*NUCLEAR AND MITOCHONDRIAL DNA FROM WILD-TYPE AND PETITE YEAST: CIRCULARITY, LENGTH, AND BUOYANT DENSITY**

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Abstract.—Purified mitochondrial and nuclear DNA from diploid isogenic wild-type and vegetative-petite baker's yeast were analyzed by electron microscopy and by analytical ultracentrifugation in CsCl gradients. The buoyant densities in CsCl of nuclear DNA were identical for the two strains (ρ = 1.700), but there was a difference between mitochondrial DNA from the wild type (ρ = 1.684) and the petite (ρ = 1.680). Electron microscopy revealed both circular and linear filaments for nuclear and for mitochondrial DNA of both strains. Nuclear DNA molecules included 6.5 per cent cyclic filaments principally measuring 2 μ or less in contour length, and linear filaments showing a unimodal, disperse length-distribution centered at about 2 to 3 μ, for both strains. Mitochondrial DNA for wild type varied depending upon the method used to extract and purify the molecules; showing only 7.5 per cent circular molecules from CsCl-subfractionated samples, as compared with 15 per cent circles from chloroform-extracted DNA not subjected to CsCl and up to 50 per cent circles from osmotically-lysed mitochondria, as reported in an earlier study. Modal lengths of circles occurred at about 2, 5, and 10 μ. Increasing shear degradation also was evident in comparisons of the length-distribution patterns of linear molecules using the three preparative methods. Petite mitochondrial DNA contained 36–38 per cent circular molecules which measured 0.3–5.3 μ, but principally in the range of 0.3 to 2.0 μ whether from chloroform-extracted populations or from ones subfractionated in CsCl. A previous study of osmotically lysed mitochondria had shown a maximum of 8 per cent circles, which we now attribute to a failure, at that time, to detect circles measuring less than 1 μ, a substantial component encountered in the purified DNA samples in the present study. Linear filaments presented a unimodal length distribution in every case.

Despite the variation in molecule populations derived from the three different preparative methods, there were consistent differences between mitochondrial DNA from wild-type and petite yeast in frequencies and size of circular molecules, as well as in length distribution patterns.

Circular DNA molecules measuring approximately 5 μ in contour length characterize the mitochondria of various metazoans,[1–3] whereas longer noncyclic filaments have been reported for a protozoan[4] and a flowering plant.[5] Despite efforts in several laboratories, there is no agreement on the molecular length and conformation of mitochondrial DNA from wild-type yeast. Avers,[6] Avers *et al.*,[7] and Guérineau *et al.*[8, 9] have found both circular and linear molecules from wild-type preparations, whereas Sinclair *et al.*,[10] Shapiro *et al.*,[11] and Van Bruggen

et al.[1] failed to find significant amounts of circular molecules, or interpreted those cyclic filaments they did see as probable nuclear contamination. Since our previous study[7] indicated that varied results occurred when different methods were used for mitochondrial DNA extraction and purification, we continued the comparative study of wild-type and petite mitochondrial DNA to include molecules subjected to CsCl gradients. The data from the present study confirmed our earlier observations on the substantial heterogeneity of lengths and circularity of mitochondrial DNA from wild-type yeast,[6, 7] and further demonstrated the effect upon filament length and conformation of shear degradation introduced during DNA extraction and purification. We also report here further data[7] on the differences in circularity, length, and buoyant density of a vegetative-petite strain isogenic with the wild type.

Materials and Methods.—The diploid isogenic strains iso-N (wild-type) and DP-28 (vegetative-petite) of *Saccharomyces cerevisiae* have been described.[12, 13] Mitochondria were isolated from spheroplast lysates[6] of 16- or 24-hr midstationary phase liquid cultures[14] by centrifugation for 20 min at 10,000 *g*. They then were washed in 20% sucrose—1 mM EDTA in 0.05 *M* potassium phosphate buffer (pH 6.8), and treated with deoxyribonuclease-I.[6, 7]

Isolation of DNA: After deoxyribonuclease treatment, the mitochondria were washed twice in saline-EDTA, and the DNA was extracted using the chloroform-isoamyl alcohol method[15] with minor modifications.[6] Whole-cell DNA was obtained from spheroplast lysates by the same method as used for mitochondrial DNA.

CsCl centrifugation: Preparative equilibrium centrifugation was carried out at 18° in a Spinco model L ultracentrifuge using the Type-40 fixed-angle rotor.[16] The samples were overlaid with mineral oil and centrifuged for 60 hr at 33,000 rpm. After centrifugation, the bottom of the tube was punctured and fractions were collected dropwise. Absorption at 260 nm was measured in the Gilford-220 spectrophotometer using a Beckman microcell (50 μl capacity).

Analytical sedimentation was carried out according to the method of Meselson *et al.*[17] in a Spinco model E ultracentrifuge at 25°. DNA from the *Bacillus subtilis* phage SP-2 served as the density marker (density 1.723 gm/cm^3). The buoyant densities in CsCl of the DNA samples were expressed relative to the density (1.710 gm/cm^3) of *Escherichia coli* DNA.[18]

Monolayering, shadowing, and electron microscopy: DNA was monolayered on an ice-cold distilled water hypophase according to the protein-monolayer technique of Kleinschmidt,[19] as described earlier.[6, 7] Samples were picked up on parlodion-coated, carbon-stabilized grids and then were dehydrated for 10 sec in absolute ethanol. The grids were drained dry and then were shadowed with Pt/Ir (80:20) as described previously.[7] The grids were scanned and photographed at initial plate magnifications of 6700 and 9500 times with an RCA-3G electron microscope operated at 50 kv. Photographs were enlarged 4.2 times and then were projected at a further magnification of 5.1 times to permit tracings of DNA filaments for subsequent measurements with a calibrated Dietzgen map-measurer. Microscope magnifications were calibrated periodically with a grating replica (28,000 l/in.; Fullam) as a reference.

Results.—Whole-cell DNA was separated into mitochondrial and nuclear fractions using CsCl preparative centrifugation. As shown in Figure 1, wild-type (iso-N) mitochondrial DNA obtained by this method was 95 per cent pure, while mitochondrial DNA of the petite (DP-28) was 94 per cent pure. Nuclear DNA from both strains showed no evidence of contamination by mitochondrial DNA. Buoyant density determinations of purified mitochondrial and of whole-cell DNA samples revealed peak density values of 1.684 gm/cm^3 for wild-type

mitochondrial DNA and 1.680 gm/cm³ for petite mitochondrial DNA (Fig. 1). Nuclear DNA of both strains (Fig. 1) was 1.700 gm/cm³.

DNA obtained from the mitochondrial fraction collected after differential centrifugation at 10,000 *g* was completely free of nuclear contamination (Fig. 1*E*). Therefore, there was no need to further purify the mitochondria by equilibrium centrifugation in sucrose or renografin density gradients.[11, 20]

Wild-type mitochondrial DNA comprised 12.1 per cent of the total-cell DNA, whereas petite mitochondrial DNA accounted for only 5.1 per cent of the whole-cell DNA. These values were determined from measurements of the area under the mitochondrial and nuclear peaks after analytical ultracentrifugation of whole-cell DNA.

Circular and linear molecules were found in wild-type mitochondrial DNA prepared by CsCl centrifugation of whole-cell DNA (Fig. 2). The circular filaments constituted 7.5 per cent of the molecules measured (Fig. 2*C*). The contour lengths of these molecules varied, with modes occurring at 2.0 to 2.5 and 5.0 to 6.0 μ, and with a few other circles between 8 and 10 μ. Molecules with two free ends varied in length from 0.9 to 14.5 μ, with at least one mode at 5.0 to 5.5 μ.

Mitochondria from the petite yielded the highest frequency of circular filaments (Fig. 2). They constituted 36–38 per cent of the molecules measured, regardless of whether the DNA was isolated from a mitochondrial fraction (see Fig. 2*F*) or was purified by CsCl preparative centrifugation of whole-cell DNA (cf. Fig. 2*G*). The circles varied from 0.3 to 5.3 μ (Fig. 3), but most were between 0.3 and 2.0 μ. They formed a disperse distribution with obvious modes occurring at 0.35 to 0.45, 0.75 to 0.85, and 1.05 to 1.15 μ, suggesting a multiple-length distribution (Fig. 4). Linear filaments from petite mitochondria presented a unimodal distribution which was skewed toward the shorter lengths centering at about 1.5 to 3.5 μ (Fig. 2*E-G*).

Nuclear DNA preparations (Fig. 2*D*) from wild-type and petite strains yielded

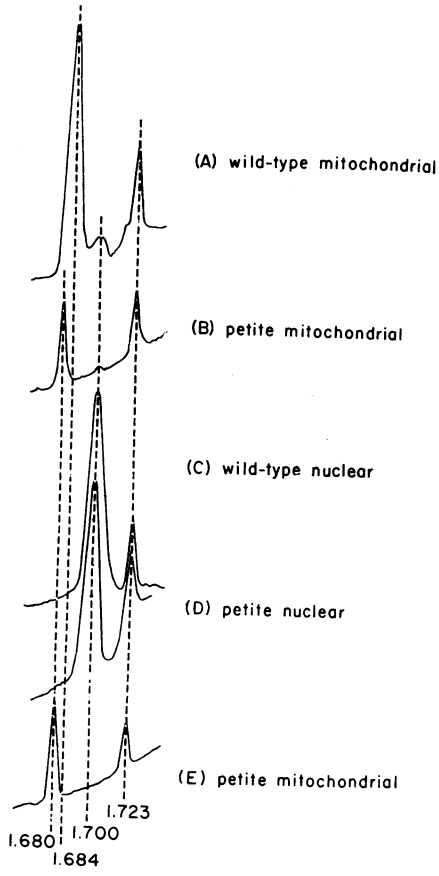

FIG. 1.—Microdensitometer tracings of DNA photographed after analytical ultracentrifugation of samples collected from regions of mitochondrial (*A*, *B*) and nuclear (*C*, *D*) densities of whole-cell DNA which was purified by CsCl preparative centrifugation. The sample used in *E* was obtained by extracting DNA from a mitochondrial fraction of the vegetative-petite strain. The density marker was DNA from the *Bacillus subtilis* phage SP-2 (density 1.723 gm/cm³).

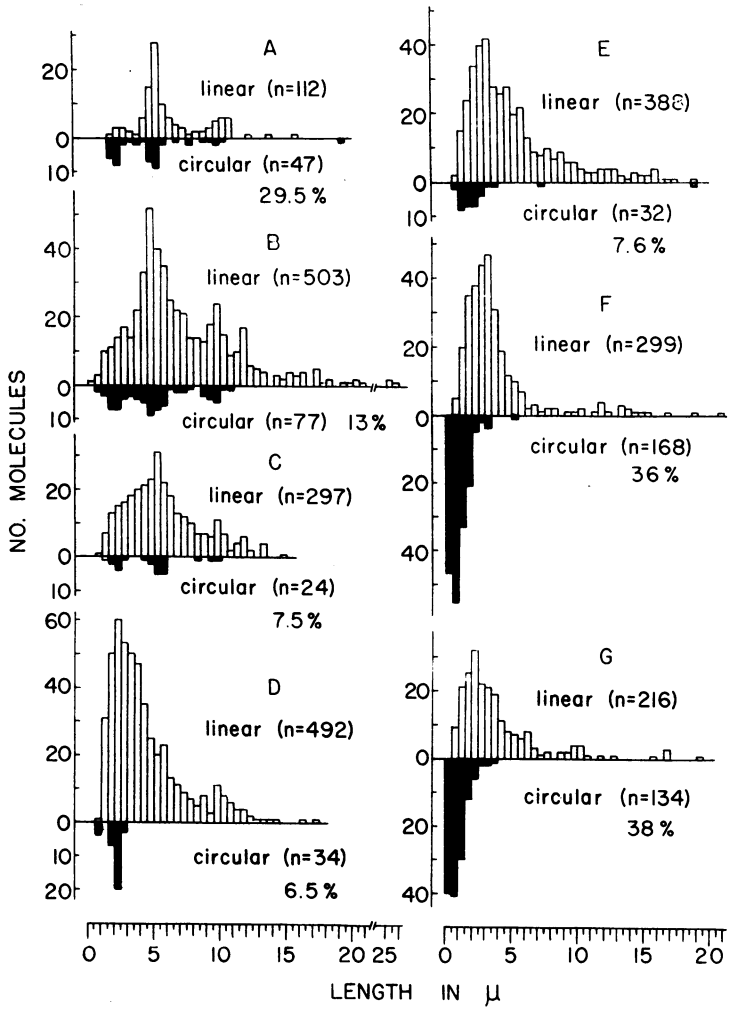

FIG. 2.—Length-distribution histograms showing frequencies of linear and circular DNA molecules from the wild-type strain (*A–D*): (*A*) mitochondrial DNA from osmotically-lysed mitochondria,[7] (*B*) mitochondrial DNA extracted and purified from a mitochondrial fraction,[7] (*C*) mitochondrial DNA purified on CsCl, and (*D*) nuclear DNA purified on CsCl; and from the petite strain (*E–G*): (*E*) mitochondrial DNA from osmotically-shocked mitocnondria,[7] (*F*) mitochondrial DNA extracted and purified from a mitochondrial fraction, and (*G*) mitochondrial DNA purified on CsCl. (A) (B), and (E) are from an earlier paper[7] and are included here for comparison.

6.5 per cent circular filaments, but most were between 1.5 and 2.5 μ, and none was longer than 3.0 μ. Linear molecules presented a unimodal distribution which was skewed toward the shorter lengths centering at about 1.5 to 4.0 μ.

All experiments were replicated from two to four times. Replicates of the same experimental procedure always were similar, whereas consistent differences were obtained when different extraction methods were analyzed.

To determine whether there were some 2-μ circles of mitochondrial origin but

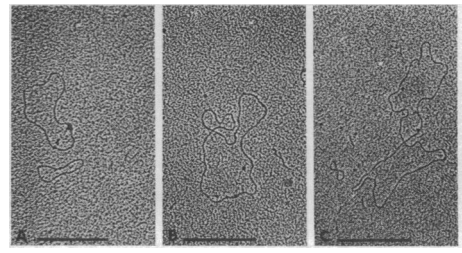

FIG. 3.—Circular DNA molecules from petite yeast mitochondria measuring (*A*) 0.7 and 1.2 μ, (*B*) 2.0 μ, and (*C*) 0.4 and 3.5 μ. The bars are 0.5 μ.

of nuclear density, a sample of wild-type DNA extracted from deoxyribonuclease-treated mitochondria was centrifuged on a CsCl preparative density gradient. DNA from the area of nuclear density was then monolayered, photographed, and measured. In this preparation, 30 per cent of the total molecules measured were circles, all of which were 1.5–2.5 μ long; whereas circular molecules of this size made up only 5.3 per cent of the total molecules when purified nuclear DNA preparations were examined (see Fig. 2*D*).

Discussion.—It has been difficult to reconcile the observations reported by several laboratories concerning the length and conformation of yeast mitochondrial DNA.[1, 6–11] The difficulty apparently lies in the differences in methods used for DNA extraction and purification. Earlier, we[6, 7] had studied the effects upon filament length and conformation of such variables as the method used to isolate mitochondria, the deproteinizing agents used, the deproteinizing procedure, and the degree of osmotic shock to the mitochondria during monolayering. We found that the greatest circle frequency occurred in preparations of intact mitochondria which had been subjected to the least degree of osmotic shock during monolayering. In some wild-type preparations, up to 50 per cent of the DNA filaments lying close to, but not attached to, membranes were circular. This frequency could be reduced to 8 per cent by lysing the mitochondria in 7 *M* ammonium acetate, rather than 5 *M* ammonium acetate.[7] Since the salt concentration only influences physical rupture of the mitochondria, it seemed likely that nuclease or ligase variations were not responsible for the observed differences in circle frequency. We also found that in wild-type mitochondrial DNA preparations purified by chloroform extraction there was a maximum circle frequency of 15 per cent. Therefore, we believe that mechanical shear was the likeliest cause for the variations observed in samples prepared under these different conditions.

Based upon these earlier observations, we expected that mitochondrial DNA subjected to CsCl centrifugation subsequent to chloroform extraction would yield an even lower frequency of circular molecules. The results of the present study substantiated our prediction that greater shear degradation forces during CsCl centrifugation would lead to fewer circular molecules and shorter linear filaments. When we used CsCl to purify wild-type mitochondrial DNA we found that circular filaments now constituted only 7.5 per cent of the molecules measured—a reduction by one-half compared to DNA not purified in CsCl. As the DNA was subjected to increased preparative manipulation, the sharpness of the modes for linear molecules decreased and the circle frequency was reduced. Others[10, 11]

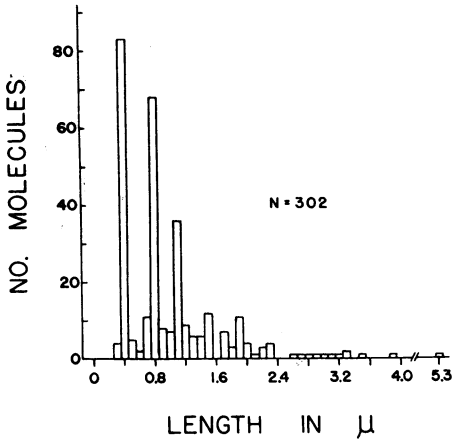

FIG. 4.—Circular molecules of petite mitochondrial DNA from Fig. 2*F* and 2*G* combined, shown in class intervals of 0.1 μ.

also have found few circular filaments in mitochondrial DNA that had been subfractionated in CsCl, whether from purified DNA[10, 11] or from lysates directly.[10] Both groups reported few linear filaments longer than 7 μ. Guérineau *et al.*[9] reported finding 20–30 per cent cyclic molecules after CsCl centrifugation of wild-type mitochondrial lysates. However, about three-fourths of the circular molecules which were described measured 3 μ or less. This distribution is incompatible with our own findings.

Based on all of these observations, we believe that the most accurate determination of the *in vivo* length and conformation of yeast mitochondrial DNA is made from mitochondria which are gently lysed during monolayering. If purified mitochondrial DNA is required, the method of choice would appear to be chloroform extraction.[7] Using the mitochondrial fraction collected after differential centrifugation at 10,000 *g*, we obtained DNA that was completely free of nuclear contamination. There was a minimum of 5 to 6 per cent nuclear contamination in every mitochondrial DNA sample collected after CsCl preparative centrifugation of whole-cell DNA.

Nuclear DNA of the wild-type and petite strains yielded identical length-distributions for circles and linear filaments. We found only 6.5 per cent cyclic filaments, most of which (or 5.3 per cent of all filaments measured) were between 1.8 and 2.2 μ long.

After CsCl centrifugation of chloroform-extracted wild-type mitochondrial DNA, we found that 30 per cent of the molecules recovered from the area of nuclear density consisted of circles about 2 μ long. This frequency was significantly greater than the 5.3 per cent found in this same region when purified nuclear DNA was analyzed. It is most probable that the additional 2-μ circles represent a contribution by the mitochondria to the region of nuclear density. If true, wild-type yeast mitochondria contain at least two classes of 2 μ-long circles which differ in chemical composition, assuming that buoyant density differences reflect differences in average base composition of the DNA molecules.[18]

Mitochondria from the petite strain yielded only small DNA circles along with linear filaments, as had been found earlier.[7] But, improved shadowing techniques allowed the resolution of many very small circles (< 0.5 μ), resulting in a higher circle frequency than had been reported earlier. Microscope grids for the present study were selected for use on the basis of thinness and homogeneity of the support film and the fineness of the platinum deposit. Then, molecules on such grids were photographed at random. This insured a high degree of resolu-

tion and aided in the interpretation of these photographs. In this study, mitochondrial DNA from petites contained 36–38 per cent circular molecules, and this frequency did not vary between DNA from purified mitochondria or that from whole-cell DNA subfractionated by CsCl centrifugation. By weight, these circles constituted 13.3 per cent of the total mass of molecules measured (circular and linear).

The contour lengths of the circular DNA molecules from petite mitochondria suggest a multiple-length distribution, with at least three modes occurring at 0.4, 0.8, and 1.1 μ. We cannot determine from the present data whether or not the multiple-length distribution of these cyclic filaments is due to the joining of cohesive ends,[11] to a recombination[21] or replication[22] phenomenon, or to *in vivo* heterogeneity.[6, 7]

It is significant that similar length distributions for linear molecules were found for petite mitochondrial DNA prepared using three different methods. In every case the petite DNA filaments with two free ends presented a unimodal distribution skewed toward the shorter lengths. By comparison, linear filaments from wild-type molecules were always distributed across a broad range with more than one obvious mode. Also, wild-type molecules showed a definite response to the method of preparation. As these molecules were subjected to increased preparative manipulation, the sharpness of the modes decreased, and the circle frequency was reduced. Since both strains were subjected to the same three preparative methods (osmotic lysis during monolayering, chloroform extraction of DNA from purified mitochondria, and CsCl purification from whole-cell DNA), the differences in length distribution and circularity *between* strains cannot be a function of the method of preparation. In all cases there were consistent differences between wild-type and petite mitochondrial DNA in length distribution, circle frequency, and contour lengths of circular molecules. We also found a lower buoyant density in CsCl of petite mitochondrial DNA as compared with its isogenic wild type, although it is known that absolute values vary from one strain to another.[23–25] In sharp contrast with these observations, nuclear DNA from both strains were identical in *all* characteristics measured.

There are an estimated 45–50 mitochondria per cell in stationary-phase cultures of iso-N and 15–20 mitochondria in the petite cell.[26] Since there are approximately 37 per cent as many mitochondria in the petite cell as compared with wild type, and approximately 42 per cent as much mitochondrial DNA in the petite, this correlation suggests that in the petite cell there may be about as much DNA per mitochondrion as there is in the wild-type cell. Such calculations, though crude, do not provide support for the existence of substantial deletions in the petite mitochondrial DNA complement. Deletions have been suspected because of the irreversible and pleiotropic nature of this mutation.[27]

Although it still is unknown whether the differences in mitochondrial DNA reported here lead to specific phenotypic differences, it is significant that mitochondrial DNA prepared by diverse methods shows consistent variations between isogenic wild-type and vegetative-petite strains whose mitochondria differ substantially both in structure and in function.[12, 13, 26]

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