MITOCHONDRIAL DNA FROM *ASPERGILLUS NIDULANS*

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1. Introduction

Extranuclear mutants have been isolated recently in the Ascomycete fungus *Aspergillus nidulans* [1,2], and cycloheximide-resistant incorporation of labelled amino acid into mitochondrial proteins has been demonstrated [3]. Although the size and shape of mitochondrial DNA in yeast [4] and *Neurospora crassa* [5,6] has been determined, there has been no similar report for mitochondrial DNA of *Aspergillus*.

In the present paper, we report the presence in Aspergillus mitochondria of DNA which appears to be smaller than that reported in yeast and Neurospora.

2. Methods

Strain R21 (a *p*-aminobenzoic acid auxotroph) was grown as described previously [3]. When labelled mitochondrial DNA was required, strain UP9 (an adenine auxotroph [1]) was used, and 2 [^{3}H] adenine (0.5 μCi/ml) plus 50 μg/ml unlabelled adenine were added to the medium with the inoculum. Mitochondria were isolated [7] and purified [3] as described previously.

Total cell DNA was isolated from 5 g of squeezed mycelium previously washed in distilled water and ground to a powder in liquid N_2. The powder was incubated in saline-SDS (0.15 M NaCl, 0.1 M EDTA, 6% sodium dodecyl sulphate, pH 9.0) for 30 min at 70°C, deproteinized with phenol–cresol and the DNA spooled out according to Wood and Luck [8]. This was dissolved in 2 ml 0.1 X SSC, treated with RNase, deproteinized, re-spooled and finally dissolved in 4 ml of 0.1 X SSC. It was then dialyzed against the same buffer.

For mitochondrial DNA, mitochondria purified from 100 g mycelium were resuspended in 4 ml of mitochondrial isolation buffer [7], pancreatic DNase I was added to 50 μg/ml, $MgCl_2$ to 7 mM [9] and the mixture was incubated at 4°C for 30 min. The mitochondria were pelleted, washed with isolation buffer (10 ml) and dissolved in 4 ml of saline-SDS at 70°C. After a 5 min incubation, the suspension was extracted with phenol–cresol and precipitated with ethanol. The precipate was dissolved in 4 ml 0.1 X SSC, incubated with RNase [8] and dialysed as for total DNA.

Following addition of CsCl to a density of 1.70 g/ml, the DNA was centrifuged to equilibrium at 105 000 g_{av} for 40 h.

Renaturation experiments were carried out in 0.1 X SSC (10–15 μg DNA/ml) according to Bultmann and Laird [10] at 65°C for phage λ DNA, 59°C for phage T4 DNA and 57°C for mitochondrial DNA following heat denaturation and shearing of the DNA to an average size of 4×10^6 daltons as assessed by sedimentation velocity in sucrose gradients. Phage T4 and Phage λ DNA were purified by the method of Freifelder [11]. Mitochondrial DNA was prepared for electron microscopy by an osmotic shock procedure, spreading a crude mitochondrial fraction from 4 M ammonium acetate onto a hypophase of chilled water [12].

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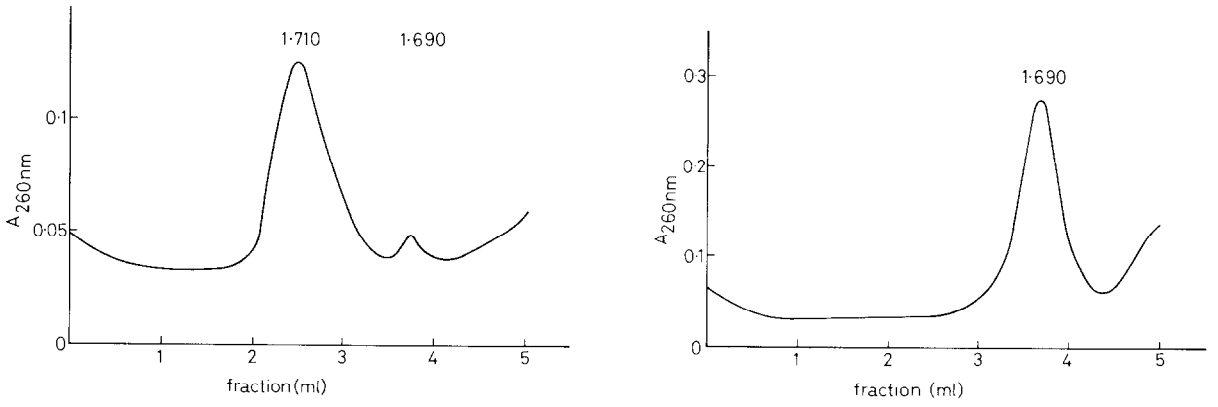

Fig.1. Ultracentrifugation of DNA on CsCl gradients. Following centrifugation, the gradient was pumped through a Uvicord and the absorbance monitored at 260 nm. The densities were measured in a refractometer at 25°C. (a) Total DNA. (b) Mitochondrial DNA.

3. Results and discussion

The buoyant density distribution of total DNA (fig.1a) shows a major peak of density 1.710 g/ml and a minor one of density 1.690 g/ml. This minor peak corresponds to the purified mitochondrial DNA (fig.1b). These densities, calculated from a preparative CsCl gradient, are in good agreement with those of Edelman et al. [13] obtained by analytical ultracentrifugation. The amount of the mitochondrial DNA is about 1–2% of the total DNA and shows a hyperchromicity of 36–40%. Contamination of the mitochondrial DNA by nuclear DNA was not observed even if the DNase treatment of purified mitochondria was omitted.

The renaturation constant was used as an approach to determine the genetic complexity of the mitochondrial DNA. Second order rate plots of the renaturation of the mitochondrial and two reference DNAs (phages λ and T4) are represented in fig.2. There is no evidence for the presence of fragments renaturing more rapidly than the bulk of the DNA, suggesting that there is no major repetition of genes in this DNA. The interpretation of the results (table 1) is not straightforward, since the renaturation constants of the reference DNAs are not exactly indirectly proportional to the molecular weight. It has been pointed out that the GC content of the DNA is important in low salt concentrations [4], thus the value for mitochondrial DNA was calculated using the T4 standard since the GC contents

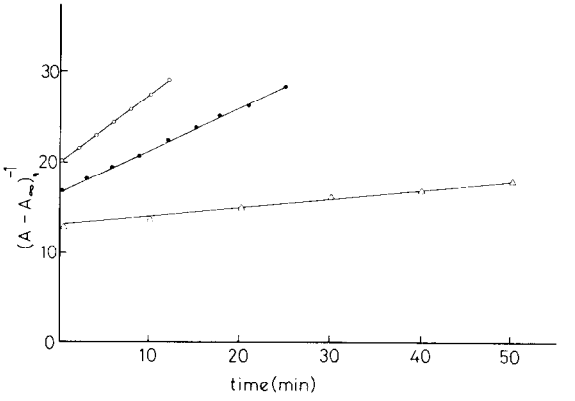

Fig.2. Second order plot of the renaturation of phages T4, lambda and mitochondrial DNA. The results are expressed according to Wetmur and Davidson (14). (○), Phage lambda. (●), Mitochondrial. (△), Phage T4.

are similar. This gives an approximate molecular weight of 26×10^6 for the mitochondrial DNA.

The sedimentation velocity of purified mitochondrial DNA in a preparative sucrose gradient indicated a mol. wt of less than 10×10^6, probably due to the fragmentation of the DNA during the purification. To overcome this problem, 2 [^{3}H] adenine-labelled mitochondrial DNA was centrifuged following direct lysis of whole purified mitochondria on a CsCl gradient (fig.3). This DNA was centifuged in an ethidium bromide-CsCl gradient (fig.4) and showed a minor

Table 1
Renaturation constants of lambda, T4 and mitochondrial DNA

DNA	Renaturation constant $l \cdot mole^{-1} \cdot sec^{-1}$	Genome size daltons	GC content %
Phage λ	65	33×10^6	49
Phage T4	7.0	13×10^7	34
Mitochondrial	35		31

The figures given are the average of 3 determinations in each case. The molecular weight and GC content of the phage DNA were obtained from Wetmur and Davidson [14] and Thomas and McHattie [15].

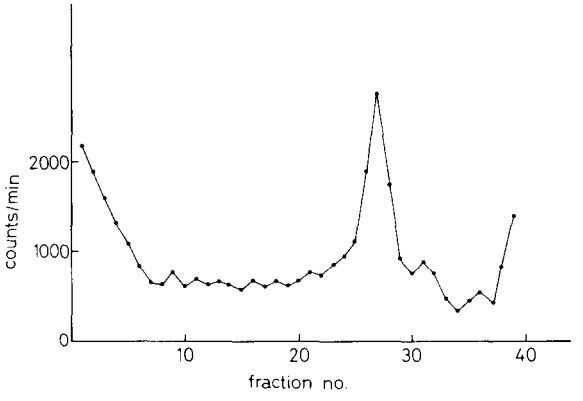

Fig.3. CsCl ultracentrifugation of labelled mitochondrial DNA. Frozen pellets of purified mitochondria were dissolved in 4 ml saline-SDS at 70°C and incubated for 30 min. The SDS was removed by centrifugation following chilling to 4°C. The supernatant was adjusted to a density of 1.70 g/ml with CsCl and ultracentrifuged. Estimation of radioactivity was carried out according to Grinsted et al. [16].

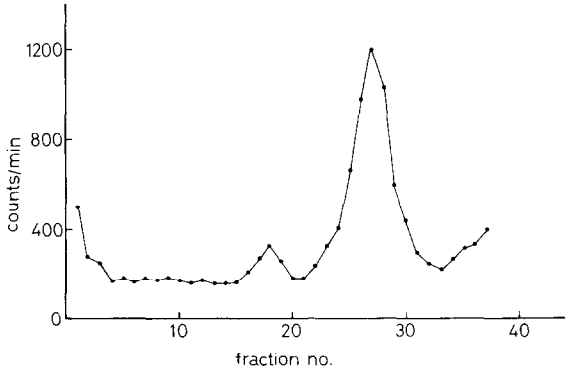

Fig.4. Ethidium bromide/CsCl centrifugation. The labelled mitochondrial DNA obtained as described in fig.3 was recentrifuged in an ethidium bromide/CsCl gradient [16].

band on the denser side of the main band. The sedimentation velocity of these bands was determined by centrifugation on sucrose gradients (fig.5) using Staphylococcal plasmid reference DNAs of 34s and 49s [17]. The minor band gave rise to a 53s peak and the major band to 34 and 23s peaks. The appearance of the small band on the CsCl/ethidium bromide gradient suggests that at least a small amount of the mitochondrial DNA is in the form of covalently closed circular DNA, and assuming that the 53s peak corresponded to this DNA and the 34s peak to open circular DNA, a mol wt of $20–25 \times 10^6$ is indicated [18]. The 23s peak may represent degraded linear DNA, though it cannot be ruled out that other species of mitochondrial DNA may be present.

Electron microscopy on DNA released by the osmotic shock procedure revealed the presence of a few

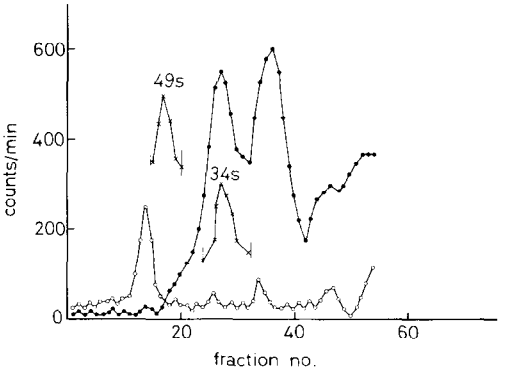

Fig.5. Sedimentation velocity of mitochondrial DNA. The minor peak (○) and major band (●) from the ethidium bromide/CsCl gradient were layered on sucrose gradients with 49s and 34s reference DNA's (X—X) [17] and centrifuged according to Grinsted et al. [16].

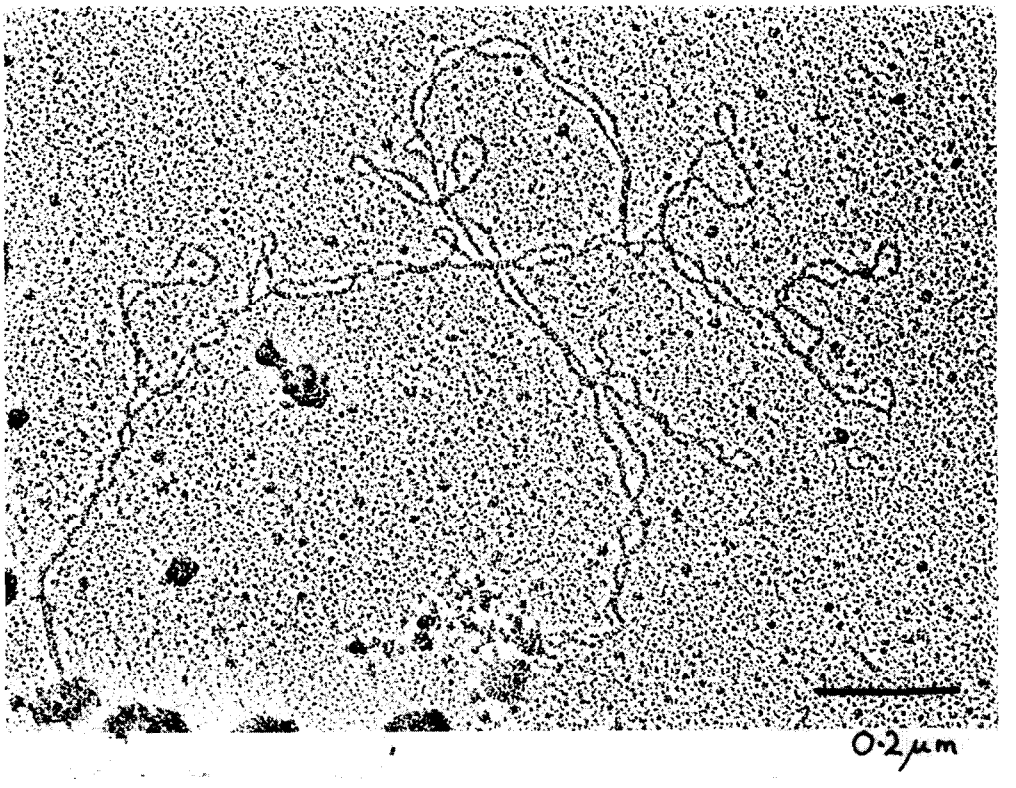

Fig.6. Electron micrograph of twisted circular DNA molecule prepared as described in the text. Length 10.6 μm. (Preparation by Dr A. C. Arnberg, Biochemistry Laboratory, University of Groningen, Holland.)

twisted circles (fig.6), though these were rare and usually partially covered by debris making accurate measurement of the total length difficult. Nevertheless, the measured length was in the range of 10–10.6 μm, indicating a mol wt of approx. 20–21 × 10^6.

The three approaches used point to a mol. wt in the range of 20–26 × 10^6 for the mitochondrial DNA of *Aspergillus nidulans*, which is substantially smaller than figures obtained for *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis* (50 × 10^6 [4]) and *Neurospora crassa* (40 × 10^6 [5,6]), other Ascomycetes, and even smaller than *Tetrahymena pyriformis* mitochondrial DNA (35 × 10^6 [19]). It has been suggested that the downward trend in the size of mitochondrial DNA from simple eukaryotes such as yeast to that of higher animal cells represents a gradual evolutionary loss of mitochondrial autonomy [20], though our results suggest that *Aspergillus nidulans* does not fit easily into this pattern.

It is interesting to note that closed circular mitochondrial DNA smaller than that observed in *Saccharomyces cerevisiae* has recently been observed in several 'petite-negative' yeast species [21].

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