Circular DNA molecules measuring approximately 5 μ in contour length have been reported to occur in mitochondrial preparations from bird,1, 2 amphibian,3 and mammalian species.4–6 Examination of other species including invertebrates and various plant groups must be accomplished before a generalization can be made regarding circularity and length of mitochondrial DNA molecules. During the course of our studies on mitochondrial heterogeneity in baker's yeast,6, 7 we analyzed the DNA from mitochondria of a wild-type and of a vegetative-petite mutant using a modified Kleinschmidt8 technique for monolayering DNA from osmotically disrupted organelles.5 While it was not entirely unexpected that the DNA's of wild-type and petite would be different,9 there were some surprising results in these experiments. There was considerable variation in DNA filament length within the wild-type-strain samples, which contrasted sharply with the relative uniformity of contour length found for vertebrate mitochondrial DNA.1–5 Also, whereas wild-type molecules exhibited a predominantly circular configuration, the DNA from petite mitochondria occurred principally in the form of two-ended rods of varying lengths. Although there has been some controversy over the occurrence of DNA in mitochondria from petites of yeast,9–12 the present data confirm its occurrence at least in the one strain which was examined. The existence of a heterogeneous population of DNA molecules in mitochondria has obvious genetic implications in the central problem of the role of mitochondrial DNA in the cellular economy.

Materials and Methods.—The wild-type haploid strain D310-4D (kindly provided by Dr. F. Sherman) and an acriflavine-induced vegetative petite, D310-2A-184 of the essentially isogenic strain D310-2A (also from F. Sherman), were used in all experiments. The petite proved to be suppressive since approximately 50% of the hybrid wild-type × petite zygotes plated directly from the mating mixture were of the petite phenotype,13, 14 as were a high proportion of the colonies from vegetative progenies of single-colony isolates taken from the mating mixture plates. Stock cultures of the two haploids were kept at 5°C on agar slopes containing 1% yeast extract, 1% peptone, and 2% dextrose,15 and were transferred serially at frequent intervals. An inoculum from the slant culture was placed in 100 ml of a semisynthetic medium containing 0.004% adenine sulfate16 to meet the auxotrophic strain requirements, and grown for 24 hr at room temperature on a rotary shaker to provide preinoculum for the experimental cultures. About 106 cells from the 24-hr liquid culture preinoculum were added to 800 ml of fresh semisynthetic-adenine sulfate medium in 2-liter Erlenmeyer flasks for growth on the rotary shaker at room temperature for 24 hr. The cell yield was about 10 gm per liter for the wild-type and half that much for the petite strain.

Isolation of mitochondria: Cells were sedimented from the culture fluid and washed with glass-distilled water prior to preparations for cell breakage. In two experiments, the washed cells were broken with the Nossal shaker operating at 6000 oscillations per min for 1 min using the method of Tewari et al.,12 and in the remaining experiments cells were converted to spheroplasts before being lysed.16 In the latter method, washed cells were incubated for 30 min at 33°C in 2.5 vol. of 0.14 M mercaaptoethylamine-HCl-0.04 M ethylenediaminetetraacetate (EDTA),16 then sedimented for incubation at 33°C for 45 min in 1.7 vol. of 0.72 M sorbitol in 0.02 M KPO4-citrate buffer, pH 6.8, plus 1 ml ghuslase (Endo Labs.) per 10 gm cells.17 After snail enzyme
treatment the cells were sedimented, washed with 3 vol 0.9 M sorbitol, and suspended in 2 vol of 0.25 M sucrose-1 mM EDTA in 0.05 M KPO₄ buffer, pH 6.8, to be lysed using a Vortex mixer. The brei was centrifuged twice at 2000 × g to remove intact cells and debris, and the resulting supernatant was sedimented at 10,000 × g for 20 min to collect the mitochondrial particles. The 10,000 × g particles were washed once with 20% sucrose-1 mM EDTA in 0.05 M KPO₄ buffer, pH 6.8, and resedimented at 10,000 × g. The washed particles were suspended in 50 μg per ml deoxyribonuclease-I-0.25 M sucrose-5 mM MgCl₂ and incubated at 37°C for 30-40 min to remove any contaminating nuclear DNA. The particles then were sedimented at 10,000 × g and washed with saline-EDTA, pH 8.0, to inhibit further enzyme action. After this, the particles were centrifuged again at 10,000 × g and suspended in a small volume of a 1:10 dilution of SSC (SSC = 0.15 M NaCl, 0.015 M Na citrate) for immediate use in monolayering.

All operations were performed at 0-4°C except for the mercaptoethylamine and glusulase steps.

Isolation of mitochondrial DNA: In one experiment DNA was extracted from wild-type mitochondria using a modification of the method of du Buy et al. Mitochondria were collected as described above and treated for 30 min at 37°C with 50 μg per ml deoxyribonuclease-I in 0.25 M sucrose-5 mM MgCl₂, washed with saline-EDTA, and lysed in 1:10 SSC containing 1% sodium lauryl sulfate during heating at 60°C for 10 min. The suspension was cooled to 37°C using an ice bath, and 100 μg per ml pronase (Calbiochem) was added for incubation overnight at 37°C. An equal volume of chloroform-isomyl alcohol was added to the mitochondrial suspension and shaken vigorously for 30 min. The suspension then was centrifuged at 13,000 × g for 5 min and the aqueous phase was removed. Concentrated SSC was added to the 1:10 SSC of the aqueous phase to constitute the standard SSC concentration and pancreatic ribonuclease (previously heated to 80°C for 10 min) was added to obtain 50 μg per ml of enzyme for incubation at 37°C for 30 min to remove RNA. This was followed by a second Sevag deproteinization, and the aqueous phase was dialyzed overnight against two changes of SSC. The DNA was collected in SSC from the dialysis bag and its concentration was determined by absorption at 260 μm. Aliquots of the DNA solution were used for monolayering.

Monolayering DNA: Freshly isolated mitochondria were monolayered essentially according to Nass. The mitochondria were diluted with ice-cold 4 M ammonium acetate-0.01% cytochrome c. Approximately 0.1 ml of the mitochondrial dilution was allowed to flow down from a 1-ml long-tipped pipette along a vertically held glass rod onto a hypophase of ice-cold glass-distilled water in a Teflon-lined 8 × 8 × 2-in. pan. The edges of the expanding film were determined by the tare boundary on the hypophase. Ten μg per ml deoxyribonuclease-I and 5 mM MgCl₂ were added to some preparations prior to spreading, and remained in contact with the osmotically released mitochondrial DNA for 3-5 min before film samples were collected on grids. Slightly different conditions were used for isolated DNA. The DNA solution was diluted to 2 μg per ml in 2 M ammonium acetate-0.01% cytochrome c and about 0.1 ml of the dilution was allowed to flow down a glass slide, inclined at 30°, onto the ice-cold distilled water hypophase in a paraffin-coated glass Petri dish. Talc particles served to delineate the hypophase boundary.

Platinum shadowing of grids: Samples of DNA were picked up from the monolayer films, after they had stopped expanding, by touching the film with collodion-coated, carbon-stabilized, 400-mesh nickel grids. The grid samples were dehydrated in absolute ethanol for 30 sec, drained on lens paper, and stored for no longer than 1 hr prior to shadowing. When a sufficient number of grids was obtained they were arranged on a specimen holder (Delaware Sci. Co.) and placed on a rotary turntable (Ladd Res. Instr., Inc.) so that they were 6.0-7.5 cm from the tungsten wire holder on which 4 cm of Pt/Pd (50:20) wire was wound, and at an angle of 7°. The Pt was evaporated in a Cenco instrument for 2-3 min in a vacuum of less than 10⁻⁴ mm Hg, while the grids rotated at 12 rpm.

Electron microscopy: The grids were scanned with an RCA EMU-3G electron microscope operated at 50 or 100 kv at an initial plate magnification of 10,000 times. Photographs were enlarged up to 42,000 times for measurements of molecule length using a Dietzgen map measurer. The magnifications were calibrated with a replica of a diffraction grating (Fullam; 28,800 L/in.).

Results.—Circular DNA filaments were found in all preparations of wild-type (Fig. 1) and petite mitochondria (Fig. 2). The two experiments using the Nossal shaker to break wild-type cells yielded only three circles among the 29 filaments which could
be measured from photographs. The 26 rod-shaped filaments were of various lengths. In contrast with this result, the DNA from three experiments using spheroplast lysates was predominantly circular in conformation (Figs. 1 and 3).

The distribution of filament lengths was quite different in samples of osmotically disrupted mitochondrial DNA which had been exposed to deoxyribonuclease in the monolayer film (Fig. 1). Of the 28 molecules measured, 25 were rod-shaped fibers
Fig. 3.—Circular DNA filaments from osmotically disrupted wild-type mitochondria obtained from spheroplast lysates. (a) Contour length of 9.2 μ. ×29,000. (b) Contour length of 4.0 μ. ×50,000.

Fig. 4.—Petite DNA filaments showing typical tangles, with one that has no free ends visible (arrow). ×44,400.

Fig. 5.—Wild-type DNA filaments from osmotically lysed mitochondria. ×46,400.
between 0.5 and 4.9 μ in length, with a mode of 1.7 μ. The three circles were small, but they were within the range of sizes obtained in all other experiments. The deoxyribonuclease-treated fibers were smaller and more homogeneous than the two-ended filaments observed in any other experiment.

Isolated wild-type mitochondrial DNA provided only 20 measurable filaments, of which 12 were circular (Fig. 1). The size distribution of the two-ended DNA molecules was similar to that which was found in the three experiments using osmotically disrupted mitochondrial DNA from spheroplast lysates.

Two separate experiments were performed using 10,000 X g particles isolated from spheroplast lysates of the suppressive petite D310-2A-184. There were six circles among the 38 measured filaments, varying in contour length from 0.6 to 8.8 μ (Fig. 2). These circular molecule sizes were the same as some which were found among the wild-type DNA preparations from spheroplasts. The size distribution of DNA filaments with two free ends was within the broad range found for all wild-type preparations, and considerably more variable than the deoxyribonuclease control sample of the wild-type isogenic strain. The number of measurable circles was small since most of the filaments were considerably twisted (Figs. 4 and 5).

In some earlier experiments using 5000 X g particles, large circular molecules were observed occasionally. A number of these were photographed and measured although they clearly were different from the DNA fibers observed in the same preparations. The circles generally had a contour length of 20–25 μ. Treatment with deoxyribonuclease during monolayering resulted in the absence of these circular

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Fig. 6.—Large circular fiber of possible nucleoprotein nature, measuring 24 μ in contour length. Note the much finer filaments of DNA at arrow. This preparation was shadowed from two sides. X11,000.
forms. The large circles always were thicker than the DNA fibers also observed (Fig. 6) and showed irregularity of molecule width in contrast with the uniform thickness of DNA fibers.

Discussion.—Although most of the data were collected from DNA released from osmotically disrupted mitochondria, there was remarkable uniformity between similar experiments for the same strain. Because of this consistency, it is unlikely that the heterogeneity of DNA molecules was due to the occurrence of contaminating microorganism DNA. This might be responsible for some of the observed molecules, however, since aseptic conditions are not observed during any of the handling steps before or during monolayering. By far the greatest amount of material in any preparation was mitochondrial, and it is logical that most or all of the observed DNA was from this source.

There was a striking difference between results from Nossal preparations and those from spheroplast lysates. The occurrence of 90 per cent two-ended filaments in mechanically disrupted cell materials and only 22 per cent rods in spheroplast preparations can be interpreted most easily as a consequence of different degrees of mitochondrial damage during isolation. Other studies have shown clearly that mechanically broken cell preparations exhibit marked damage when analyzed using conventional biochemical and physiological assays.

The occurrence of six measurable circular DNA molecules among a total of 38 photographed filaments from the two experiments using petite spheroplasts, at least suggests that some if not all petite mitochondrial DNA is circular. These filaments were of the same lengths as had been found in wild-type preparations, which makes it more probable that they were petite mitochondrial molecules rather than contaminant DNA circles from other microorganisms. It is possible that mitochondrial DNA from petites is more fragile than from wild-type organelles, but it is equally possible that a proportion of the mitochondrial DNA molecules from petites exists as rods in vivo. As was shown so beautifully by Spiegelman and his co-workers for φX174, there is a functional difference between intact and fragmented circular DNA molecules. Perhaps the petite mitochondrial phenotype is a consequence of impaired DNA function during transcription, which is one of the demonstrated mitochondrial activities.

Further experiments can be performed to test these alternative possibilities. Under the conditions which were used, it is unlikely that the circular molecules were formed by the joining of cohesive ends of linear molecules as has been demonstrated for some bacteriophages.

There have been conflicting reports on the existence of mitochondrial DNA in petites. Mounolou et al. and Tewari et al. have characterized petite mitochondrial DNA, whereas Corneo et al. and Moustacchi and Williamson failed to find a specific mitochondrial DNA component in petite strains of Saccharomyces cerevisiae or S. carlsbergensis. The present data confirm the occurrence of DNA at least in one suppressive petite strain of S. cerevisiae.

Although there has been no specific observation made of circular molecules from nuclear preparations of any eucaryote, their existence may be inferred from two reports. Hotta and Bassel found circular DNA molecules in pronase-treated boar sperm, but no attempt was made to identify the subcellular sources of the various molecules measured. Since vertebrate mitochondrial DNA appears to be approximately 5 µ in contour length, it is possible that circles as long as 16.8 µ were of
nuclear rather than mitochondrial origin in their material. Sinclair and Stevens\textsuperscript{4} mentioned that circular DNA of various lengths was found in whole-cell preparations of yeast, but they did not specify the range of measurements of these molecules. Our own observations of relatively large circles of uneven thickness may be interpreted as evidence of circular nucleoprotein molecules, presumably of nuclear origin. Although it is impossible to make accurate comparisons from results obtained using radically different methods, it is interesting that nucleoprotein fibers from chromosomes always have an uneven width which gives them a knobby appearance.\textsuperscript{27, 29} The large circles sometimes observed in our earlier studies of low-speed particle fractions were very similar in general aspect to knobby chromosome fibers from various organisms. It is possible to test this interpretation by further experiments using monolayered nuclear or whole-cell brei fractions.

Nass\textsuperscript{5} has calculated that there may be five or six circular molecules of DNA, of an average contour length of 5 \(\mu\), in each mitochondrion of mouse L-cells. Using the same bases for calculation, if the average yeast mitochondrion contains \(3 \times 10^{-17}\) gm of DNA,\textsuperscript{12} or an average of \(18 \times 10^6\) daltons of DNA per mitochondrion, this would permit up to 9 or 10 \(\mu\) (assuming the B configuration and a mass per unit length of \(1.92 \times 10^6\) daltons per \(\mu\)) of DNA fiber per mitochondrion. Since the amount of satellite DNA may vary with the growth phase of the culture,\textsuperscript{11} and since determinations of molecular weight from chemical measurements are not always reliable,\textsuperscript{12} no certainty can be attached to estimates of the numbers of DNA molecules per mitochondrion. But, the largest circle measured in the present experiments was 10.1 \(\mu\), which is within the expected maximum range of filament lengths. If there is less DNA per mitochondrion in yeast than in some other species,\textsuperscript{5, 30} then different DNA molecules well may occupy different mitochondria. While some mitochondria may contain a single molecule of 9- or 10-\(\mu\) contour length, others may contain two or more smaller molecules such as those which have been observed in the monolayered preparations.

A central question which has still to be answered is whether or not mitochondrial DNA has a genetic function.\textsuperscript{31} Since most of the necessary elements of a genetic apparatus have been identified in mitochondria,\textsuperscript{25, 32–34} the most pressing requirement now is to establish at least a correlation between genotype and phenotype. Mitochondria of a single cell may vary phenotypically in ultrastructure\textsuperscript{35} and/or enzymatic activities.\textsuperscript{6, 7} With the present demonstration of the existence of identifiably different DNA molecules, it becomes possible to search for a direct relationship between a specific genetic material and a specific phenotypic consequence. Such studies now are in progress.\textsuperscript{36}

Summary.—Monolayered DNA from osmotically disrupted mitochondria was predominantly circular when organelles were isolated from wild-type spheroplast lysates, but occurred as linear fragments of various lengths in Nossal preparations. The DNA circles measured from 0.5 to 10.1 \(\mu\) in contour length. Mitochondrial DNA from vegetative petite spheroplasts occurred mostly as rods, although some circles were measured between 0.6 and 8.8 \(\mu\) in contour length. Calculations of DNA content per mitochondrion led to the theoretical possibility that different DNA molecules might be present in different mitochondria. The implications of such diversity were discussed in relation to possible genetic properties of mitochondrial DNA in yeast.
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