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Physicochemical Characterization of Mitochondrial DNA from Soybean

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ABSTRACT

Mitochondrial DNA (mtDNA) of soybean (*Glycine max* L.) was isolated and its buoyant density was contrasted with that of nuclear (nDNA) and chloroplast (ctDNA) DNA. Each of the three DNAs banded at a single, characteristic buoyant density when centrifuged to equilibrium in a CsCl gradient. Buoyant densities were 1.694 g/cm^3 for nDNA and 1.706 g/cm^3 for mtDNA. These values correspond to G-C contents of 34.7 and 46.9%, respectively. Covalently closed, circular mtDNA molecules were isolated from soybean hypocotyls by ethidium bromide-cesium chloride density gradient centrifugation. Considerable variation in mtDNA circle size was observed by electron microscopy. There were seven apparent size classes with mean lengths of 5.9 μm (class 1), 10 μm (class 2), 12.9 μm (class 3), 16.6 μm (class 4), 20.4 μm (class 5), 24.5 μm (class 6), and 29.9 μm (class 7). In addition, minicircles were observed in all preparations. Partially denatured, circular mtDNA molecules with at least one representative from six of the seven observed size classes were mapped. In class 4, there appear to be at least three distinct denaturation patterns, indicating heterogeneity within this class. It is proposed that the mitochondrial genome of soybean is distributed among the different size circular molecules, several copies of the genome are contained within these classes and that the majority of the various size molecules may be a result of recombination events between circular molecules.

Mitochondrial DNA from a number of higher plant sources (14) has been isolated. The buoyant density in neutral CsCl of plant mtDNA is approximately 1.706 g/cm^3, suggesting a conserved base ratio for mtDNA of higher plants (22, 26). This also appears to be the case with ctDNAs from higher plants in which buoyant densities of approximately 1.697 g/cm^3 have consistently been found (26). Mitochondrial DNAs from animal sources appear to exist as circular molecules of 4 to 6 μm in size (19). A circular conformation has also been observed by electron microscopy in mtDNA from yeast (17) and *Neurospora* (1). In higher plants, circular mtDNAs of approximately 30 μm in length have been observed by Kolodner and Tewari (14) in pea, spinach, lettuce, and bean. By means of renaturation kinetics, the kinetic complexity of pea mtDNA has been estimated to be approximately 74×10^6 daltons (14), while potato mtDNA gave a larger value of approximately 100×10^6 daltons (25). In all cases, no extensively repeated segments of DNA were observed.

In this study, soybean mtDNA from 4-day hypocotyls was isolated and distinguished from nDNA and ctDNA by its characteristic buoyant density. Mitochondrial DNA was isolated as a supercoiled molecule by the dye buoyant density method (20) and observed by electron microscopy to have a circular conformation. These circular molecules were heterogeneous in size. To describe this heterogeneity in circle size further, molecules were partially denatured and examined for the distribution of AT-rich regions of the genome. Several possible reasons for the size variation of the circular molecules were discussed.

MATERIALS AND METHODS

Isolation of DNAs for Buoyant Densities. Approximatley 1 kg of hypocotyls from 4-day dark-grown soybean (*Glycine max* L.) seedlings of the variety Davis were homogenized in a Waring Blendor in 2 volumes of buffer (medium A) containing 0.3 M mannitol-0.05 M Tris-HCl (pH 8.3)-3 mM EDTA-0.1% BSA-1 mM 2-mercaptoethanol. Homogenates were filtered through four layers of cheesecloth and centrifuged at 121*g* for 10 min to obtain a crude nuclear pellet. This pellet was resuspended in 20 ml of 0.15 M NaCl-0.1 M EDTA (pH 8.5)-2% sodium laurylsulfate and the nuclei allowed to lyse for 15 min. The mixture was placed in a 60 C bath for 10 min, phenol-extracted twice, and the nDNA alcohol-precipitated and spooled onto a glass stirring rod. The spooled nDNA was resuspended in a 0.01 M Tris-HCl (pH 8) buffer, incubated with RNase (50 μg/ml) for 1 hr at 37 C, phenol-extracted, dialyzed against 0.01 M Tris-HCl (pH 8) for 48 hr, and alcohol-precipitated by placing in a freezer for 48 hr. The nDNA was resuspended in 0.01 M Tris-HCl (pH 8) buffer and the UV spectrum obtained with a Beckman DB-G spectrophotometer. The ratio of the *A* at 260 to 280 nm always exceeded 1.8.

The supernatant fraction from which the crude nuclear fraction had been isolated was first recentrifuged at 1,465*g* for 10 min and then centrifuged at 14,600*g* for 20 min for the mitochondrial fraction. This mitochondrial pellet was resuspended in 100 ml of buffer (medium B) containing 0.3 M mannitol-0.05 M Tris-HCl (pH 8)-0.01 M $MgCl_2$-0.1% BSA-1 mM 2-mercaptoethanol, centrifuged at 17,000*g* for 10 min, resuspended in 40 ml of medium B, and treated with 50 μg/ml of DNase I for 1 hr on ice. Subsequently, 100 ml of medium C, containing 0.15 M NaCl-0.1 M EDTA (pH 8), was added to the mixture which was then centrifuged at 17,300*g* for 10 min. The resulting pellet was washed twice with 100 ml of medium C, resuspended in 10 ml of buffer containing 0.1 M Tris-HCl-EDTA (pH 8), lysed with N-lauroyl sarcosine added to a final concentration of 0.5%, and the lysed mitochondria extracted twice with equal volumes of phenol saturated with 0.1 M Tris (pH 12). The mtDNA was alcohol-precipitated by placing in a freezer for 48 hr. The precipitate was collected by centrifugation and dissolved in 2 ml of medium A. The mtDNA was then incubated at 37 C for 1 hr with 50 μg/ml of RNase, extracted twice with phenol, and dialyzed against 0.01 M Tris-HCl (pH 8) for 48 hr with five changes of 1 liter each.

Chloroplast DNA was isolated from approximately 600 g of 14- to 21-day-old leaves. These leaves were homogenized in 4 volumes of buffer (medium A) in a Waring Blendor and filtered through four layers of cheesecloth. The filtrate was centrifuged twice at

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100*g* for 10 min to remove nuclei and debris and the crude chloroplast fraction pelleted by centrifugation at 1,020*g* for 15 min. Subsequently, the chloroplast fraction was treated as described above for mitochondria except that the pelleting of chloroplasts was accomplished by centrifugation at 1,020*g* for 15 min.

DNA buoyant density was determined by analytical density gradient centrifugation in neutral CsCl with a Spinco model E analytical ultracentrifuge equipped with UV optics. Densitometer tracings were made with a Gescan automatic recording and integrating scanner. *Micrococcus luteus* DNA of density 1.7310 g/cm^3 was used as a marker. Calculations of buoyant density measurements have been described by Mandel *et al.* (18).

Isolation of mtDNA for Electron Microscopy. Mitochondrial DNA was isolated as for buoyant densities as previously described. However, the lysate was immediately placed on an ethidium bromide-cesium chloride gradient and centrifuged for 24 hr at 44,000 rpm in a 65-type rotor at 20 C. The lower band which contained supercoiled molecules was added to a second ethidium bromide-cesium chloride gradient and recentrifuged for an additional 24 hr. The mtDNA was removed from the gradient with a wide mouth pipette and allowed to sit for approximately 1 hr in the refrigerator before being mounted by the method of Kleinschmidt (13) for microscopy. The lower band from samples in which the mitochondria had not previously been treated with DNase I was also examined. The spreading of mtDNA for electron microscopy was done at 24 C with the protein monolayer picked up on parlodion-coated 200-mesh grids. Grids were stained with uranyl acetate (6) and subsequently shadowed from one direction with 1 cm of Pt: 20% Pd wire (Fullam), then rotated 90° and shadowed with a second 1 cm of wire. Grids were examined with a Siemens EM1A electron microscope at a magnification of 10,000 and the size of the molecules calculated with a replica grating (Fullam, 2160 lines/mm). Selected molecules were photographed and projected on a screen where they were traced and measured.

Denaturation of mtDNA. The mtDNA used in denaturation studies was isolated as for buoyant density measurements. However, after alcohol precipitation for 24 hr, the precipitated mtDNA was placed directly on a dye-buoyant density gradient and centrifuged for 24 hr. A single band was observed which consisted of linear and open circular molecules. This band was resuspended in a second dye-buoyant density gradient and centrifuged for another 24 hr. The DNA was removed from the gradient and dialyzed in subdued light against a total of 4 liters of TES (0.05 M Tris-5 mM EDTA-0.05 M NaCl, pH 8) buffer for 3 hr with four changes. Circular mtDNA molecules were partially denatured using the isodenaturing technique of Davis and Hyman (5). The hypophase consisted of 60% formamide (99% Matheson, Coleman and Bell) and 0.01 M Tris-EDTA (pH 8.5). The spreading solution consisted of 85 to 90 μl of formamide, 5 μl of Cyt *c* (1 mg/ml), and 5 μl of mtDNA which had been diluted four times with TES buffer.

RESULTS

Buoyant Densities of DNAs. The buoyant densities of both nDNA and mtDNA have been derived from four determinations from each of four separate isolations. Each type of DNA was observed as a single peak with a density of 1.694 ± 0.001 g/cm^3 for nDNA and 1.706 ± 0.001 g/cm^3 for mtDNA (Fig. 1). Yields of ctDNA were extremely low; however, the buoyant density of ctDNA was judged to be sufficiently different from that of mtDNA and similar to the value of 1.697 g/cm^3 which is that approximated for all other plant ctDNA (26). The percentage G-C content was calculated by the method of Schildkraut *et al.* (21) to be 34.7% for nDNA and 46.9% for mtDNA. The mtDNA buoyant density is in agreement with values reported from other higher plant species (22, 26). The constancy of mtDNA base composition in different species may indicate that these DNAs are highly conserved. For soybean mtDNA, this characteristic buoyant density may be utilized both as a means of separation and identification.

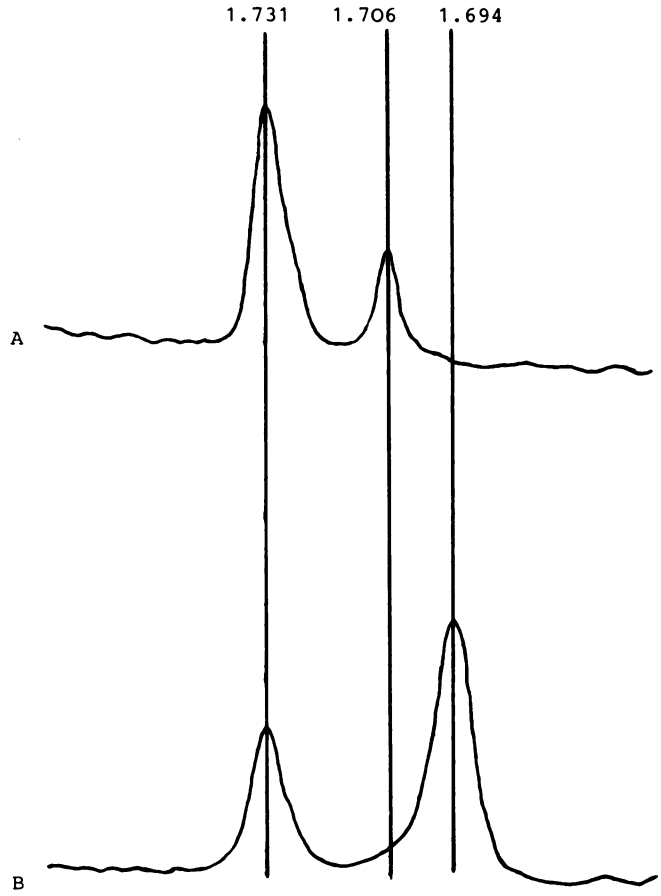

FIG. 1. Densitometer tracings after analytical density gradient centrifugation of (A) mtDNA; (B) nDNA. *Micrococcus luteus* DNA of density 1.7310 g/cm^3 was used as a marker.

Isolation of Circular mtDNA Molecules. When centrifuged to equilibrium in a dye-buoyant density gradient, the mtDNA was observed with UV light as two fluorescent bands which were separated by a distance of approximately 1 cm. After the lower band had been removed from the second ethidium bromide-cesium chloride gradient, the supercoiled form (Fig. 2) spontaneously converted to open circles and subsequently to linear molecules after storage at 0 C. One hundred seventeen open circles (Fig. 3) were photographed and measured. Size classes were arbitrarily established by pooling regions which appeared to have a concentration of similar size molecules. Circles of less than 3 μm were classified as minicircles. No supercoiled DNA band was observed from nuclei lysed and centrifuged to equilibrium in a dye-buoyant density gradient so that all circular DNAs are presumed to be organelle in origin. In addition, no difference was observed in the distributions of circular molecules between lower bands where mitochondria had or had not been treated with DNase I. This indicates that the observed circular molecules were contained within the mitochondria and that DNA from other sources did not contribute to the formation of the lower supercoiled band. The data from both types of preparations were combined (Fig. 4) and the DNase I treatment judged unnecessary. Seven apparent class sizes were observed with mean lengths of 5.9 ± 0.72 μm (class 1), 10 ± 0.78 μm (class 2), 12.9 ± 0.51 μm (class 3), 16.6 ± 1.08 μm (class 4), 20.4 ± 0.50 μm (class 5), 24.5 ± 0.45 μm (class 6), and 29.9 ± 0.41 μm (class 7). The size class which appeared to have the largest number of molecules was the 16.6-μm group. It should be noted that larger circular molecules are

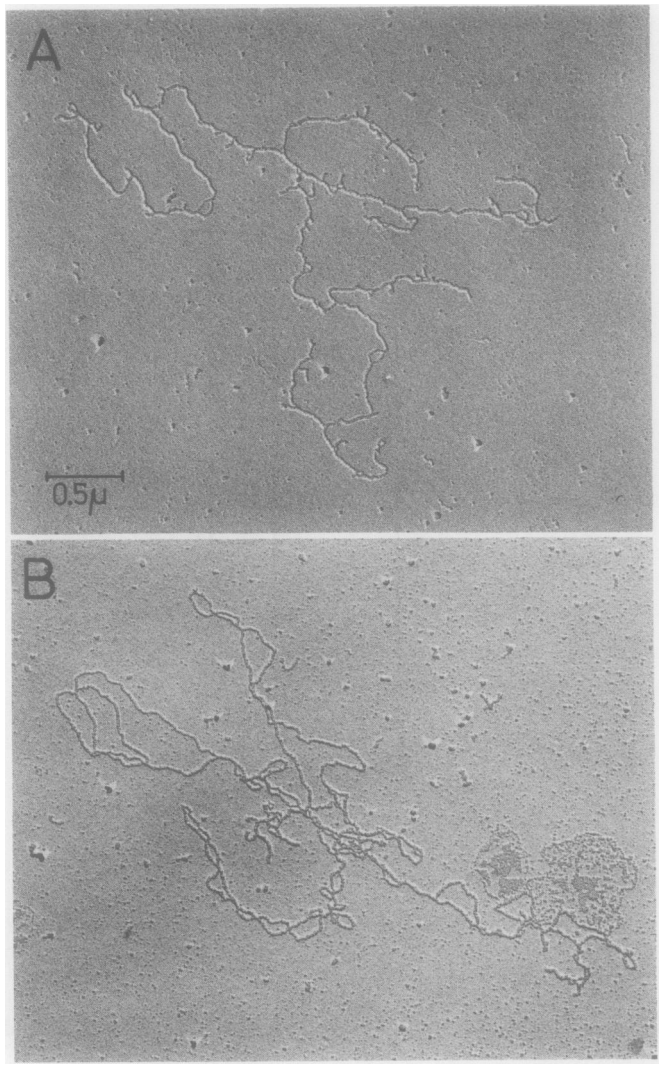

FIG. 2. Electron micrographs of a typical (A) supercoiled, circular DNA molecule; (B) supercoiled, circular DNA molecule converting to the open conformation, isolated from mitochondria.

isolated with greater difficulty. Consequently, the classes of larger molecules may actually occur with greater frequency than is shown by this distribution.

Denaturation of Molecules. Several separate mtDNA isolations were spread to obtain 19 intact, partially denatured circular molecules since more than 95% of all molecules were linear and could not be used in this analysis. With a hypophase of 60% formamide, denaturation was 2 to 15% for approximately 80% of all molecules. Molecules were photographed (Fig. 5), measured, and arbitrarily linearized. It has been shown that contour length decreases as DNA undergoes denaturation (9). Therefore, molecules were adjusted for the degree of denaturation according to the relationship between denaturation and contour length developed by Kolodner and Tewari (16). Circular molecules within each size class were also adjusted to the mean of that class and aligned for optimal fit (Fig. 6). In class 4 ($\bar{X}$ =·16.6 μm), a number of different denaturation patterns appear to exist, suggesting that these molecules have different nucleotide sequence arrangements. If it is assumed that each unique class of molecules is present in equal molar amounts, the fact that molecules of class 4 occur with the greatest frequency suggests that this class may be comprised of more than one unique class of molecules. Two molecules which have similar denaturation patterns and appear to be distinct from other molecules in class 4 were adjusted to 15 μm since their length closely approximated to that value. Other molecules in that class were adjusted to 17 μm. Despite the small number of intact, denatured molecules, several tentative observations can be made. At these levels of denaturation, there were large stretches of DNA of 2 to 14 μm in length which contained no denatured regions. These regions which are

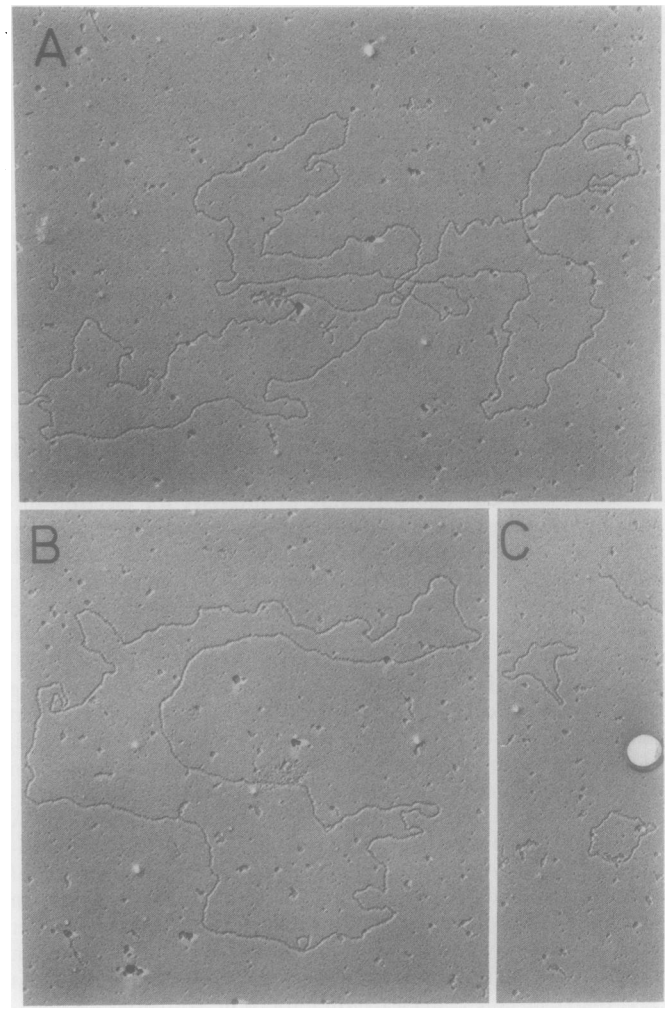

FIG. 3. Electron micrographs of circular mtDNA molecules with contour lenghts of (A) 29.2 μm; (B) 17.6 μm; (C) 1.3 and 1.7 μm.

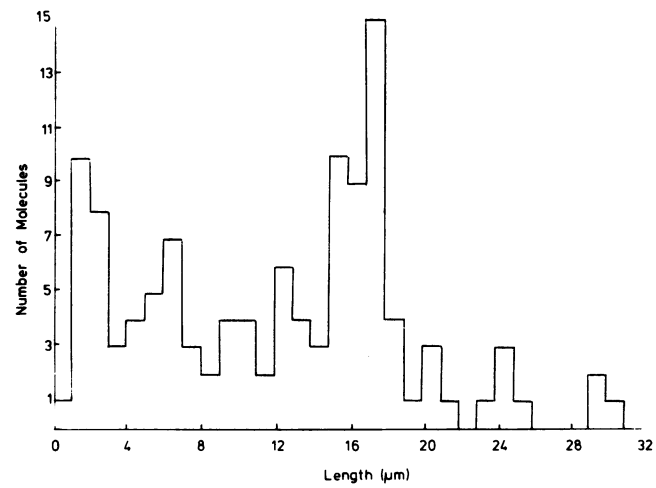

FIG. 4. Frequency distribution of circular mtDNA molecules isolated as supercoiled, circular DNA by ethidium bromide-cesium chloride density gradient centrifugation.

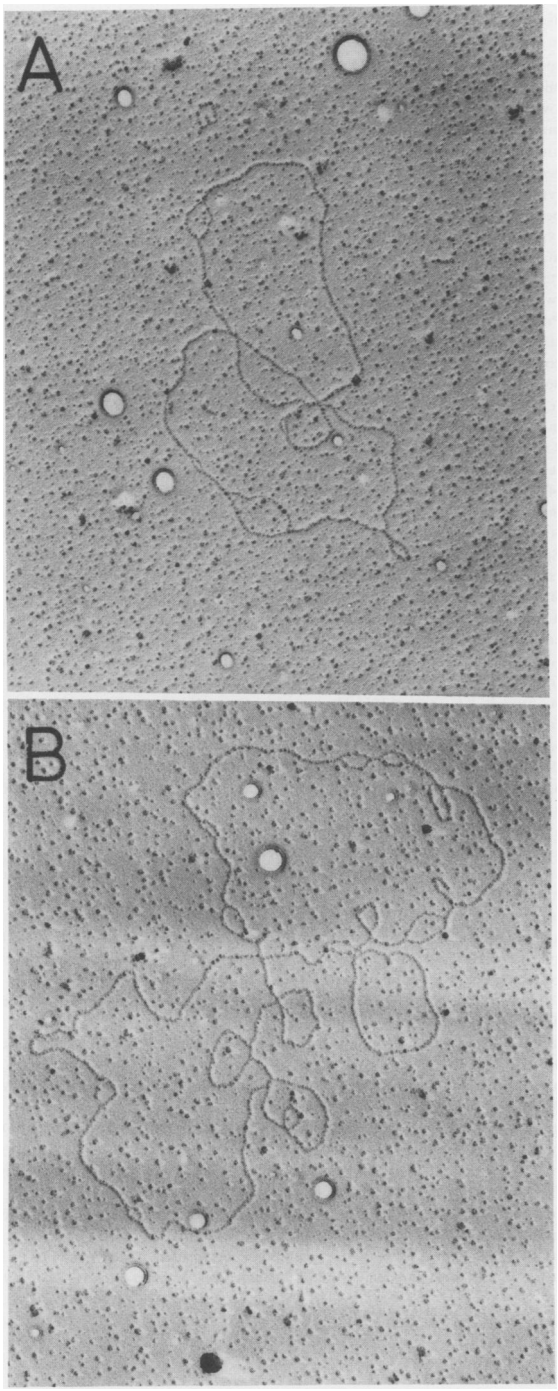

FIG. 5. Electron micrographs of partially denatured circular mtDNA molecules of (A) class 1 ($\bar{x}$ = 5.9 μm) with 11.1% denaturation; (B) class 4 ($\bar{x}$ = 16.6 μm) with 8% denaturation.

rich in G-C content may contain very similar nucleotide sequences or no similarity. In addition, some of these regions may contain small repeated units which are not observable within the undenatured stretch.

DISCUSSION

Mitochondrial DNA of soybean was isolated as a supercoiled molecule and is presumed to be maintained in this form within the mitochondria. Supercoiled mtDNA has been observed in other species of higher plants (Shah and Levings, manuscript in preparation) as well as with ctDNAs (15). It is expected, therefore, that when the proper isolation procedures are employed in other higher plant species, it will be demonstrated that their mtDNAs can be isolated as supercoiled molecules.

Kolodner and Tewari (14) reported that the mtDNAs of pea as well as spinach, lettuce, and bean could be isolated in a circular conformation with a contour length of 30 μm. Apparently, no variation in circle size was observed which is contrary to our results with soybean mtDNA. The 30-μm class is represented in our data; however, several smaller classes of circular molecules were also found in our preparations. The possibility of contaminating circular DNA molecules from bacterial sources or chloroplasts can be excluded for a number of reasons. Soybean ctDNA can be rejected because of its characteristic buoyant density, and its uniform 40-μm size (Synenki, unpublished data). A bacterial source can be rejected since there is no evidence to suggest the presence of the necessary large amounts of bacterial contamination and its absence from ctDNA. Kolodner and Tewari did observe linear molecules which were similar in length to several of our smaller classes of circular molecules. These linears may in fact have been the result of a break in the double helix of circular molecules of smaller size rather than fragments from the 30-μm circles. These different size molecules were probably not produced during the isolation procedures, since the generation of different size circular molecules after lysis of the mitochondria would require ligation and conversion to the supercoiled conformation prior to dye-buoyant density gradient centrifugation.

Several mechanisms could account for the heterogeneity observed in soybean mtDNA. Amplification of specific DNA sequences may occur, as during oogenesis in amphibian oocytes when DNA coding for ribosomal RNA undergoes extensive amplification. Concurrent with this amplification is an oligomeric series of circular molecules which is a result of this amplification process (11). However, there appears to be no oligomeric series and amplification analogous to this situation occurring in soybean mtDNA. The size distribution pattern of mtDNA molecules does not fit an oligomeric series and denaturation patterns do not show any evidence for repeated segments nor similarities which would place molecules of different classes in one or several series.

During the replication of pea ctDNA, dimers which are approximately 80 μm in length may be produced. These have been identified through denaturation mapping to be arranged in a head to tail fashion (16). Several of the larger size molecules of our data might be proposed to be multimeric forms of smaller molecules. Such a situation may exist with the 20.4-μm class since the denaturation pattern of the molecule in that class may be interpreted as being a dimer of the 10-μm group. Other multimeric classes do not appear to exist. Since other multimeric classes or extensively repeated segments were not observed in the denatur-

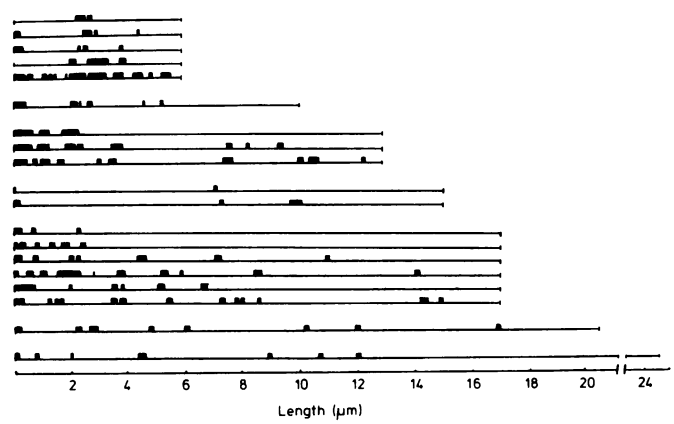

FIG. 6. Denaturation patterns of circular mtDNA molecules which have been arbitrarily linearized and aligned for optimal fit. The blocks represent denatured regions.

ation patterns, the genetic complexity of the mitochondrial genome appears to be inflated severalfold if the values of kinetic complexity for mtDNA from other plant species (14, 25) are utilized as a reasonable approximation for soybean mtDNA. Based on the heterogeneity of circle size and complexity of the denaturation patterns, it is proposed that the mitochondrial genome is dispersed among the different classes of circular molecules. Since there are several times more DNA than expected, several copies of the mitochondrial genome are probably contained among the various size molecules.

One possible method by which circular molecules of different sizes may be generated is through recombinational events with an unequal exchange of material between molecules. Recombination between plasmids in bacterial systems commonly occurs (2) and therefore, this phenomenon should be considered. It is generally accepted that recombination requires homology between nucleotide sequences although this is not necessary for specialized types of recombination (3, 4). Since homologies likely exist between regions of different size circles, exchange of material among circles may occur with sufficient frequency to generate new classes of circles. This recombination could function in a homogeneous population of mitochondria, or in a heterogeneous population where a single mitochondrion contains a single size of circular DNA. Recombination in the latter case would occur only after fusion of the mitochondria had occurred. Some heterogeneity may exist among mitochondria as a result of recombination and segregation of these circular molecules. It seems, however, that if a mitochondrion is to survive, it would have to contain at least a minimal amount of genetic information. More heterogeneity than has been detected may actually exist since the limits of resolution of the denaturation technique and the low number of intact molecules obtained for analysis may not have revealed all variation which was present. In fact, the lack of discrete size classes as shown in the distribution of circular molecules suggest that this is the case.

The significance of minicircles in soybean is unknown. Minicircles have previously been observed in a variety of eucaryotes (14, 23, 24, 28) and in most cases, are heterogeneous in size. Only in yeast, where oligomycin resistance appears to be due to the presence of a 2.2-μm molecule, has a function been shown to be associated with minicircles (8). This yeast minicircle also has been shown to contain two inverted repeats of the same sequence (7, 10), similar to transposable elements in bacterial systems. It has been suggested that this sequence organization may enable insertion of DNA at random sites in the genome. Similarly, the soybean minicircles may function in some recombinational capacity.

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