Isolation and Characterization of Mitochondrial Deoxyribonucleic Acid of *Acanthamoeba castellanii*

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DNA was prepared from isolated mitochondria of *Acanthamoeba castellanii* and was shown to behave as a single component in density gradients, on 'melting' and on renaturation. From measurements of renaturation kinetics, sedimentation coefficient and electron micrographs the genome size of the mitochondrial DNA was calculated to be about 3.4×10^7 daltons. A small proportion of the preparations could be isolated as relaxed circular molecules of mean contour length 16.2 μm .

The genome size of mitochondrial DNA in a variety of organisms has been established either by measurements of the physical size of the DNA molecule or by determination of its kinetic complexity (Wetmur & Davidson, 1968) or by both methods (Ashwell & Work, 1970). Although mitochondrial DNA of higher animals universally occurs as closed circular molecules of contour length approx. 5 μm with a corresponding kinetic complexity of 10^7 daltons, in organisms outside of this group a much larger mitochondrial DNA seems to be present. Sizes found range from 3.0×10^7 to 3.5×10^7 daltons for *Tetrahymena pyriformis* (Flavell & Jones, 1970b) through about 5×10^7 daltons for yeast (Hollenberg *et al.*, 1970) to about 7×10^7 daltons for higher plants (Kolodner & Tewari, 1972). Of those mitochondrial DNA species that have been studied in detail, *Tetrahymena* mitochondrial DNA appears to be unique in that no circular molecules have been isolated (Clegg *et al.*, 1974). It is not known whether the unusual size and structure of *Tetrahymena* mitochondrial DNA is typical of protozoa although its genome size is similar to that of another ciliate protozoan *Paramecium aurelia* (Flavell & Jones, 1971). The studies reported here on the mitochondrial DNA of *Acanthamoeba castellanii*, a member of a quite distinct group of the protozoa, show that it has a genome of similar size to *Tetrahymena* which can be isolated in a circular form.

Partial purification and characterization of *Acanthamoeba* mitochondrial DNA has been reported by Adam *et al.* (1969).

Experimental

Growth of *Acanthamoeba castellanii*

A. castellanii (Neff strain) was obtained from the Culture Collection of Algae and Protozoa,

Cambridge, U.K. and grown axenically in the medium of Neff & Neff (1964). All cultures were incubated on a gyrotory shaker at 28–30°C. Stock cultures (100 ml) contained in 500 ml conical flasks were subcultured every 3 days. Large cultures (2 litres) contained in 4-litre conical flasks were inoculated with about 30 ml from a stock culture and harvested after 3 days when in late-exponential phase. A yield of 20–25 ml of packed cells from each flask was usually obtained. Cultures were checked as a routine for bacterial contamination by plating on nutrient agar.

Preparation of mitochondria

Cultures were harvested by centrifuging at 500g for 10 min at room temperature. All subsequent operations were conducted at 2–4°C. The cells were washed once in water, resuspended in 0.3M-sucrose containing 10mM-Tris, 0.2M-EDTA and 0.1% (w/v) bovine serum albumin adjusted to pH 7.4 with HCl, and centrifuged at 500g for 10 min. The washed cells were resuspended in 5 vol. of the same buffer and homogenized by passing through a domestic cream homogenizer; approx. 50% cell breakage was obtained. The homogenate was centrifuged at 500g for 10 min, then the pellet of unbroken cells was resuspended in 5 vol. of buffer and again homogenized. After centrifuging at 500g for 10 min the supernatants were combined. Overall about 90% cell breakage was obtained. The combined supernatants were centrifuged at 5500g for 10 min, and the crude mitochondrial pellet thus obtained was resuspended gently in buffer. After centrifugation at 500g for 10 min to remove remaining cell debris, the mitochondria were washed twice by centrifugation at 5500g for 10 min. In some preparations, the mitochondria were incubated with 50 μg of DNAase*/ml

*Abbreviations: DNAase, pancreatic deoxyribonuclease; RNAase, pancreatic ribonuclease; SSC buffer, 0.15M-NaCl–0.015M-trisodium citrate, pH 7.0.

for 30 min in a buffer consisting of 0.3M-sucrose-10mM-Tris-HCl-5mM-MgCl₂, pH7.4, to remove contaminating nuclear DNA. After this treatment, the mitochondria were washed three times by centrifugation in the extraction buffer.

Extraction of mitochondrial DNA

Method 1. DNAase-treated mitochondria from 20 ml of packed cells were suspended in 20 ml of saline-EDTA (0.15M-NaCl-0.015M-EDTA, pH8.0) and diethylpyrocarbonate and sodium dodecyl sulphate were added to final concentrations of 0.2% (w/v) and 0.5% (w/v) respectively. Lysis occurred immediately and 20 ml of phenol saturated with saline-EDTA was added. The mixture was shaken gently at 4°C for 15 min, 20 ml of chloroform was added and shaking continued for 10 min. The phases were separated by centrifugation and the treatment with phenol and chloroform repeated on the aqueous phase. Phenol was extracted from the aqueous layer with ether, RNAase (free of DNAase) was added to a final concentration of 20 µg/ml and then the solution was incubated at 25°C for 30 min. The extraction with phenol was repeated. Further purification of mitochondrial DNA was achieved by chromatography on hydroxyapatite (Flavell & Jones, 1970a).

Method 2. Mitochondria that had not been treated with DNAase were resuspended in saline-EDTA and lysed by the addition of sodium dodecyl sulphate to a final concentration of 1% (w/v). Solid CsCl was added to give a density of 1.60 g/ml, and the solution was incubated at room temperature for 1 h. The solution was centrifuged at 12000g for 30 min and the floating layer of protein that formed was discarded. The clear solution was divided into two portions: one was adjusted to $\rho = 1.662$ g/ml and the other to $\rho = 1.758$ g/ml by the addition of solid CsCl. The light solution (6.5 ml) was carefully layered over 5.5 ml of the dense solution in a 40 ml polypropylene centrifuge tube which was then completely filled with liquid paraffin. The tubes were centrifuged at 42000 rev./min at 5°C for 20 h in the 8 × 40 ml angle rotor of the MSE SuperSpeed 65 ultracentrifuge. The tubes were pierced and fractions collected manually. Those fractions containing mitochondrial DNA were combined, diluted at least fourfold with SSC buffer and the DNA was recovered by centrifugation at 150000g for 12 h. Pellets were dissolved in 0.1 × SSC buffer.

Method 3. Mitochondria, isolated from 5 ml of packed cells without a DNAase treatment, were suspended in 3 ml of saline-EDTA and lysed by the addition of sodium dodecyl sulphate to a final concentration of 1% (w/v). The lysate was extracted once with phenol saturated with saline-EDTA, and the residual phenol extracted with ether. The solution

was layered on a 17.5 ml isokinetic sucrose gradient and centrifuged in the 3 × 23 ml rotor of the MSE SuperSpeed 65 ultracentrifuge for 20 h at 24000 rev./min at 5°C. The sucrose gradient contained 50mM-Tris-HCl-10mM-EDTA, pH7.3, and extended from 27 to 37% (w/v) sucrose. The tube was pierced and 1 ml fractions were collected manually while monitoring E_{254} continuously. Selected fractions were combined, diluted fourfold with buffer and the DNA was recovered by centrifugation at 150000g for 12 h.

Whole-cell DNA

Whole-cell DNA of *Acanthamoeba* was prepared as described for *Tetrahymena pyriformis* by Flavell & Jones (1970a).

Electron microscopy

DNA was prepared for electron-microscopical examination by the method of Davis *et al.* (1971). Monolayers were spread on a hypophase of 0.25M-ammonium acetate and picked up on formvar films supported on nickel grids (200 mesh). Grids were rotary shadowed with 14 mg of Pt-Pd (80:20, w/w) from a distance of 10 cm at an angle of 7°. Electron micrographs were made with a Jeol Jem 7A electron microscope. Magnifications were calibrated with a diffraction grating replica (2160 lines/mm) or by the inclusion of relaxed circular bacteriophage PM2 DNA in the specimen.

Sedimentation coefficients of native DNA were determined by the method of Bruner & Vinograd (1965). Measurements of thermal denaturation, renaturation kinetics, buoyant density of DNA and sedimentation coefficients of sheared DNA at alkaline pH, and preparations of bacteriophage T4 DNA and *Micrococcus lysodeikticus* DNA were done as described by Flavell & Jones (1970a,b). Bacteriophage PM2 DNA was prepared as described by Espejo & Canelo (1968).

Results

Whole-cell DNA of *Acanthamoeba* showed two components of buoyant densities 1.694 g/ml and 1.720 g/ml when examined in CsCl gradients in the analytical ultracentrifuge (Fig. 1a). DNA isolated from mitochondria without DNAase treatment also contained both components, but the lighter component was enriched about fivefold (Fig. 1b). Only the lighter component was present after treatment of the mitochondria before lysis with DNAase (Fig. 1c). We therefore attribute the density of 1.694 g/ml to mitochondrial DNA and the density of 1.720 g/ml to nuclear DNA in agreement with the observations of

Fig. 1. Analytical CsCl density gradients

Microdensitometer tracings of u.v. photographs of DNA from *Acanthamoeba* at equilibrium in CsCl density gradients. All samples contained *Micrococcus lysodeikticus* DNA (ρ 1.731) as a reference, had an initial density of 1.710 g/ml and were centrifuged for 22 h at 45000 rev./min at 25°C. (a) Whole-cell DNA; (b) DNA from washed mitochondria; (c) DNA from mitochondria that had been treated with DNAase.

Adam *et al.* (1969). These buoyant densities correspond to base compositions of 35% G+C and 61% G+C respectively.

The yield of mitochondrial DNA was somewhat dependent on the concentration of bovine serum albumin in the extraction medium, showing a maximum of about 7 $\mu\text{g}/\text{ml}$ of packed cells at 0.1% (w/v) bovine serum albumin. The yield was decreased by about 50% when albumin was omitted or when its concentration was raised to 0.2% (w/v).

Mitochondrial DNA of similar purity although in somewhat lower yield could be prepared by method 2.

Fig. 2. Thermal denaturation of mitochondrial DNA

A DNA solution (20 $\mu\text{g}/\text{ml}$) in SSC buffer was heated at each temperature for 10 min. The temperature was measured with a calibrated thermocouple in the sample cell.

Fig. 2 shows a thermal-denaturation profile of purified mitochondrial DNA. The hyperchromicity of 40% indicates that the DNA is free from contamination with RNA and other u.v.-absorbing species. T_m is 83°C which corresponds to a base composition of 34% G+C, in good agreement with the value deduced from the buoyant density. The sharpness of the thermal transition suggests that the mitochondrial DNA is relatively homogeneous in base composition.

Denaturation of mitochondrial DNA, followed by rapid cooling caused an increase of buoyant density to 1.710 g/ml, whereas after incubation of the denatured DNA at 65°C for 2 h at a concentration of about 20 $\mu\text{g}/\text{ml}$, the buoyant density fell to 1.696 g/ml, which was close to the native DNA value. The renatured DNA exhibited a thermal transition essentially indistinguishable from that of native DNA. These experiments indicate that *Acanthamoeba* mitochondrial DNA, in common with other mitochondrial DNA species, is of relatively low complexity.

Quantitative measurements of the rate of renaturation of denatured sheared DNA were made as described by Flavell & Jones (1970b). Fig. 3 shows the renaturation of mitochondrial DNA and of bacteriophage T4 DNA, plotted in terms of a second-order reaction, from which the second-order rate constant

(k_2) can be calculated (Wetmur & Davidson, 1968). The mitochondrial DNA renatures as a single component up to at least 80% renaturation. The sedimentation coefficient of the sheared DNA at pH13 was determined and the observed values of k_2 were corrected to a sedimentation coefficient of 5S, by assuming that k_2 is proportional to $(s_{20,w}^{pH13})^{1.25}$ as shown by Wetmur & Davidson (1968). Bacteriophage T4 DNA was chosen for comparison since its base composition is almost identical with that of mitochondrial DNA; Wetmur & Davidson (1968) showed that the relationship between complexity and rate constant depends on the base composition and

Fig. 3. Second-order rate plot of the renaturation of mitochondrial DNA

DNA solutions (20 µg/ml) in 1M-NaCl-0.2mM-EDTA-5mM-Tris-HCl, pH7.8, were denatured in the spectrophotometer by heating to 99°C for 5 min, cooled quickly to 66°C and E_{260} was measured continuously. $E_{260(\infty)}$ and $E_{260(t)}$ are the E_{260} values for the fully renatured DNA and for the partially renatured DNA at time t (min) respectively. ○, *Acanthamoeba* mitochondrial DNA; ●, bacteriophage T4 DNA.

hence, to avoid ambiguity, it is essential to use, as a kinetic standard, a DNA of closely similar base composition. From the ratio of the rate constants for bacteriophage T4 DNA and mitochondrial DNA, and by assuming a complexity of 1.3×10^8 daltons for bacteriophage T4 DNA (Abelson & Thomas, 1966), the kinetic complexity of mitochondrial DNA can be calculated to be 3.4×10^7 daltons (Table 1). The measurements in the two buffers agree reasonably well and are probably within the experimental error of the method. The rate constant observed for bacteriophage T4 DNA in the buffer containing 1M-Na⁺ agrees well with reported values (Wetmur & Davidson, 1968; Wells & Birnstiel, 1969; Flavell & Jones, 1970b).

Sedimentation coefficients of various preparations of mitochondrial DNA were determined by the method of Bruner & Vinograd (1965) by using bulk solutions of CsCl of densities between 1.250 g/ml and 1.400 g/ml, and converted into $s_{20,w}$ values for DNA (sodium salt). Mitochondrial DNA prepared by method 1 sedimented as two components, the major component (about 60–75%) at 20S and the minor component at 36S. The proportion of the 36S component was quite variable and decreased rapidly after storage of the DNA. Preparation of DNA by method 2 was introduced in an effort to decrease the degradation which we assumed to be occurring during the phenol extractions and hydroxyapatite chromatography involved in method 1. Sedimentation analyses of these preparations showed a much greater proportion of the 36S component, in some cases as much as 95%. The mean value of $36 \pm 0.4S$ for DNA (sodium salt) calculated from measurements in CsCl, may be compared with a value of $35.0 \pm 1.0S$ measured directly in NaCl solutions.

A sedimentation coefficient of 36S corresponds to a mol.wt. of 3.5×10^7 (Studier, 1965) if we assume that this component consists of linear molecules (see below), and the good agreement of this molecular weight with the kinetic complexity of the DNA leads

Table 1. Renaturation kinetics of *Acanthamoeba* mitochondrial DNA

Measurements were made at $T_m - 25^\circ C$. Na⁺ buffer is 1.0M-NaCl-0.2mM-EDTA-5mM-Tris-HCl, pH7.8.

DNA sample	Buffer	$s_{20,w}^{pH13}$	DNA(µg/ml)	k_2		$10^{-7} \times$ Kinetic complexity
				Observed (litre · mol ⁻¹ · s ⁻¹)	Corrected*	
Mitochondrial DNA	1M-Na ⁺	5.9	20	114.5	93	3.17
	2 × SSC	5.2	10	48.2	45.8	3.50
	2 × SSC	5.2	15	48.6	46.2	3.46
	2 × SSC	5.2	20	48.9	46.6	3.42
Bacteriophage T4	1M-Na ⁺	5.5	20	25.5	22.7	
	2 × SSC	4.8	20	11.6	12.3	

* Values corrected to a standard sedimentation coefficient of 5S.

EXPLANATION OF PLATE I

Electron micrograph of relaxed circular mitochondrial DNA molecules

The 41S fraction of mitochondrial DNA was prepared as described under 'Method 3'. The bar represents 1 μ m.

Fig. 4. Frequency distribution of molecular lengths

DNA molecules were examined in the electron microscope as described in the Experimental section. (a) Mitochondrial DNA prepared by method 2; (b) DNA from lysate of mitochondria; (c) relaxed circular DNA prepared by method 3.

us to believe that the 36S component corresponds to genome-size linear molecules.

In most of the preparations made by method 2, a minor component sedimenting at 40–45S was present. In no case was the amount of this component greater than about 5% of the total. This component was isolated by sucrose-density-gradient centrifugation (method 3) and its sedimentation coefficient determined to be 41S. Since a DNA molecule of mol. wt. 3.5×10^7 would sediment at about 40S as a relaxed circle (Hudson *et al.*, 1968) it seemed probable that a small proportion of the preparations consisted of relaxed circles. No component sedimenting more rapidly than 45S, which might be closed circular DNA, was ever observed.

Electron microscopy

Mitochondrial DNA, prepared by method 1, contained few molecules in excess of $5 \mu\text{m}$ in length and was obviously extensively degraded, as suggested by its sedimentation coefficient. However, when DNA prepared by method 2 was examined, molecules ranging in length up to $18 \mu\text{m}$ were found; Fig. 4(a) shows a frequency distribution of lengths for such a preparation based on measurements of about 80 molecules. It can be calculated that at least 50% by weight of this preparation consists of molecules between 15 and $18 \mu\text{m}$ in length. The two groups of lengths probably represent full-length and half-length molecules. When a lysate of mitochondria was prepared for electron microscopy without any prior purification, a much higher proportion of molecules between 15 and $18 \mu\text{m}$ was observed (Fig. 4b). The mean length of the molecules was $16.7 \mu\text{m}$, corresponding to a mol. wt. of about 3.3×10^7 , which is in good agreement with the estimates made by sedimentation and renaturation. In none of these preparations could any circular molecules of $16 \mu\text{m}$ contour length be unambiguously identified. A few attempts were made to observe DNA molecules released from mitochondria by osmotic shock, but although linear molecules of about $16 \mu\text{m}$ were found, no circular molecules were seen.

When the 41S component isolated from a sucrose density gradient was examined it was found to consist almost entirely of relaxed circular molecules. A typical molecule is shown in Plate 1 and the length distribution in Fig. 4(c); the mean length was $16.2 \mu\text{m}$. No precise estimate of the quantity of this component present in the original preparation could be made, but it is certainly less than 5%.

Discussion

The isolation of mitochondrial DNA from purified mitochondria of *Acanthamoeba* has not been reported, although a minor component of buoyant density 1.693 g/ml was isolated from whole cell DNA by Adam *et al.* (1969) and partially characterized. However, this component showed biphasic 'melting' and renaturation and was almost certainly contaminated with nuclear DNA. In contrast, our preparations behave as a single component in analytical CsCl gradients, on 'melting' and on renaturation.

The physical estimates of molecular size of the least degraded preparations agree well with the kinetic complexity and it is clear that, on the one hand, no repetitive sequences are present within the genome, and on the other, no significant sequence differences exist within the population of mitochondrial DNA molecules. Since none of our estimates of size is absolute, the mean value of about 3.4×10^7 daltons is probably indistinguishable from those reported for

Tetrahymena pyriformis (Flavell & Jones, 1970b) and *Paramecium aurelia* (Flavell & Jones, 1971). Preliminary studies by Manning *et al.* (1971) of *Euglena gracilis* mitochondrial DNA suggest that this may also be of a similar size. Studies of a wider range of protozoa are obviously necessary before any conclusions can be reached about the significance of this unusual size of mitochondrial DNA.

The isolation of genome-sized molecules in good yield proved to be difficult. Since a molecule of 3.4×10^7 daltons should not be unduly sensitive to shear and the usual precautions against shear were taken, it seems unlikely that this is a major cause of degradation. It is much more probable that endogenous nuclease action is responsible since, in common with most protozoa, *Acanthamoeba* seems to have high nuclease activity (Adam *et al.*, 1969). Another possible source of damage to the molecule is common to all preparations of mitochondrial DNA: depurinated sites and 'nicks' seem to be produced by free-radical reactions during mitochondrial isolation (Bode, 1967). Since even linear molecules were usually partly degraded, it is perhaps not surprising that such very low amounts of circular molecules could be isolated and that all circular molecules which were observed, were relaxed molecules. An alternative explanation, which our results do not exclude, is that breakage of a circular replication intermediate is a normal event in these mitochondria. This is, however, rendered unlikely by the work of Bohnert (1973), who found that up to 35% of the light component of whole-cell DNA (i.e. probably mitochondrial DNA) consisted of relaxed circular molecules. Some closed circular molecules were also present in his preparations. It seems most probable therefore that limited degradation during mitochondrial isolation is the cause of the low proportion of circular molecules isolated.

Whatever the proportion of circular molecules may be *in vivo*, it is clear that they are present. *Tetrahymena* remains unique as the only system in which it has been necessary to postulate wholly linear

intermediates in mitochondrial DNA replication (Clegg *et al.*, 1974).

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