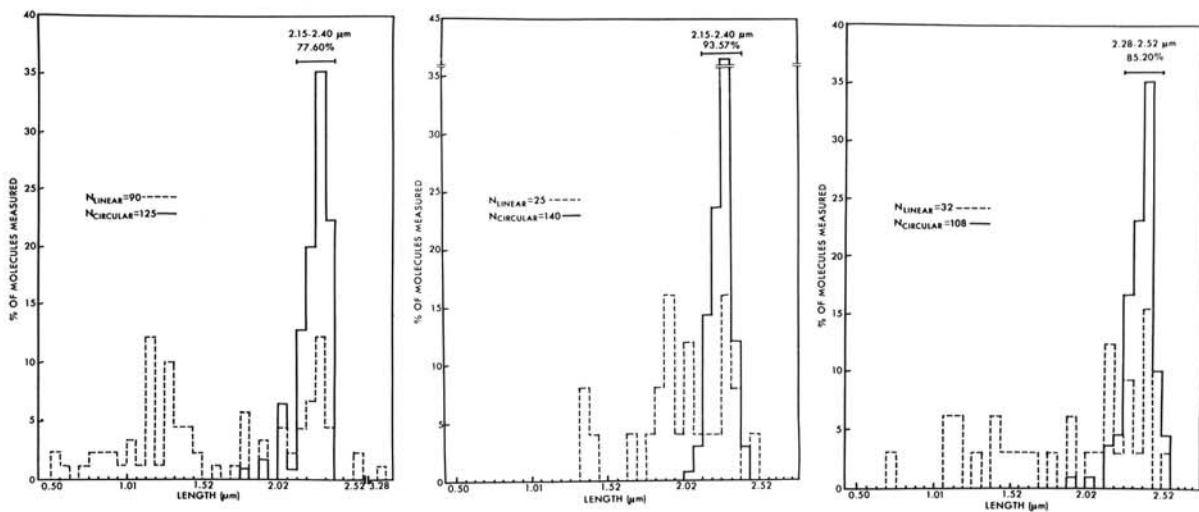

Fig. 7. Length distributions of linear and circular forms of cauliflower mosaic virus nucleic acid in three separate preparations.

TABLE 2. Distribution and length of molecular forms of the electrophoretic components of cauliflower mosaic virus nucleic acid recovered from 2.2% polyacrylamide-0.5% agarose composite gels

Component	Number and percentages of molecules									Length of molecules	
	Linear	Circular	Number of crossovers in circular forms							Linear	Circular
			0	1	2	3	4	5	5+		
A	0	24	0	0	1	0	2	3	18	. . .	2.18-2.34 µm
			. . .	. . .	4.2%	. . .	8.3%	12.5%	75%		
B	1	21	7	3	0	4	5	2	0	. . .	2.08-2.40 µm
			33.3%	14.5%	. . .	19.1%	23.8%	9.5%	. . .		
C	10	0	. . .	. . .	. . .	. . .	. . .	. . .	. . .	1.89-2.37 µm	. . .

studies. Ribonucleotide sequences could be linked to CauMV-NA through hydrogen bonds (i.e., as in DNA-RNA hybrids). More likely, covalently inserted ribonucleotides could link single-strand breaks or gaps in the DNA of mature CauMV genome (9, 19, 27).

The CauMV-NA consists of at least three distinct electrophoretic species in composite acrylamide-agarose gels. As determined by electron microscopy, the minor, most rapidly migrating component consists only of linear forms while the slower-migrating bands contain primarily circular forms. Furthermore, only the slower-migrating bands are infectious. Therefore, the biologically functional structure of the CauMV genome appears to be a twisted or untwisted circular form of DNA with specifically located alkali-labile sites. These sites are possibly covalently linked ribonucleotide sequences. We were unable to demonstrate by alkaline sucrose density gradient or by CsCl-EthBr equilibrium density gradient centrifugation that classical covalently closed circular DNA forms with or without superhelicity and distinct from linear and nicked open circular form are associated with CauMV-NA. Nevertheless, PAAGE components A and B are morphologically distinct. The structural difference(s) between these 2 forms in CauMV-NA preparations appears to be quite stable. Topological constraints on the NA other than covalent closure (e.g., the presence of tightly bound protein or other non-DNA components) could be responsible for these forms. The structure of CauMV-NA molecules recovered from each PAAGE component was similar to the structure of the molecules in the corresponding sucrose density gradient fractions that contained the gel components. Molecules in the most rapidly migrating gel component were linear and morphologically similar to those in the slowest sedimenting component in sucrose density gradients. The slowest moving gel component contained molecules morphologically identical to the most rapidly sedimenting molecules which contained many crossovers or twists. Since molecules recovered from gels that retain structure similar to the molecules in sucrose density gradient fractions as well as in unfractionated preparations there is no obvious structural modification of the molecules during electrophoresis.

We cannot preclude the possibility that the heterogeneity in centrifuged sucrose density gradients results from contamination of CauMV-NA preparations with proteins, polyamines, or other non-DNA materials (24). Indeed, this might also explain why the most rapidly sedimenting molecules correspond to the slowest electrophoretic molecules in composite polyacrylamide-agarose gels. Nevertheless, the sedimentation of CauMV-NA in sucrose density gradients as describe herein is extremely reproducible.

Although we routinely precipitated CauMV-NA with ethanol, we did not observe the compact masses of molecules previously associated with this treatment (25). Furthermore, the short fibrous masses lacking a uniform diameter (25) were absent in our CauMV-NA preparations

In two experiments we measured 15 and 22% linear molecules in the total populations from sucrose density gradients. About 41% of the molecules in a third experiment were linear.

The linear forms present in CauMV-NA preparations

could be generated from the circular forms by breaks in both strands of the duplex. The structural differences accounting for the distinct electrophoretic mobilities of the two slow-migrating components in composite acrylamide-agarose gels appear to be related to the twisting or untwisting of the molecules. The occurrence or absence of supercoiling in circular molecules of CauMV-NA was not clearly established previously because of the occurrence of compact and tangled masses associated with the circular DNA (25, 26). We also failed to establish conclusively the occurrence of supercoiled CauMV-NA although some highly twisted forms were present in specific fractions from centrifuged sucrose density gradients and PAAGE component A.

The difference in electrophoretic mobility might be due to PAAGE component A being a twisted circular structure which is stabilized in a manner other than by covalent closure of both strands of the duplex while PAAGE component B might be a relaxed circle or an open circle with at least one nick or break in one strand. PAAGE component B might then be expected to migrate slightly faster in the gels due to its slightly greater flexibility. The nature of the twisted forms is being examined. Although the relative infectivity of PAAGE component A seemed to be slightly greater than that of PAAGE component B, the level of infectivity of these components recovered from the gels was too low to conclusively determine their relative infectivities. However, if the twisted form is the native structure of the CauMV genome, it might be expected that preparations of PAAGE component A would be more infectious than those of PAAGE component B. Unless the genome of CauMV is segmented, no enhancement of infectivity would occur when the PAAGE components are mixed in all possible combinations.

LITERATURE CITED

1. AAIJ. C., and P. BORST. 1971. The gel electrophoresis of DNA. Biochim. Biophys. Acta 269:192-200.
2. BISWAL, N., B. K. MURRAY, and M. BENYESH-MELNICK. 1974. Ribonucleotides in newly synthesized DNA of herpes simplex virus. Virology 61:87-99.
3. BRAKKE, M. K. 1960. Density-gradient centrifugation and its application to plant viruses. Adv. Virus Res. 7:193-224.
4. DUGAICZYK, A., H. W. BOYER, and H. M. GOODMAN. 1975. Ligation and circular structures. J. Mol. Biol. 96:171-184.
5. HARRISON, B. D., J. T. FINCH, A. J. GIBBS, M. HOLLINGS, R. J. SHEPHERD, V. VALENTA, and C. WETTER. 1971. Sixteen groups of plant viruses. Virology 45:356-363.
6. HAYWARD, G. S., and M. G. SMITH. 1972. The chromosome of bacteriophage T5. I. Analyses of the single-stranded DNA fragments of agarose gel electrophoresis. J. Mol. Biol. 63:383-395.
7. HELINSKI, D. R., and D. B. CLEWELL. 1971. Circular DNA. Annu. Rev. Biochem. 40:899-942.
8. HELLING, R. B., H. M. GOODMAN, and H. W. BOYER. 1974. Analysis of endonuclease R-ECoRI fragments of DNA from lambdoid bacteriophages and other viruses by agarose gel electrophoresis. J. Virol. 14:1235-1244.
9. HIRSCH, I., and V. VANKA. 1974. Ribonucleotides linked to DNA of herpes simplex virus. J. Virol. 13:1163-1168.
10. HULL, R., R. J. SHEPHERD, and J. D. HARVEY. 1976. Cauliflower mosaic virus: An improved purification

procedure and some properties of the virus particles. J. Gen. Virol. 31:93-100.

11. IFFT, V. B., D. H. VOGT, and J. VINOGRAD. 1961. The determination of density distributions and density gradients in binary solutions at equilibrium in the analytical centrifuge. J. Phys. Chem. 65:1138-1145.

12. KELLY, D. C., and J. S. ROBERTSON. 1973. Icosahedral cytoplasmic deoxyriboviruses. J. Gen. Virol. 20:17-41.

13. KLEINSCHMIDT, A. K., A. BURTON, and R. L. SINSHEIMER. 1963. Electron microscopy of the replicative form of the DNA of the bacteriophage X174. Science 142:961.

14. KLEINSCHMIDT, A., and R. K. ZAHN. 1959. Über deoxyribonucleinsaure-Molekeln in protein-mischfilmen. Z. Naturforsch. 146:770-779.

15. KOLLER, T., A. G. HARFORD, Y. K. LEE, and M. BEER. 1969. New methods for the preparation of nucleic acid molecules for electron microscopy. Micron 1:110-118.

16. LEWIS, H. A., R. C. MILLER, JR., J. C. STONE, and R. A. J. WARREN. 1975. Alkali lability of bacteriophage W-14 DNA. J. Virol. 16:1375-1379.

17. PETTERSSON, U., C. MULDER, H. DELIUS, and P. A. SHARP. 1973. Cleavage of adenovirus Type 2 DNA into six unique fragments by endonuclease R.RI. Proc. Nat. Acad. Sci., USA 70:200-204.

18. RAYMER, W. B., and T. O. DIENER. 1969. Potato spindle tuber virus: A plant virus with properties of a free nucleic acid. Virology 37:343-350.

19. ROSENKRANZ, H. S. 1973. RNA in coliphage T5. Nature 242:327-329.

20. RUSSELL, G. J., E. A. C. FOLLETT, and J. H. SUBAK-SHARPE. 1971. The double-stranded DNA of cauliflower mosaic virus. J. Gen. Virol. 11:129-138.

21. SHARP, P. A., B. SUGDEN, and J. SAMBROOK. 1973. Detection of two restriction endonuclease activities in Haemophilus parainfluenze using analytical agarose-ethidium bromide electrophoresis. Biochemistry 12:3055-3063.

22. SHEPHERD, R. J. 1970. Cauliflower mosaic virus. No. 24 in Descriptions of plant viruses. Commonw. Mycol. Inst., Assoc. Appl. Biologists, Kew, Surrey, England.

23. SHEPHERD, R. J. 1976. DNA viruses of higher plants. Adv. Virus Res. 20:305-332.

24. SHEPHERD, R. J., G. E. BRUENING, and R. J. WAKEMAN. 1970. Double-stranded DNA from cauliflower mosaic virus. Virology 41:339-347.

25. SHEPHERD, R. J., and R. J. WAKEMAN. 1971. Observation on the size and morphology of cauliflower mosaic virus deoxyribonucleic acid. Phytopathlogy 61:188-193.

26. SHEPHERD, R. J., R. J. WAKEMAN, and R. R. ROMANKO. 1968. DNA in cauliflower mosaic virus. Virology 36:150-152.

27. SPEYER, J. F., J. CHAO, and L. CHAO. 1972. Ribonucleotides covalently linked to deoxyribonucleic acid in T4 bacteriophage. J. Virol. 10:902-908.

28. ZEIGER, R. S., R. SOLOMON, C. W. DINGMAN, and A.C. PEACOCK. 1972. Role of base composition in the electrophoresis of microbial and crab DNA in polyacrylamide gels. Nat. New Biol. 238:64-69.