Evidence for Deletion and Changed Sequence in the Mitochondrial Deoxyribonucleic Acid of a Spontaneously Generated Petite Mutant of *Saccharomyces cerevisiae*†

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ABSTRACT: Mitochondrial DNA (mtDNA) from a grande (wild-type) and a spontaneously derived cytoplasmic petite (respiratory-deficient) strain of yeast has been compared using a DNA–DNA filter hybridization technique. Hybridizations were carried out in 36% formamide–1 × SSC at 35° for 24 hr. Self-hybridization of [^{3}H]mtDNA in solution was minimal under the conditions used. When filter mtDNA was progressively increased, a plateau of binding of [^{3}H]mtDNA was reached which was always higher for the isohybrids (grande [^{3}H]mtDNA *vs.* filter grande mtDNA, petite [^{3}H]-mtDNA *vs.* filter petite mtDNA) than for the heterohybrids (grande mtDNA *vs.* petite mtDNA). Isohybrids bound 75–85% of the [^{3}H]mtDNA in solution. Filters charged with petite mtDNA bound only 70% as much grande [^{3}H]mtDNA as did grande mtDNA filters. In the reciprocal reaction with petite [^{3}H]mtDNA in solution, hybridization to grande mtDNA was 85% of that to petite mtDNA. Such a finding is incompatible with simple deletion; this conclusion was confirmed by competition experiments. A two-filter system was used in which the mtDNA on one filter is progressively increased to compete with a constant amount of mtDNA, on a second filter, for available [^{3}H]mtDNA in solution. Heterocompetitions were always less effective than isocompetitions. The thermal stabilities of the filter-bound isohybrids and heterohybrids were similar, and $T_{m,i}$ values indicated well-matched duplexes, providing evidence for the absence of scattered sequence changes in petite mtDNA. We concluded that the petite mtDNA is changed in two ways: (1) by deletion of grande mtDNA, and (2) by generation of localized stretches of "new" nonhomologous base sequences.

Characterization of the cytoplasmic "petite" mutation in yeast has classically depended on the respiratory-deficient phenotype and the segregation of this phenotype among the ascospores after sporulation (Ephrussi, 1953). More recently, analyses of the mtDNA[1] of grande (wild-type) and petite yeast have shown correlations between the presence of the mutation and altered physical properties of the mtDNA. Thus, mtDNA from cytoplasmic petite yeast generally has a lower buoyant density on CsCl gradients (Mounolou *et al.* 1966; Carnevali *et al.*, 1969), and a lower G + C base content and thermal stability (Bernardi *et al.*, 1970, 1972). In extreme cases, such as in petite cells formed by prolonged mutagenesis with ethidium bromide, mtDNA is not detectable (Goldring *et al.*, 1970).

On the basis of the above correlations it is generally assumed that the cytoplasmic petite phenotype is the result of an altered mtDNA. However, the methods used can detect only relatively gross changes in the mtDNA; in some cytoplasmic petite strains, for example, the mtDNA has no detectable CsCl buoyant density change. Part of our general aim, therefore, is to define more sensitive procedures for the comparison of mtDNA from different organisms, and from different strains of yeast. Such procedures should allow a closer examination of mtDNA, a more exact correlation between its physical alteration and genetic content, and some insight into the mechanism of the petite mutation.

Recent studies in this laboratory and others have provided a more complete examination of some properties of mtDNA from grande and petite yeast (Rabinowitz and Swift, 1970; Borst, 1972). In the present paper we report on the application of a DNA–DNA filter hybridization technique for determining the extent of sequence homology between the mtDNA of a wild-type (grande) yeast strain and that of a spontaneously mutated cytoplasmic petite strain. The technique of DNA–DNA filter hybridization has been used previously to examine base sequence homologies in a variety of organisms (McCarthy and Bolton, 1963; McCarthy and McConaughy, 1968). We have found that this technique can be used successfully with mtDNA, and that conditions that result in a very specific hybridization can be defined. The results obtained from hybridization procedures suggest that the grande and petite mtDNA examined contain approximately 30% nonhomologous sequences. We discuss how these findings may be related to kinetic and analytic data and to the mode of petite mtDNA formation.

Methods

Strains and Culture Conditions. The two strains of yeast used are haploid chromosomal isogenic strains originally obtained from the collection of Dr. P. P. Slonimski (Centre de Génétique Moléculaire de C.N.R.S., Gif-sur-Yvette, France). The petite strain, a subclone of D243-2B-R1-6 denoted R1-6/1, is a spontaneously mutated cytoplasmic petite derived from the grande strain, D243-2B-R1. These strains have been described previously (Mounolou *et al.*, 1966; Fukuhara *et al.*, 1969; Bernardi *et al.*, 1970; Casey *et al.*, 1972; Cohen *et al.*, 1972).

Yeast strains were grown to late exponential phase in

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[1] Abbreviations used are: mtDNA, mitochondrial DNA; nDNA, nuclear DNA; SSC, 0.15 M NaCl–0.015 M sodium citrate.

liquid medium containing (%, w/v): galactose (2); Difco yeast extract (1); and Difco bactopeptone (2), as described previously (Cohen *et al.*, 1972). For growth of the petite strain, 0.1% glucose was added to the medium. To prepare cells with labeled DNA, we added 3–4 mCi of [2-^{3}H]adenine (approximately 22 Ci/mmol, Schwarz Bio-Research) to each liter of growth medium, which in this case contained only 0.25% yeast extract. This procedure did not reduce the exponential growth rate of the cultures, or the final cell yield.

Mitochondrial Isolation and DNase Treatment. Mitochondria were isolated from both grande and petite strains of yeast after snail enzyme digestion of the cell walls, using the procedure of Duell *et al.* (1964) with modifications (Kovac *et al.*, 1968; Rabinowitz *et al.*, 1969). Washed cells were preincubated for 15 min at 30° in a solution containing 0.1 M 2-mercaptoethanol–20 mM EDTA–100 mM Tris (pH 9.3). Cells were then collected, washed once in 1.2 M sorbitol, and digested in 3–5 ml of snail enzyme solution/g wet-wt of cells. The snail enzyme solution contained 1.2 M sorbitol, 0.1 M citrate–potassium phosphate (pH 5.8), 1 mM EDTA, and 2 ml of snail digestive juice (glusulase, Endo Laboratories, N. Y.) per 100 ml of solution. After cell wall digestion, protoplasts were washed three times in 1.2 M sorbitol, resuspended in five volumes of 0.7 M sorbitol, and incubated for 15 min prior to breakage in a Waring blender (full speed, 30 sec). Cell debris was removed, and a mitochondrial pellet was collected, as described previously (Rabinowitz *et al.*, 1969). Except where noted, all steps were carried out at 0–4°.

To minimize contamination by nDNA, the mitochondrial pellet was suspended in buffer (0.9 M sorbitol–0.01 M $MgCl_2$–0.01 M Tris-HCl, pH 7.5) containing 50 μg/ml of DNase I (Worthington Biochemicals), and incubated at 30° for 30–60 min. DNase was removed from the mitochondria by three centrifugation washes, each at 27,000*g*, for 10 min in a solution containing 0.9 M sorbitol, 2 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 0.1% (w/v) bovine serum albumin (fraction V). The washed pellet was kept frozen at −16° until it was used to prepare mtDNA.

DNA Extraction. The method of preparation of mtDNA from DNase-treated mitochondria has been described previously (Fauman, 1970; Casey *et al.*, 1972), and is based on a procedure developed by Bernardi (1971). Essentially, the mitochondrial pellet was lysed with sodium dodecyl sulfate and deproteinated with chloroform–octanol (9:1), and the DNA was purified by chromatography on hydroxylapatite. The nucleic acid extract of DNase-treated mitochondria from approximately 120 g wet-wt of cells, in 0.1 M sodium phosphate buffer (pH 6.8), was loaded onto an 8- to 10-g column of hydroxylapatite (Bio-Rad) equilibrated in the same buffer. The column was washed with 0.22 M phosphate buffer (pH 6.8) until $OD_{260\ nm}$ of the eluate was zero. The mtDNA was then eluted with 0.4 M phosphate buffer (pH 6.8); peak fractions were pooled and dialyzed against 0.1 × SSC at 4° overnight. The purity of the mtDNA was checked by CsCl isopycnic centrifugation. Contamination of the mtDNA with nDNA was less than 1%. On alkaline sucrose gradients mtDNA from both grande and petite strains banded in the same region, with the main portion sedimenting around 14 S (J. Locker, personal communication). Yeast nDNA was extracted from the cell debris–nuclear pellet (0–1600-g fraction after protoplast breakage) by a method similar to that described above for mtDNA. It was purified by chromatography on hydroxylapatite, as described by Bernardi *et al.* (1972).

Hybridization Procedures. Nitrocellulose filters (24 mm, Bact-T-flex, type B6) were loaded with DNA according to the method of Gillespie and Spiegelman (1965). DNA in 0.1 × SSC was denatured by the addition of 0.2 ml of 1 M NaOH/ml of DNA solution and incubated at 35° for 20 min. After cooling, the solution was neutralized with cold 1 M HCl; hyperchromicity was approximately 30%. Alternatively, DNA was denatured by heating at 90° for 4 min. The two procedures led to similar hybridization results. The denatured DNA solution was adjusted to 4 × SSC and loaded onto filters that had been soaked and washed in 4 × SSC. Following loading, which was accomplished under very light suction (5–7 cm of H_2O), the filters were washed with 5–10 ml of 4 × SSC under suction of about 150 mm of Hg. After drying on filter paper for 1 hr at room temperature, the loaded filters were preincubated in Denhardt's solution (Denhardt, 1966) for 1–2 hr and then further dried at room temperature for 1 hr. The filters were dried overnight in a vacuum oven at approximately 80°.

A microhybridization system was developed by means of which a high hybridization efficiency could be obtained. In this method, the DNA-containing or blank filter(s) and the incubation solution containing labeled, denatured mtDNA are sealed between two pieces of parafilm, and the unit is immersed in a water bath (Fauman, 1970).

The [^{3}H]mtDNA, in 0.1 × SSC, was sonicated, using a Branson Model S-75 sonicator with 0.5-in. tip (4 × 30 sec pulses at maximum output), and then alkali denatured as described above and neutralized. Sedimentation analysis in the Beckman Model E ultracentrifuge gave a molecular size of 5–6 S for both grande and petite mtDNA (Fauman and Rabinowitz, 1973). The neutralized solution was buffered to a pH of 6.5–7.0 with 0.01 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid. The labeled DNA solution was adjusted so that it contained 2 × SSC and 36% formamide; this solution served as the incubation solution for hybridization. The formamide lowered the T_m of the mtDNA by about 25°, to approximately 50°, according to the relationship defined by McConaughy *et al.* (1969).

The hybridization unit ordinarily contained only one filter; in special cases, as described later, two-filter units were used. For each single-filter unit 0.067 ml of incubation solution was added, and the sealed unit incubated in a water bath maintained at 35°, *i.e.*, at approximately 15° below the adjusted T_m. After incubation (usually 24 hr), the filters were washed at room temperature for 2 hr in several changes of 100 ml of 2 × SSC. Filters were finally washed with 40 ml of 2 × SSC under suction, dried in a vacuum oven, and counted in 5 ml of toluene-base scintillation fluid. Trial incubations and washings, carried out with filters containing labeled grande and petite mtDNA, indicated that filter retention of mtDNA was greater than 95%.

Thermal Stability Measurements. Melting curves were obtained for DNA–DNA hybrids bound to filters, and for native DNA in solution. For the former, the filters were washed and dried after hybridization, and then incubated in successive vials with 0.6 ml of 1 × SSC containing 36% formamide. Temperature increments were 3–5°, and each temperature was maintained for 10 min. For counting 0.4 ml of H_2O and 15 ml of Triton scintillation fluid were added to each vial. Each liter of Triton fluid contained 5.0 g of 2,5-diphenyloxazole, 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, 250 ml of Triton X-100 (Emulsion Engineering, Inc., Elk Grove Village, Ill.), and 750 ml of toluene. The counts released from the hybrid at successive temperatures were used to construct melting curves.

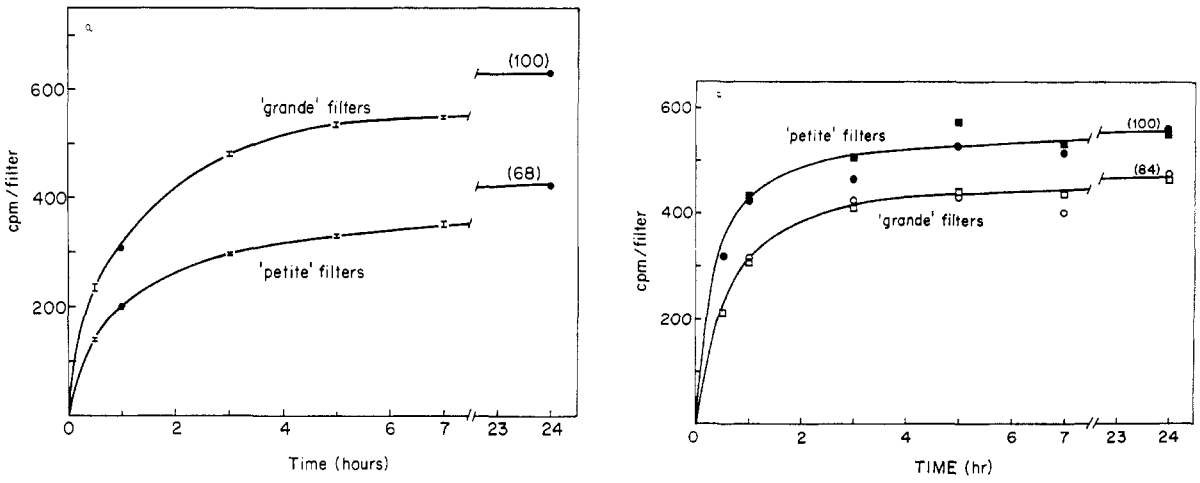

FIGURE 1: Time course of hybridization. (a, left) With grande [^{3}H]mtDMT in solution. Filters containing 2 μg of either grande mtDNA (grande filters) or petite mtDNA (petite filters) were incubated at 35° in single-filter hybridization units with grande [^{3}H]mtDNA (0.067 ml containing 0.089 μg of mtDNA at 9450 cpm/μg). At various times after the start of the reaction, filters were removed (in duplicate) and washed, dried, and counted, as described in Methods. Bars represent the range of the duplicate incubations; solid circles indicate determinations not carried out in duplicate. Values in parentheses are the relative extents of reaction after 24 hr. (b, right) With petite [^{3}H]mtDNA in solution. Reaction conditions are described in part a. The specific activity of petite [^{3}H]mtDNA was 8600 cpm/μg. Squares indicate filters containing 1 μg of mtDNA; circles indicate 2-μg mtDNA filters. Again, values in parentheses show the relative extent of the two reactions after 24 hr.

To determine the optical melting curves of native DNA, we gradually heated the mtDNA, in 1 × SSC, in quartz cuvets in a Gilford Model 2400 recording spectrophotometer, at a heating rate of 12°/hr, and graphed the optical hyperchromicity, monitored at 260 nm, as a function of temperature.

Results

Hybridization Reaction Conditions and Thermal Stability of Reaction Products. To facilitate description of the DNA–DNA hybridization system, we denote the hybrid products of the reaction between grande [^{3}H]mtDNA in solution and grande mtDNA or petite mtDNA on filters as grande isohybrids and grande heterohybrids, respectively. Similarly, products of the reaction between petite [^{3}H]mtDNA in solution and petite or grande mtDNA on filters are denoted petite iso- or heterohybrids. We sought first to establish hybridization conditions under which the reaction would be both specific and nearly complete.

The concentration of labeled DNA in solution was kept low (1.33 μg/ml) so as to minimize its renaturation. Very small volumes (0.067 ml/filter) of labeled solution DNA were used. The ratio of filter DNA to DNA in solution was thus maintained at high levels (10–100) to force the reaction toward completion. Under these conditions, 75–85% of the labeled DNA added to the hybridization unit was recovered in the grande or petite isohybrid. One can calculate from the renaturation rates of the grande and petite mtDNAs (Fauman and Rabinowitz, 1973) that less than 10% of the DNA in solution would have renatured after 24-hr hybridization. Thus, renaturation of DNA in solution is relatively small and apparently interferes very little with the interpretation of the hybridization results. Hybridization was carried out in 36% formamide at 35°.

The time course of the reaction with grande [^{3}H]mtDNA in solution is shown in Figure 1a, and that with petite [^{3}H]-mtDNA in solution in Figure 1b. The reactions were relatively rapid with the binding, after 1 hr 50–80% of that after 24 hr. Double-reciprocal plots (1/hybridization *vs.* 1/time, analogous to DNA–RNA hybridization plots presented by Bishop, 1969; Bishop *et al.*, 1969; Birnstiel *et al.*, 1972) of these data indicate that the reactions were 95–98% complete at 24 hr. With either petite or grande [^{3}H]mtDNA in solution, at 24 hr the extent of heterohybrid formation was 15–30% less than that of isohybrid formation.

The thermal stabilities of the hybrids formed on the filters were examined by measuring the release of labeled DNA from the filters as a function of temperature (McCarthy and McConaughy, 1968). All the hybrids formed during a 24-hr reaction period had relatively sharp melting profiles. The $T_{m,i}$ values, defined as the temperatures at which half the labeled DNA is released from the filters (McCarthy and McConaughy, 1968), were 50–52.5° when measured in solutions containing 36% formamide and 1 × SSC (Figures 2 and 3), approximately 15° above the temperature used for hybridization. The differences in the $T_{m,i}$ values between iso- and heterohybrids were small and are probably within the error of the method, especially when the different slopes and amounts of low-melting material are considered (Figures 2 and 3). The slopes of melting curves are greater for the isohybrids than for the heterohybrids; this may be a reflection of greater homogeneity in the isohybrids formed. Hybrids formed after a 1-hr reaction have the same $T_{m,i}$ values as hybrids formed after 24 hr.

For comparison, the optically monitored melting profiles of native grande and petite mtDNA, measured in 1 × SSC in the absence of formamide, are shown in Figure 4. Petite mtDNA had a T_m of 73.2°, somewhat lower than that of grande mtDNA (75.2°). The addition of 36% formamide lowered the T_m of grande and petite mtDNA to 53.5 and 51°, respectively (Table I). The decrease in T_m was about 4° less than that predicted from the correction offered by McConaughy *et al.* (1969). The $T_{m,i}$ values obtained for the filter-bound hybrids agree well with these T_m results, although the petite hybrids might have been expected to melt with relatively lower $T_{m,i}$ values.

On the basis of these data, all subsequent hybridization reactions were carried out at 35° for 24 hr.

DNA–DNA Hybridization: Petite vs. Grande mtDNA. The observation that isohybrid formation was more extensive than

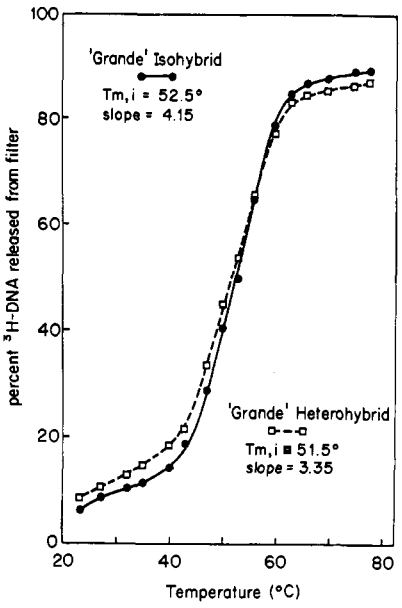

FIGURE 2: Thermal stability of grande filter-bound hybrids. Hybrids formed after 24-hr reaction were washed and dried, then incubated in successive vials containing 0.6 ml of 36% formamide and 1 × SSC with temperature increments between each incubation, as described in Methods. Grande iso- and heterohybrids are defined in the text. The 100% values were 450 and 305 cpm for the iso- and heterohybrids, respectively.

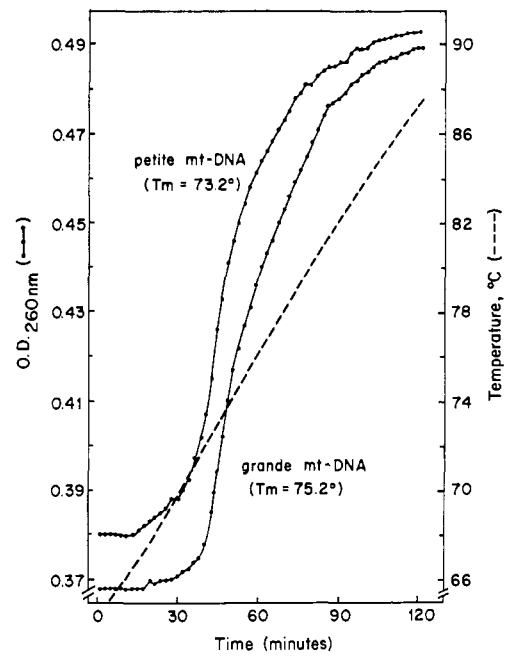

FIGURE 4: Thermal stability of native grande and petite mtDNA. Grande and petite mtDNA were dialyzed against 1 × SSC for 30 hr, with one change. The mtDNAs were then slowly heated (12°/hr) and the hyperchromicity was measured with a Gilford recording spectrophotometer, Model 2400. Temperature programming was provided by a Haake PG11 programmer coupled to a Haake water bath. The upper curve shows the hyperchromicity of petite mtDNA.

heterohybrid formation is of particular interest, for it provides an indication of the extent and nature of the change in mtDNA during petite formation. However, because of the kinetic complications of the two-phase hybridization system used, it was necessary to define the reaction parameters more precisely before more definite conclusions could be drawn. Figure 5a,b shows the effect of the filter content of mtDNA on the extent of hybrid formation, while the initial solution concentration of either grande [³H]mtDNA (Figure 5a) or petite [³H]mtDNA (Figure 5b) is held constant. The [³H]mtDNA bound is maximal for a filter mtDNA content above about 2 μg. The relative height of the plateau attained, however, depends on the source of the DNA. With either grande or petite mtDNA in solution, the isohybrid plateau is significantly higher than the heterohybrid plateau (Figure 5a,b). The plateau levels of hybridization from several saturation-type experiments with several different preparations of grande and petite mtDNA are shown in Table II. With grande [³H]mtDNA in solution, the isohybrid plateau is 30% higher than the heterohybrid plateau, whereas the difference is only 15% with petite [³H]mtDNA in solution. Analysis of the data indicates that the difference between grande and petite heterohybridization levels (30% *vs.* 15%), as well as differences between iso- and heterohybridizations, are statistically significant (Table II).

Isohybrid exceeds heterohybrid formation over a range of solution DNA concentrations (Figure 6). With constant filter

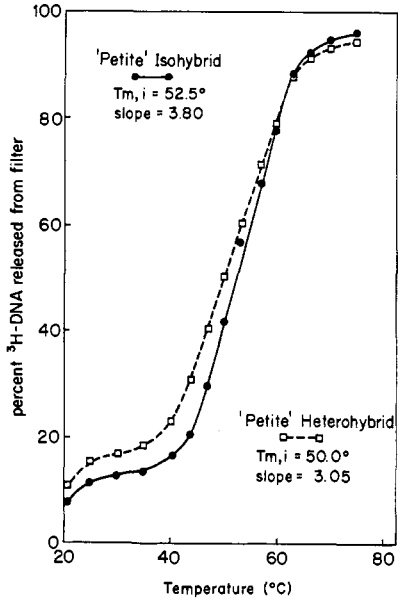

FIGURE 3: Thermal stability of petite filter-bound hybrids. Hybrids formed after 24-hr reaction were heated in formamide–SSC solution, as described in the legend of Figure 2. Petite isohybrid and heterohybrid are defined in the text. The 100% values were 496 and 436 cpm for the iso- and heterohybrids, respectively.

TABLE I: Thermal Stability of mtDNA.

Solution	T_m Values	
	Grande	Petite
36% Formamide, 1 × SSC[a]	53.5	51.0
1 × SSC	75.2 (49.5)	73.2 (47.5)

[a] Absorbance measured at 270 nm. Values in parentheses are corrected to 36% formamide, using the relationship of McConaughy *et al.* (1969).

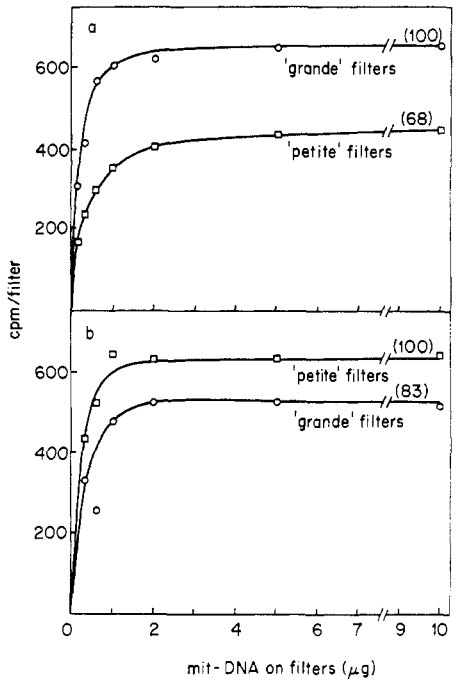

FIGURE 5: Effect of filter DNA content on the extent of hybridization. Filters containing various amounts of grande (○) or petite (□) mtDNA were incubated at 35° with either (a) grande [^{3}H]mtDNA (0.067 ml/filter, 0.089 μg/filter, 9450 cpm/μg) or (b) petite [^{3}H]-mtDNA (0.067 ml/filter, 0.089 μg/filter, 8600 cpm/μg). After 24-hr reaction, filters were removed and prepared for counting. It should be noted that each point results from an independent incubation carried out in a single-filter hybridization unit, and all were exposed to the same initial concentration of labeled solution mtDNA. Values in parentheses represent relative amounts of binding to grande and petite filters, at the highest filter DNA concentrations used.

DNA concentrations, the extent of binding to the filters is directly and linearly proportional to the concentration of mtDNA in solution over the range tested (Figure 6a,b). Again, the difference between iso- and heterohybrid formation

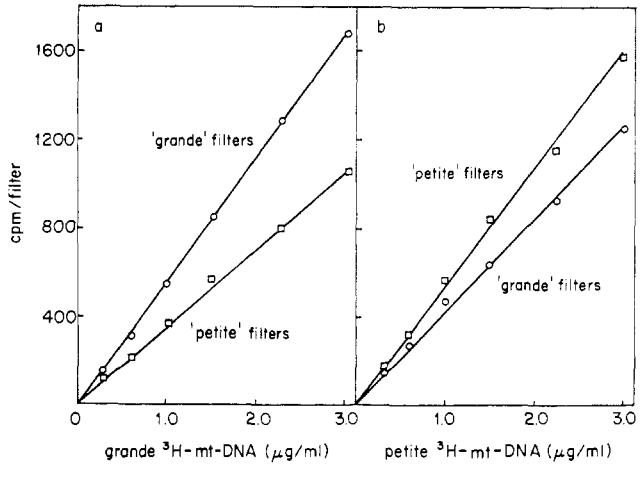

FIGURE 6: Dependence of filter binding on solution mtDNA concentration. Filters containing 2 μg of grande mtDNA (○) or petite mtDNA (□) were incubated at 35° for 24 hr in single-filter hybridization units with 0.067 ml of either (a) grande [^{3}H]mtDNA (9450 cpm/μg) or (b) petite [^{3}H]mtDNA (8600 cpm/μg) at the concentration indicated. Counts bound to filters with petite [^{3}H]mtDNA in solution have been corrected for, so that they are comparable to the counts shown in part a.

TABLE II: DNA-DNA Hybridization: Experimental Variation.[a]

Grande mt-DNA Filters		Petite mt-DNA Filters		Hetero-hybrid/ Iso-hybrid ×100
DNA preparation	Binding (cpm)	DNA preparation	Binding (cpm)	
A. Grande [^{3}H]mtDNA in Solution				
1	670	1	540	80.5
1	640	1	530	83.0
2	630	1	425	67.5
2	625	1	420	67.5
2	600	2	400	67.0
3	655	2	415	64.0
Mean ± SD	636 ± 24		455 ± 62	
B. Petite [^{3}H]mtDNA in Solution				
1	485	1	550	88.5
1	540	1	655	82.0
2	530	1	660	80.5
2	500	2	600	83.5
3	525	2	575	91.0
Mean ± SD	516 ± 22 (568 ± 25)[b]		608 ± 48 (668 ± 53)[b]	

[a] Values are taken from independent saturation-type experiments, and represent the plateau hybridization levels achieved. Three different preparations of unlabeled grande mtDNA and two of petite mtDNA were used. The specific activities of the [^{3}H]mtDNA preparations were 9450 cpm/μg (grande) and 8600 cpm/μg (petite). With grande [^{3}H]mtDNA in solution, the difference in binding to filters containing grande or petite mtDNA was significant to a level of $p < 0.001$. With petite [^{3}H]mtDNA in solution the difference in binding to petite and grande mtDNA on filters was $p < 0.01$. Grande heterohybrid binding was significantly lower ($p < 0.01$) than petite heterohybrid binding when the different specific activities of the labeled mtDNA's were taken into account. [b] Values in parentheses were corrected for the specific activity difference between the grande and petite [^{3}H]mtDNA, so that petite saturation levels could be compared directly to grande levels.

is 15–30%. The constant percent binding (75–85%) of [^{3}H]-mtDNA at all concentrations shows that renaturation of labeled DNA in solution is negligible under our conditions. Binding to filters containing 5 μg of *E. coli* DNA (Fauman and Rabinowitz, 1973) or 5 μg of yeast nDNA (not shown) was about 0.5% of the binding to mtDNA filters, and was difficult to distinguish from background levels of binding.

The results suggest that the mtDNAs from the grande and petite strains studied differ by about 30% in their nucleotide sequences. Furthermore, the binding patterns seen in Figures 5 and 6 seem to be inconsistent with simple deletion as the only difference between grande and petite mtDNA. If the petite mtDNA were a simple deletion mutant, all petite mtDNA sequences would be represented in grande mtDNA. In a "saturation-type" experiment (where filter DNA content is gradually increased, Figure 5), the binding of petite [^{3}H]-mtDNA would thus be the same with either grande or petite mtDNA at high-filter DNA concentrations. This pattern of behavior can be illustrated in a reconstruction experiment.

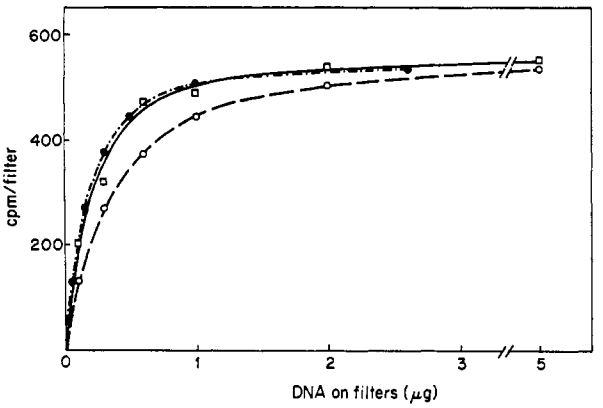

FIGURE 7: Effect of nonhomologous sequences on [^{3}H]mtDNA binding to filters. Filters were loaded with various amounts of either petite mtDNA or a 1:1 mixture of petite mtDNA and yeast nDNA. The filters were then incubated for 24 hr at 35° in single-filter hybridization units with petite [^{3}H]mtDNA (0.067 ml, 0.089 μg; 8600 cpm/μg). (□) Binding to filters containing petite mtDNA. (○) Binding to filters containing petite mtDNA and yeast nDNA ("reconstructed grande" filters). (●) Binding to "reconstructed grande" filters, as a function of the petite mtDNA portion of the filter DNA.

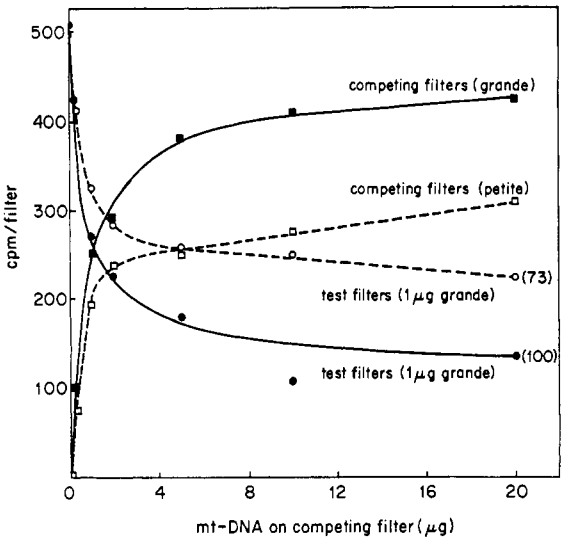

FIGURE 8: Competition of grande and petite mtDNA on filters for grande [^{3}H]mtDNA in solution. Two-filter hybridization units were constructed so that each contained a test filter and a competing filter, as described in the text, and 0.133 ml of grande [^{3}H]mtDNA (0.089 μg, 9450 cpm/μg). All the test filters contained 1 μg of grande mtDNA, while the competing filters contained 0–20 μg of grande or petite mtDNA. Hybridization units were incubated at 35° for 24 hr, then prepared for counting (see Methods). Values in parentheses indicate efficacy of competition relative to isocompetition (grande *vs.* grande). (●) Binding to test filters, with competition from grande filters. (■) Binding to grande competing filters. (○) Binding to test filters, with competition from petite filters. (□) Binding to petite competing filters.

If it is assumed, for example, that the petite mtDNA is a simple 50% deletion, then 50% of the sequences in the grande mtDNA would be absent in the petite. Hence a model grande mtDNA can be reconstructed by adding an equal quantity of nonhomologous sequences to petite mtDNA. Results of such an experiment in which the nonhomologous sequences added were yeast nDNA are shown in Figure 7.

Binding to "reconstructed grande" mtDNA is exactly as would be expected from the quantity of petite DNA present on the filter (compare broken line, solid circles, with unbroken line in Figure 7). The final plateau reached is approximately the same for both the pure petite mtDNA and the reconstructed grande mtDNA. The hybridization system thus behaves in the expected manner: the presence of nonhomologous DNA on filters does not interfere with the binding of petite [^{3}H]mtDNA in solution to petite mtDNA on filters. It follows that the results shown above (Figures 5 and 6; Table II) are not due to complexities of the filter hybridization system. Also, it is unlikely that simple deletion is the only mechanism responsible for petite mtDNA formation.

Competition Experiments. Further support for this conclusion was obtained with competition-type experiments. It is not possible to carry out DNA–DNA hybridization competition experiments as is normally done in DNA–RNA hybridization studies, *i.e.*, by adding increasing amounts of a competing, nonlabeled species to the labeled species in solution, since the renaturation of the solution DNA increases as its concentration is raised. Instead, we have devised a two-filter hybridization unit in which the DNA immobilized on one filter competes with DNA on the other filter for the available solution DNA. In this way, any significant increase in self-hybridization of the solution DNA is avoided.

Figure 8 shows the results of a typical competition experiment, which consists of a control set and an experimental set. In the control set, the two-filter hybridization units contained (1) a filter with 1 μg of grande mtDNA (the test filter), and (2) a filter with 0–20 μg of grande mtDNA (competing filter). DNAs on the two filters compete for the available solution DNA, which in this case was grande [^{3}H]mtDNA. As the amount of grande mtDNA on the competing filter is increased, the binding to the 1-μg test filter is decreased (Figure 8, solid lines). In the experimental set, the test filters again contained 1 μg of grande mtDNA, but the competing filter contained 0–20 μg of petite mtDNA (Figure 8, broken lines). In this case the competition is approximately 30% less effective than in the control. Other experiments, not presented here, show that yeast nDNA does not compete.

The competition experiment was repeated with petite [^{3}H]mtDNA in solution (Figure 9). Both sets of test filters contained 1 μg of petite mtDNA; in the control set competing filters contained 0–30 μg of petite mtDNA (Figure 9, broken lines). Again, competition for the available solution DNA was observed. When the competing filters contain grande mtDNA (Figure 9, broken lines), the effectiveness of the competition is decreased. Maximum competition is approximately 80% that of the control.

The competition experiments, like the saturation plots described above (Figure 5, Table II), suggest that both grande and petite mtDNA contain sequences that are nonhomologous one to another. Nonhomologous sequences appear to account for about 15–20% of the petite mitochondrial genome. Hence the alteration of the petite mtDNA, at least in the strain studied, cannot be explained by a simple deletion only, although a 15% deletion appears to be present as well. If deletion has been the only change, grande mtDNA, at high concentrations, should have been able to compete as effectively as petite mtDNA for the petite mtDNA in solution.

Discussion

Substantial changes in buoyant density (Mounolou *et al.*, 1966; Carnevali *et al.*, 1969), melting characteristics (Bernardi *et al.*, 1970), and base composition (Bernardi *et al.*, 1970) of mtDNA from cytoplasmic petite mutants of yeast have been

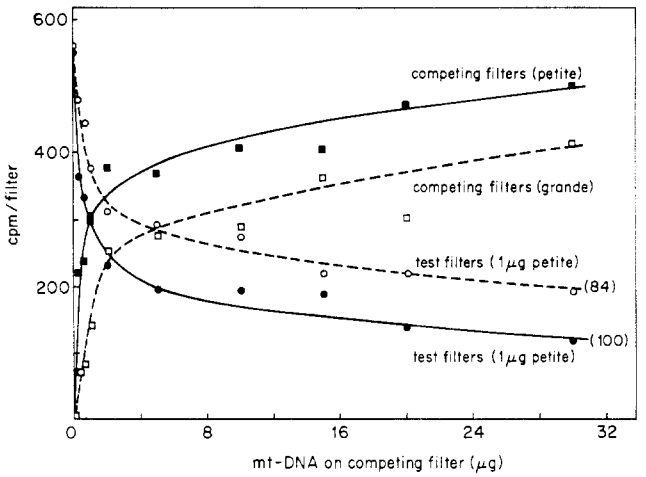

FIGURE 9: Competition of grande and petite mtDNA on filters for petite [^{3}H]mtDNA in solution. Two filter hybridization units were constructed as described in the text, and in legend to Figure 8. In this case the test filters contained 1 μg of petite mtDNA, and each hybridization unit contained 0.133 ml of petite [^{3}H]mtDNA (0.089 μg, 8600 cpm/μg). Values in parentheses indicate relative efficacy of competition. (●) Binding to test filters, with competition from petite filters. (■) Binding to petite competing filters. (○) Binding to test filters, with competition from grande filters. (□) Binding to grande competing filters.

documented; nevertheless, the mechanism leading to these changes is still unclear. Alterations in petite mtDNA may be explained by extensive deletion, by gross alterations in the nucleotide sequence of grande mtDNA, or by a combination of both processes. The possibility that petite mtDNA may contain reiterations of small or large segments of the mtDNA has also been raised (Mehrotra and Mahler, 1968; H. Fukuhara, 1971, personal communication; Casey *et al.*, 1969; Cohen *et al.*, 1972).

That petite DNA may contain nonhomologous sequences was first suggested by the DNA–RNA hybridization studies of Fukuhara *et al.* (1969), in which chased total cell grande RNA hybridized to a greater extent with grande than with petite mtDNA; and conversely, petite RNA hybridized better with its own mtDNA than with that of the grande. Similar experiments by Fauman (Fauman, 1970; Fauman *et al.*, 1973), supported by competition studies, also indicated that stable RNA sequences that were not present in the grande were transcribed in the petite.

The hybridization data presented in this paper provide a clear demonstration of the presence of nonhomologous nucleotide sequences in a grande and a derived cytoplasmic petite strain of yeast. With saturating amounts of mtDNA on filters, isohybrid formation was always greater than heterohybrid formation. This result would not be expected for a simple deletion, *i.e.*, if partial deletion of grande mtDNA is the only change. In that case all the petite sequences would still be represented in the wild-type mitochondrial genome, and hybridization with petite [^{3}H]mtDNA in solution should give a final binding plateau that is the same for filters containing sufficiently high amounts of either grande or petite mtDNA. Such a result was not observed; this does not appear to be secondary to kinetic difficulties inherent in the two-phase hybridization system (see below).

Two-filter competition experiments (Figures 8 and 9) strongly support the above evidence. If a simple deletion were the only mechanism, at high competing mtDNA concentrations grande mtDNA should then have been able to compete as effectively as petite DNA for petite [^{3}H]mtDNA in solution. The greater competition by petite than by grande mtDNA leads us to conclude that the petite mtDNA contains a class of sequences not present in grande mtDNA.

Thermal stability studies of the iso- and heterohybrids show that all the hybrids formed are well base paired, with stabilities similar to those of native DNA. These data strongly indicate the absence of scattered base alterations, although small changes cannot be excluded. Hence, the new class of sequences in the petite must be localized, possibly contiguous, and not randomly scattered throughout the genome.

Considerable evidence has accumulated that supports the presence of large deletions in mtDNA of ethidium bromide induced petites. For example, increasing periods of exposure to ethidium bromide result in petites having progressively smaller mtDNA, and ultimately in its disappearance (Goldring *et al.*, 1970, 1971). Furthermore, the second-order renaturation velocity constants (K_2) of mtDNAs from different ethidium bromide induced petites are 2–600 times faster than that in the grande, indicating a marked reduction in complexity and suggesting considerable deletion (F. Michel and H. Fukuhara, unpublished data). It has been assumed that a similar deletion mechanism acts during the formation of spontaneous petites (Borst, 1972). The data summarized in Table II show that the hybridization results from saturation-type experiments were only partly reciprocal. With grande [^{3}H]mtDNA in solution, binding to filters containing petite mtDNA was only 70% of that to homologous filters. On the other hand, with petite [^{3}H]mtDNA in solution, the homology was 85%. We suggest that the difference between grande and petite mtDNA may be accounted for in part by petite-unique sequences and in part by a deletion of grande mtDNA. It should be emphasized that these petite-unique sequences have been detected as sequences sufficiently changed so as not to bind in the hybridization assay. Extensive base substitution in a localized region of the grande mtDNA or reiteration of a small segment may explain the presence of these sequences. However, until the sequences can be isolated and characterized it is not possible to definitely postulate any mechanism, or biological role for these sequences.

The hybridization results appear to be inconsistent with more trivial explanations. Reconstruction experiments (Figure 7) show that the presence of nonhomologous sequences on the filter does not interfere with the binding to homologous sequences. Grande mtDNA sequences homologous to petite mtDNA should therefore have been available for hybridization. It must be emphasized that the hybridization changes observed in the spontaneously mutated petite strain studied were relatively small (15–30%). Problems related to inaccessibility of homologous sequences on filters would possibly be encountered in studies of petites having greatly altered mtDNA, as it would then be necessary to apply large amounts of grande mtDNA to filters to achieve adequate levels of sequences homologous to petite mtDNA.

Another possible difficulty in the use of the filter DNA–DNA hybridization system is that variable self-hybridization of solution mtDNA might confuse the interpretation of the results. This problem could become particularly important in petites with greatly increased renaturation rates, *e.g.*, in the series of petites with renaturation rates up to 600 times that of the parent wild-type (F. Michel and H. Fukuhara, personal communication). The moderate twofold increase in renaturation rate displayed by the mtDNA of the petite strain used in the present study (Fauman and Rabinowitz, 1973) appears to

present no serious complication. When the concentration of solution DNA was increased over a range up to tenfold that usually used, hybridization increased linearly with solution DNA concentration. Isohybrid remained greater than heterohybrid formation at all concentrations of solution DNA. The linearity of the response, and the fact that 75–85% of input labeled DNA was recovered in stable hybrids at all concentrations of solution DNA, show renaturation of solution DNA to be of relatively little significance. Calculations of the expected renaturation support this conclusion.

The mtDNA of the petite strain studied shows a twofold increase in the renaturation rate of the main portion of the DNA, relative to grande mtDNA (Fauman and Rabinowitz, 1973; J. Casey, unpublished observations). This observation suggests a 50% decrease in complexity of the petite mtDNA; this change is somewhat greater than observed with DNA–DNA hybridization. However, the physical properties of yeast mtDNA are aberrant in some ways (Borst, 1972; Bernardi *et al.*, 1970, 1972; Piperno *et al.*, 1972), and renaturation kinetic measurements have given anomalous results (Hollenberg *et al.*, 1970; Christiansen *et al.*, 1971). Furthermore, the filter DNA–DNA hybridization measurements may overestimate the degree of homology, due to the formation of filter-bound hybrids that have labeled single-stranded ends. These factors may explain the quantitative discrepancy between the hybridization and renaturation measurements.

In summary, the spontaneously derived petite mutant studied in this paper appears to contain mtDNA that is changed relatively little. About 70% of the sequences are homologous to the parent grande mtDNA. The hybridization data indicate that the decrease in homology is explained in part by deletion and in part by the presence of "new" sequences in the petite mtDNA. More recently we have compared the DNA–DNA hybridization properties of mtDNA isolated from a series of four petites including R1-6/1 (P. Gordon and M. Rabinowitz, in preparation). All strains show deletion of grande mtDNA; only in R1-6/1 can "new" sequences be detected. It remains to be determined whether petite-unique sequences occur only in spontaneously mutated cytoplasmic petites, or whether they are also present in ethidium bromide induced mutants.

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