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Respiratory-Deficient Mutants of *Torulopsis glabrata*, a Yeast with Circular Mitochondrial Deoxyribonucleic Acid of 6 μm

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Purified mitochondria from the petite positive yeast *Torulopsis glabrata* contain a circular deoxyribonucleic acid (DNA) with a length of 6 μm and a buoyant density of 1.686 g/cm³. This DNA is absent from ethidium bromide-induced respiratory-deficient mutants. Additionally, a second circular DNA component is present in both mutant and wild-type cultures with a length of 3 μm and buoyant density of 1.710 g/cm³. Spontaneously arising respiratory-deficient mutants occur at a frequency of 10^{-5}, which is 1,000-fold lower than in *Saccharomyces cerevisiae*. The mutants in *T. glabrata* each have mitochondrial DNA lower in buoyant density than the wild type and this change is accompanied by the appearance of a large number of small circular molecules. In considering factors responsible for the decreased frequency of spontaneous mutants in *T. glabrata*, a suggestion is made that the mitochondrial genome in *S. cerevisiae* may be the result of two independent dimerizations of a basic 6-μm molecule.

Evidence has accumulated that the cytoplasmically inherited petite mutation resulting in respiratory deficiency in the yeast *Saccharomyces cerevisiae* is accompanied by deletions from the circular mitochondrial deoxyribonucleic acid (DNA) (6, 14, 15). It has been proposed that these deletions occur by way of internal recombination at sites of homology, resulting in the excision of smaller circular DNA molecules (8, 18). The homologous sites are thought to be located in, or associated with, the adenine and thymine (AT)-rich noncoding sequences known to constitute about half the DNA and to be heterogeneously distributed around the circular mitochondrial genome (2, 18, 19). Studies with petite-negative yeasts have been undertaken to test these proposals. This class of yeasts is so termed because cytoplasmically inherited respiratory-deficient mutants have not been isolated from them (3, 10, 11). Mitochondrial DNA from four petite-negative yeasts have circular genomes ranging from only a quarter to half the length of that from *S. cerevisiae* (17, 21). The correlation between the smaller mitochondrial genome size and petite negativity prompted us to suggest that one explanation for petite negativity may be that the yeast lack the AT-rich noncoding sequences and, by implication, the homologous sites which are thought to occur in the *S. cerevisiae* genome (1, 17).

As a continuation of our approach to testing the idea that there may be a correlation between genome size, presence of AT-rich sequences and the ability to form petites, we have examined the size and sequence heterogeneity of a number of petite-positive yeasts which are not closely related to *S. cerevisiae*. A surprising result is that *Torulopsis glabrata*, an asporogenous yeast, has circular mitochondrial DNA of only 12.8×10^6 daltons, a quarter the size of the *S. cerevisiae* mitochondrial genome. Nevertheless, spontaneously arising respiratory-deficient mutants from *T. glabrata* can have mitochondrial DNAs of altered buoyant densities and smaller genome sizes, thereby implying that they are formed by a similar mechanism to petites in *S. cerevisiae*. We conclude from these results that there is no simple relationship between genome size and presence or absence of AT-rich sequences in mitochondrial DNA. The implications of these considerations to the mechanism of the petite mutation are discussed.

MATERIALS AND METHODS

Organism. *T. glabrata* strain 138 was obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands and maintained on GYP slopes (see below).

Media. The basic yeast extract, peptone medium (YP), contains per liter: 3 g of KH_2PO_4, 5 g of yeast

extract (Difco), and 10 g of peptone (Difco). To this is added for GYP medium, 20 g of glucose; Gly YP medium, 40 g of glycerol; for EtYP medium, 40 ml of 96% ethanol; and GGYP medium, 40 g of glycerol and 2 g of glucose. Media are solidified by the addition of 15 g of agar (Difco).

EtBr-induced respiratory-deficient mutants. Cells of *T. glabrata* were exposed to the highest concentration of ethidium bromide (EtBr) compatible with growth to ensure the complete loss of mitochondrial DNA. This was achieved as previously detailed (4), by subculturing from the margin of growth after a loop of culture had been streaked through a drop of concentrated (10 mg/ml) EtBr on a GYP plate.

Spontaneous respiratory-deficient mutants. *T. glabrata* was grown for 24 h in EtYP liquid medium, plated on GGYP medium, and putative respiratory-deficient colonies were selected by small size after 3 days growth at 30 C. The putative mutants were resuspended in 0.5 ml of sterile water and portions were dropped onto GYP and GlyYP plates. Subsequently it was found necessary to streak some of the original isolates onto GGYP plates to obtain respiratory-deficient mutants free from contaminating respiratory-competent cells.

Growth rates. These were determined by following the change in optical density at 640 nm of cultures growing in 50 ml of GYP medium in 125-ml conical flasks shaken at 200 rpm. The cultures were maintained at 30 C in a gyratory water bath shaker. Dry weight can be related to optical density using a value of 0.022 mg (dry weight) per ml, giving an optical density of 0.1.

Absorption spectra determinations. Cultures of 100 ml in GYP medium were harvested by centrifugation in late-exponential or early-stationary phase, and the cells were resuspended in 3 ml of 50% (vol/vol) glycerol-0.05 M sodium phosphate buffer, pH 7.0. The absorption spectrum was determined against a starch flour paste (13) using a Cary 14 spectrophotometer fitted with a scattered transmission accessory. Dry weights of washed samples were determined after 24 h at 110 C on preweighed aluminum foil cups.

Isolation and characterization of circular DNAs. Culture conditions, fractionation of cells, dye-buoyant-density centrifugation, analytical ultracentrifugation, and electron microscopy have all been described in detail elsewhere (4, 5). Briefly, strains were grown overnight at 30 C in GYP medium, harvested by centrifugation, resuspended in sorbitol buffer (0.5 M sorbitol, 0.05 ethylenediaminetetraacetic acid [EDTA] and 0.5 g of EtBr/liter) and disrupted with glass beads in a Braun homogenizer. A mitochondria-enriched pellet was obtained after three successive centrifugations to remove unbroken cells, debris, and nuclei. The mitochondria-enriched pellet was resuspended in 2% Sarkosyl, added to a CsCl-EtBr gradient, and then centrifuged to equilibrium in a Ti 50 rotor. The DNA band was visualized under ultraviolet light and extracted by side-puncture with an 18-gauge needle. The DNA was freed from EtBr by extraction with iso-amyl alcohol and then dialyzed overnight against 0.1× standard saline citrate (SSC, 0.15 M NaCl, 0.015 M sodium citrate). The buoyant-density analyses were performed in an MSE Centriscan 75 ultracentrifuge under standard conditions described previously (4) using *Micrococcus luteus* DNA as a marker.

For visualization in the electron microscope, DNA was spread on a hypophase of 0.25 M ammonium acetate, picked up on parlodion films, stained in 0.05 mM uranyl acetate, and observed at a magnification of ×9,800 in an Hitachi electron microscope. For molecular weight measurements the circular yeast DNA was spread with circularized λcI 857 DNA, which was a generous gift from A. Bellet, JCSMR, Australian National University. For calculations, the molecular weight of λ DNA was taken to be 30.8×10^6 (9). Lengths of relaxed circular molecules were measured with a map measurer, after projection of the negatives onto a screen giving an additional magnification of 17.3 times.

Purification of mitochondrial DNA. Spheroplasts of *T. glabrata* prepared as described previously for *S. cerevisiae* (7) were disrupted in a French press at 2,500 lb/in², and after removal of unbroken cells and debris by two centrifugations at $2,500 \times g$, a crude mitochondrial pellet was obtained by centrifugation at $8,500 \times g$ for 20 min. The mitochondrial pellet was resuspended in 0.6 M sorbitol-0.05 M EDTA, and 2-ml samples were layered onto 25 to 60% linear sucrose gradients [i.e., 25 g of sucrose plus 75 ml of 0.01 M tris(hydroxymethyl)aminomethane, pH 7.0, and 0.01 M EDTA and similarly 60 g of sucrose plus 40 ml of solvent] and centrifuged at $81,000 \times g$ for 1 h at 10 C in a Beckman SW27 rotor. A single particulate band half-way down the tube was collected by side puncture with an 18-gauge needle, diluted to 20 ml with 0.5 M sorbitol, 0.05 M EDTA, and the mitochondria were collected by centrifugation as above. Subsequently mitochondrial DNA was isolated by centrifugation in a CsCl-EtBr gradient, as described above. The mitochondrial DNA so isolated contained a single component with less than 10% nuclear DNA contamination as determined by equilibrium buoyant-density centrifugation.

Renaturation kinetic analysis. Isolated mitochondrial DNA was sheared to an $s^{pH\ 13}_{20,w}$ of 6.05 by passage through a French pressure cell at 15,000 lb/in² and then dialyzed for 24 h against two changes of 1 M NaCl. The DNA was denatured in a Teflon-stoppered quartz cuvette for 5 min in a boiling-water bath, cooled by immersion in ice for 5 s, and the rate of renaturation was measured at 60 C by monitoring the 260-nm absorption in a Gilford spectrophotometer. The absorption at infinite time ($A\infty$) was taken to be the absorption at 60 C before denaturation. The renaturation rate constant k_2 is obtained from the plot of $1/(A - A\infty)$ with time using the relationship of Wetmur and Davidson (23) whereby

$$\frac{1}{(A - A\infty)} = 2.04 \times 10^4\, k_2 t(\mathrm{s}) + \frac{1}{0.36\, A\infty}.$$

The kinetic complexity in daltons (N_D) is calculated from the equation of Wetmur and Davidson (23) for k_2 values in 1 M NaCl

$$N_D = \frac{5.5 \times 10^8\, (s^{pH\ 13}_{20,w})\ 1.25}{k_2}.$$

Sedimentation analysis. The sedimentation rate of the sheared DNA used in the analysis was determined in a Beckman analytical ultracentrifuge in 0.05 M NaOH-0.95 M NaCl using a single sector synthetic boundary cell centrifuged at 44,000 rpm. Sedimentation coefficients were calculated from the centrifugation data according to Studier (22).

RESULTS

Closed circular DNA from a mitochondria-enriched fraction from wild-type *T. glabrata* is shown in Fig. 1A and the size distributions of the molecules in Fig. 2A. Two discrete size classes of circles are present with lengths near 3 and 6 μm. Some circular molecules with lengths less than 3 μm and greater than 6 μm are also present. The molecular weights of the two major components have been determined by spreading the closed circular DNA in the presence of circularized λ DNA (Fig. 1A). From a comparison of the circular molecules relative to the size of the λ DNA, molecular weights of 6.6 for the smaller and 12.8 $\times$ 10^6

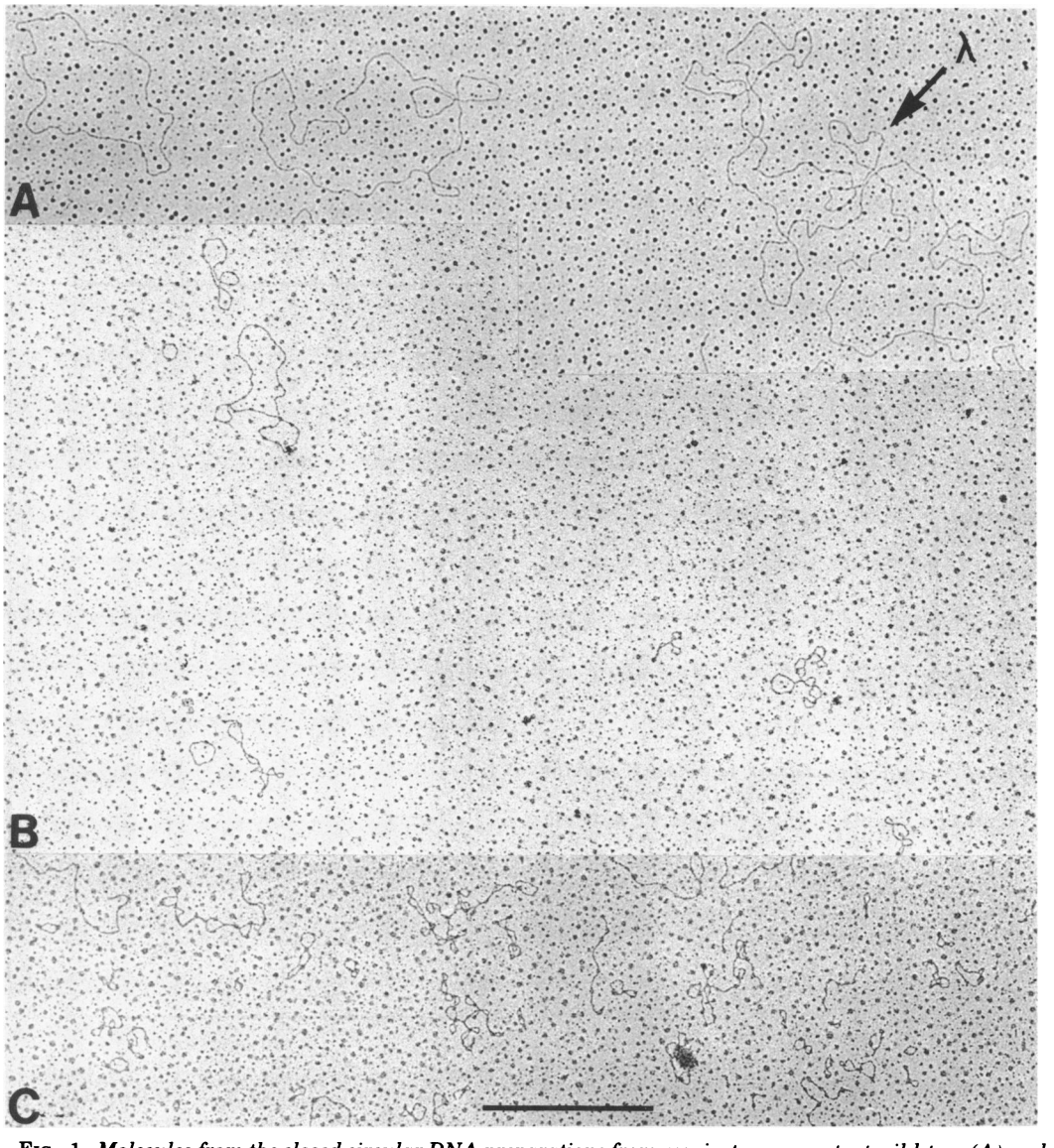

FIG. 1. *Molecules from the closed circular DNA preparations from respiratory competent wild-type (A) and two respiratory-deficient mutants sp⁻3 (B) and sp⁻19 (C). Circular molecules of the 3-μm and 6-μm size classes, together with a circular λ DNA (arrowed) are illustrated in A. The figures are at a magnification of 22,850. The bar represents 1 μm.*

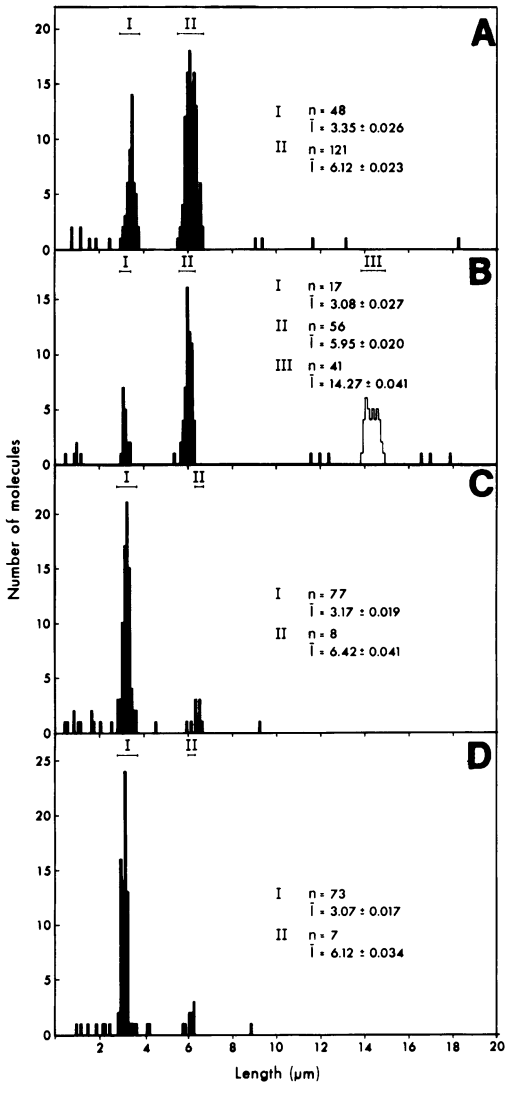

FIG. 2. *Length distribution of relaxed circular molecules from the closed circular DNA preparations from respiratory competent wild-type T. glabrata spread in the absence (A) and presence (B) of circularized λ DNA (peak III) and from two EtBr induced respiratory-deficient mutants eρ⁻1 (C) and eρ⁻4 (D). The number of molecules, mean lengths, and standard errors of each peak have been included in the figure.*

for the larger circles have been determined (Fig. 2B).

Analysis of the closed circular DNA by analytical ultracentrifugation revealed the presence of three peaks with buoyant densities of 1.710, 1.701, and 1.686 g/cm³ (Fig. 3, Table 1). An assignment of the 1.686 g/cm³ component to the 12.8 × 10⁶-daltons-size class can be inferred from the coincident loss of the 1.686-g/cm³ buoyant-density peak and large-circular molecules in EtBr-generated respiratory-deficient mutants (Fig. 2C, 2D, and 3). Although the large-circle-size class is lost from all four EtBr-induced mutants (Table 2), two mutants, eρ⁻1 and eρ⁻4, are illustrated as they have been consistently found to differ both in the ratios of 1.701 to 1.710 g/cm³ buoyant-density components in their closed circular DNAs (Fig. 3), and also in their absorption spectra (see below). The reason for these differences is unknown. Additionally the small number of circular molecules in the larger size class seen in Fig. 2C and D are most probably dimers of the 3-μm DNA species rather than remnants of the 1.686-g/cm³–12.84 × 10⁶-daltons molecules.

When mitochondria are banded on a sucrose gradient only the 1.686 g/cm³ DNA is recovered, this suggests that the mitochondrial genome is located on the 6-μm DNA molecules. An independent estimate of the mitochondrial

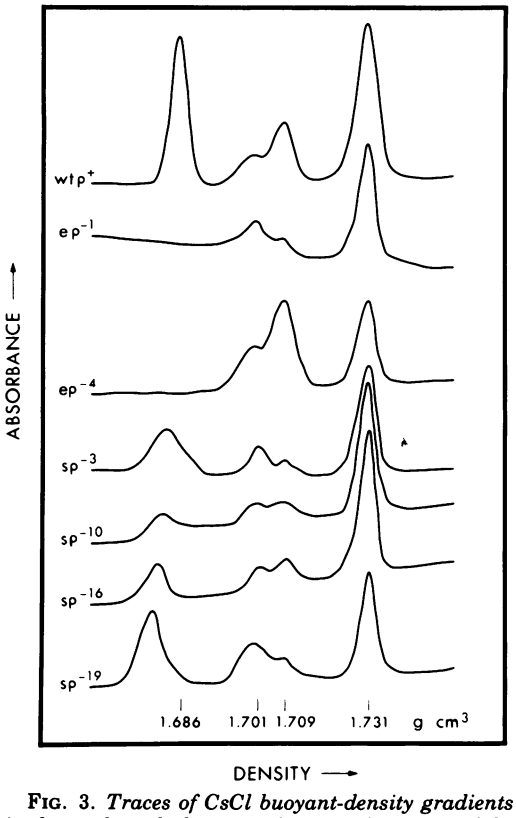

FIG. 3. *Traces of CsCl buoyant-density gradients in the analytical ultracentrifuge. Each trace is of the closed circular DNA fraction from the strain indicated at the left of the figure. DNA from Micrococcus luteus at a buoyant density of 1.731 g/cm³ is a marker.*

TABLE 1. *Results of analytical ultracentrifugation of closed circular DNA revealing three peaks*

Strain	No. of determinations	Buoyant densities of DNA components in the closed circular DNA (g/cm³)		
Wild type	8	1.6856 ± 0.0008	1.7009 ± 0.0011	1.7096 ± 0.0007
ep⁻1	2	ND[a]	1.7009	1.7093
ep⁻4	2	ND	1.7016	1.7085
sp⁻3	1	1.6818	1.6995	1.7093
sp⁻10	1	1.6805	1.7010	1.7085
sp⁻16	1	1.6801	1.7007	1.7106
sp⁻19	2	1.6784	1.7015	1.7103

[a] ND, None detectable.

TABLE 2. *Proportions of buoyant density components in the closed circular DNA preparations from four EtBr induced respiratory-deficient isolates of T. glabrata*

Mutant no.	Buoyant-density component		
	1.686 g/cm³	1.701 g/cm³	1.710 g/cm³
Wild type	57%	17%	26%
eρ⁻1	ND[a]	73%	27%
eρ⁻2	ND	49%	51%
eρ⁻3	ND	45%	55%
eρ⁻4	ND	37%	63%

[a] ND, None detectable.

genome size was made by renaturation kinetic analysis. The second order rate plot for the 1.686 g/cm³ DNA is shown in Fig. 4. The renaturation rate constant is 345 liter/mol per s at 60 C, which is approximately T_m-25 C in 1 M NaCl (Clark-Walker, unpublished observations). From this value a kinetic complexity of 14.8 × 10⁶ daltons is obtained using the determined $s^{pH,13}_{20,w}$ value of 6.05. As this sample is contaminated with approximately 7% nuclear buoyant-density DNA, and as the renaturation rate is affected by base composition, being slower for more AT-rich DNAs (23), the above result is in reasonable agreement with the molecular weight determined by electron microscopy and indicates that the 6-μm circles are unique.

A tentative buoyant density for the 3-μm circular DNA can be inferred from the correlation which exists between the amount of the 1.710-g/cm³ component and the number of 3-μm circular DNA molecules in the two respiratory-deficient isolates, eρ⁻1 and eρ⁻4. With eρ⁻4 the 1.710-g/cm³ DNA is 63% of the total (Table 2), and by electron microscopy the 3-μm circular molecules and oligomers account for 76% of the total length of all molecules, including smaller circles and linear DNA. On the other hand, with eρ⁻1 only 27% of the DNA is the 1.710-g/cm³ peak, whereas the 3-μm circular molecules and oligomers account for only 30% of the total length of DNA. These results suggest that the

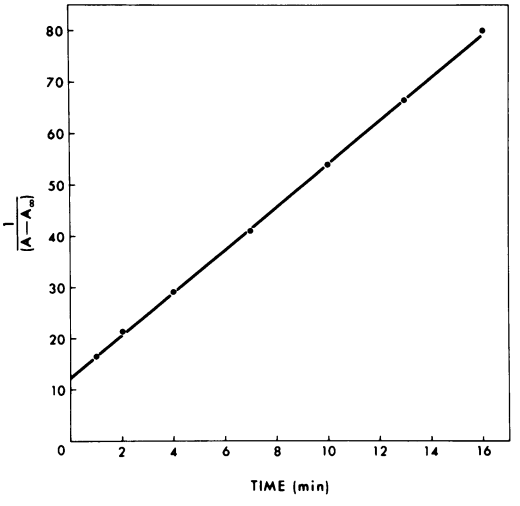

FIG. 4. *Second order rate plot for the renaturation of the 1.686-g/cm³ buoyant-density DNA.*

3-μm circular DNA size class has a buoyant density of 1.710 g/cm³.

Spontaneous respiratory-deficient mutants. One of the most unusual characteristics of petite colony formation in *S. cerevisiae* is the high frequency of spontaneous mutants (6). However, when we sought such mutants in *T. glabrata* none was found in a few thousand colonies. That this observation indeed reflects a low frequency of such mutants was clarified by plating a mixture of respiratory-competent cells and EtBr induced respiratory-deficient mutants. As seen in Fig. 5, the two colony types can be easily distinguished. Subsequently 19 spontaneously arising putative respiratory-deficient colonies were selected on the basis of small size after scanning 3 × 10⁵ colonies (Table 3). Of the initial 19 isolates, four were inviable, one grew slowly on both glycerol and glucose media, four showed only large colonies on GGYP medium, nine showed both large and small colonies on this medium, and only one, sρ⁻16, was composed entirely of respiratory-deficient cells. Subsequently, one small colony

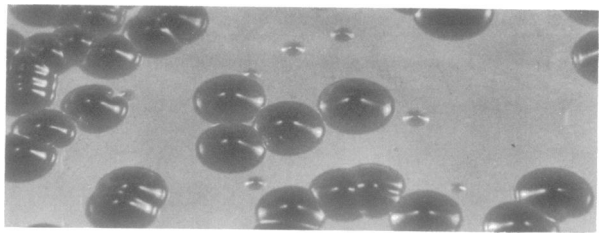

FIG. 5. *Large respiratory-competent- and small EtBr-induced respiratory-deficient mutant colonies growing on selective GGYP medium. Note the smooth margins of the large colonies.*

TABLE 3. *Putative spontaneously arising respiratory-deficient mutants of T. glabrata*

Isolate no.	First Subculture		Second subculture	
	Colony morphology on GGYP	Growth on GlyYP	Colony morphology on GGYP	Growth on GlyYP
1	Large	+[a]		
2	No growth	–[b]		
3[c]	Mixed	+	Small	–*
4	Mixed	+	Mixed	+
5	Mixed	+	Small	+
6	Large	+		
7	No growth	–		
8	Large	+		
9	Very small	+(v. slow)		
10[c]	Mixed	+	Small	–*
11	No growth	–		
12	Mixed	+	Mixed	+
13	Mixed	+	Small	+
14	Large	+		
15	Mixed	+	Mixed	+
16[c]	Small	–	Small	–*
17	No growth	–		
18	Mixed	+	Mixed	+
19[c]	Mixed	+	Small	–*

[a] +, Growth.
[b] –, No growth.
[c] *, Isolates retained for further analysis.

was selected for retesting from each of the nine "mixed" isolates on the GGYP plates. Three more purified respiratory-deficient mutants $s\rho^-3$, $s\rho^-10$, and $s\rho^-19$ were thus obtained, the remaining samples still possessing respiratory-competent cells.

Because the four respiratory-deficient isolates occurred at a frequency which suggested that they could arise from chromosomal gene mutations and as genetics tests are as yet unavailable in asporogenous yeasts, other parameters were examined to decide between the cytoplasmic or chromosomal nature of the mutations. Although growth rates and absorption spectra were determined for each isolate (see below) the most revealing tests were analyses of the buoyant densities and sizes of the closed circular DNAs.

Buoyant-density analyses showed (Fig. 3), in addition to the 1.710- and 1.701-g/cm^3 components, a lighter component in each isolate with a lower buoyant density than in the wild type (Table 1). Examination of the closed circular DNA from each mutant by electron microscopy showed the presence of a large number of small circular DNA molecules (Fig. 2B, C). With $s\rho^-3$ the size of the molecules fell into an oligomeric series based on the length of the smallest circles of 0.42 μm (Fig. 6A). The situation is more complex with the other mutants. The isolate, $s\rho^-10$, in addition to having circular DNA molecules which fall into an oligomeric series based on a monomer length of 0.41 μm, also appears to have at least one subset series of molecules indicated by arrows at a and b (Fig. 6B). The two other mutants $s\rho^-16$ and $s\rho^-19$ contained a large number of very small circular DNA molecules with no clearly defined size classes, although in $s\rho^-19$ the peak indicated by an arrow at b, with a length of 0.41 μm, could be a dimer of the peak at arrow a with a length of 0.22 μm. This cannot be so for $s\rho^-16$, because the peaks indicated by arrows at a and b have lengths of 0.20 and 0.34 μm, respectively, and the latter occurs in a higher frequency. Additionally, in the mutant $s\rho^-3$, peak VII with a length of 3.3 μm is thought to be the circular DNA with a buoyant density of 1.710 g/cm^3 which also occurs in the wild type. Using a molecular weight of 6.6×10^6 daltons for this peak a molecular weight of 0.85×10^6 daltons is obtained for the circular monomers in peak I. This value is 7.25% of the size of the original mitochondrial genome.

The growth rates of the spontaneous mutants, together with the two EtBr-induced mutants, are contrasted with wild type (Table 4). The growth rates of all respiratory-deficient mutants are lower than the respiratory-competent cells, but additionally the spontaneous mutants grow at a slower rate than the EtBr-induced mutants.

Absorption spectra of the wild type, two EtBr-induced mutants, and one spontaneous mutant, are illustrated in Fig. 7, where it is seen that the wild type has absorption peaks with maxima at 606, 561, and 551 μm, corre-

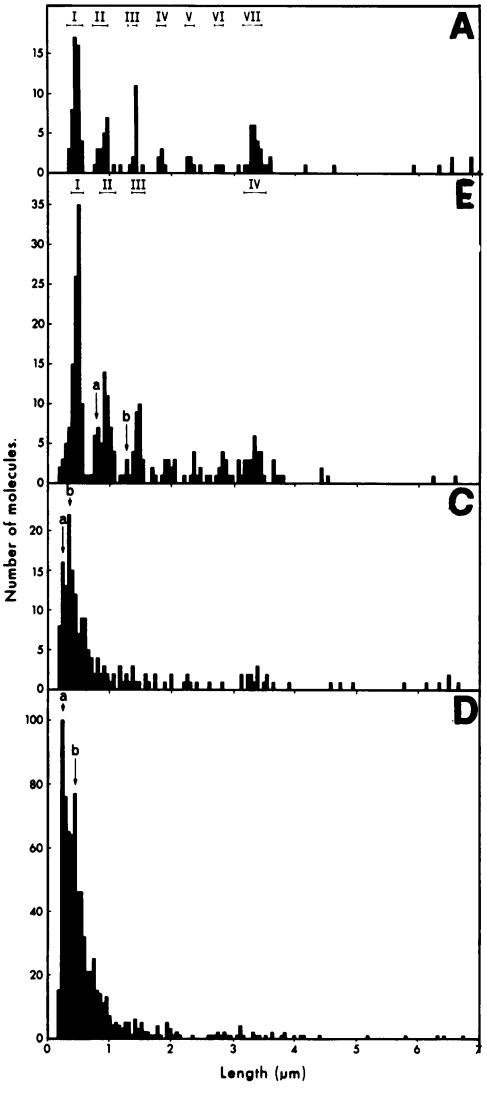

FIG. 6. *Length distributions of relaxed circular molecules from the closed circular DNA preparations from the four spontaneously occurring respiratory-deficient mutants sρ⁻3 (A), sρ⁻10 (B), sρ⁻16 (C) and sρ⁻19 (D). The peaks indicated by Roman numerals in A have numbers and mean lengths of: I, n = 48, $\bar{l}$ = 0.42 ± 0.01 μm; II, n = 19, $\bar{l}$ = 0.86 ± 0.02 μm; III, n = 14, $\bar{l}$ = 1.38 ± 0.008 μm; IV, n = 6, $\bar{l}$ = 1.80 ± 0.02 μm; V, n = 5, $\bar{l}$ = 2.26 ± 0.02 μm; VI, n = 3, $\bar{l}$, 2.74 ± 0.03 μm; VII, n = 21, $\bar{l}$ = 3.30 ± 0.01 μm. In B the peaks have numbers and mean lengths of: I, n = 103, $\bar{l}$ = 0.407 ± 0.008 μm; II, n = 54, $\bar{l}$ = 0.88 ± 0.01 μm; III, n = 30, $\bar{l}$ = 1.40 ± 0.01 μm; IV, n = 24, $\bar{l}$ = 3.30 ± 0.02 μm. The peaks indicated by arrows at a and b in B, C, and D are described in the text. Note the ordinate scale change in panel D.*

sponding to the α bands for cytochromes aa_3, *b*, and *c*, respectively. The two EtBr-induced mutants differ slightly in their absorption spectra but nevertheless lack the 606-nm absorption peak of cytochrome aa_3 and most of the cytochrome *b* absorption band at 561 nm. Two small absorption peaks at 586 and 505 nm are now present in these spectra and could be due to protoporphyrin IX.

Only the absorption spectrum of the spontaneous mutant sρ⁻16 is illustrated, as all four isolates were identical. Like the EtBr-generated mutants, these isolates all lack cytochromes aa_3 and most of the absorption band of cytochrome *b*. These parameters are compatible with a cytoplasmic origin for the spontaneous mutants.

TABLE 4. *Growth rates of the wild-type and respiratory-deficient isolates of T. glabrata*

Strain	Doubling time (min)	Doubling time (% of wild type)	Final cell yield mg(dry wt)/ml
Wild type	56		4.4
eρ⁻1	71	79%	2.4
eρ⁻4	65	86%	2.4
sρ⁻3	80	70%	2.4
sρ⁻10	80	70%	2.4
sρ⁻16	80	70%	2.4
sρ⁻19	84	67%	2.4

DISCUSSION

It is known that a number of yeasts from widely separated genera are capable of giving rise to respiratory-deficient mutants similar in phenotype to petites from *S. cerevisiae*. One of these yeasts, previously reported to be petite positive, is *T. glabrata* (3).

Circular DNA from a mitochondria-enriched membrane fraction from respiratory-competent wild-type *T. glabrata* contains three buoyant-density peaks and three types of DNA circle sizes when observed in the electron microscope. The circular DNA of buoyant density 1.686 g/cm³ and length of 6 μm, which is present in purified mitochondria, is absent from EtBr-induced respiratory-deficient mutants. This is similar to the situation in *S. cerevisiae* whereby EtBr can cause the formation of petites which have lost the 1.684 g/cm³, 25-μm mitochondrial 'DNA (12, 16). The molecular weight of the mitochondrial DNA in *T. glabrata* has been estimated to be 12.8×10^6 daltons by comparison with λ DNA used as an internal length standard. This value is supported by the estimate of 14.8×10^6 daltons obtained from renaturation studies. This finding of the small size of the mitochondrial genome in *T. glabrata* has two implications or consequences in relation to larger mitochondrial DNA molecules. Firstly it could imply that the size of larger molecules found in some yeasts, other lower eukaryotes, and higher

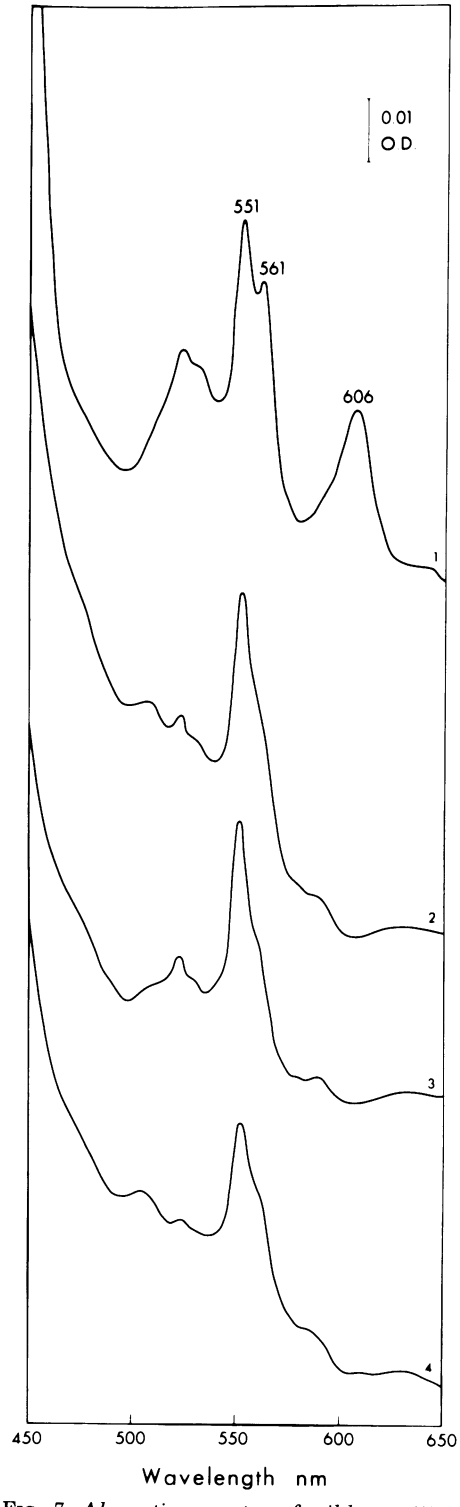

FIG. 7. *Absorption spectra of wild-type (1), two EtBr-induced mutants eρ⁻1 (2) and eρ⁻4 (3), and the spontaneously arising respiratory-deficient mutant sρ⁻16 (4). Cell densities in the cuvettes in mg (dry weight) of cells/ml were: 1, 15.8; 2, 26.5; 3, 21.3; and 4, 26.8.*

plants may not necessarily be related to their coding function and, secondly, as discussed below, it raises the question of the origin of these larger molecules.

Another size class of circular DNA having a molecular weight of 6.65×10^6 daltons must comprise most, if not all, of the 1.710-g/cm^3 buoyant-density peak. The circles with length below 3 μm and apparently heterodisperse in size are similar to those found in some other yeasts (17). Their buoyant density has not been established, but they may in part be responsible for the small peak of 1.701 g/cm^3 which is present in the supercoiled DNA of both wild-type and EtBr-generated petites.

Spontaneously arising respiratory-deficient mutants of *T. glabrata* are found to occur at the low frequency of around 10^{-5}. All four isolated mutants have mitochondrial DNA of altered buoyant density. This change is accompanied by the appearance of smaller circular molecules and the loss of the 6-μm size class. Two of the spontaneous petites, sρ⁻3 and sρ⁻10, have discrete circle size peaks falling into an oligomeric sequence reminiscent of the situation found with some EtBr-induced petites of *S. cerevisiae* (15). The other two petites, sρ⁻16 and sρ⁻19, have much smaller circular molecules with no clearly definable single monomer length. Heterodisperse circle sizes have also been observed in some petites of *S. cerevisiae* (1, 7), indicating that this is a general property of respiratory-deficient isolates. The interesting question of whether these heterodisperse circular molecules coexist within single cells is still undecided.

The occurrence of mitochondrial DNA of altered buoyant density and smaller circular size in the spontaneous mutants of *T. glabrata* indicates that the mutants are cytoplasmic in origin. Additionally the respiratory-deficient phenotype in these mutants is accompanied by a loss of cytochromes aa_3 and a slower growth rate, properties characteristic of cytoplasmically inherited respiratory-deficient mutants in *S. cerevisiae*. Although these results are strong arguments for the belief that the spontaneous mutants in *T. glabrata* are due to cytoplasmically located events, the possibility cannot at present be dismissed that these isolates carry additional chromosomal mutations affecting the respiratory-competent phenotype.

The incidence of spontaneously occurring respiratory-deficient mutants of *T. glabrata* is 1,000-fold less than in *S. cerevisiae*. This observation has a direct bearing on the proposed

mechanism of formation of petites which invokes excisions from sites of homology thought to exist in, or be associated with, AT-rich sequences. The fact that AT-rich regions exist in the mitochondrial DNA of *T. glabrata* can be inferred from the broad range of the melting profile (Clark-Walker, unpublished observations) and the 1.678 g/cm^3 buoyant density of the mitochondrial DNA from the petite sρ$^-$19. Therefore, the 1,000-fold lower incidence of spontaneously arising respiratory-deficient mutants in *T. glabrata* compared with *S. cerevisiae* is not due to the entire absence of AT-rich sequences in the mitochondrial DNA of the former yeast. Several factors need to be considered in attempting to explain the differing incidence of spontaneous mutants in the two species. Firstly, there may be more sites of homology in the mitochondrial DNA of *S. cerevisiae* than in *T. glabrata* simply because the former yeast has a genome four times the length of the latter. Additionally there may be a greater number of sites per unit length of DNA and these sites may differ in their size and the fidelity of sequence matching. Furthermore the arrangement of the sites around the circular molecule may have an influence on whether sites can be physically brought into alignment for an internal recombination event to take place.

Pertinent to these points is the fact that the mitochondrial DNA in *S. cerevisiae* is four times the length of that in *T. glabrata*. Furthermore some yeasts have circular mitochondrial DNAs either the same size as (*Schizosaccharomyces pombe*, 6 μm), or double (*Candida parapsilosis*, 12 μm; *Kluyveromyces lactis*, 12 μm) that of *T. glabrata* (17, 21). This could mean that the larger circular DNAs found in some yeasts and other organisms are the result of one or two dimerizations, as suggested for the formation of the *Escherichia coli* chromosome (24). Further support for the concept that the *S. cerevisiae* mitochondrial genome is the result of dimerization comes from the finding that the sequences coding for the small and large mitochondrial ribosomal RNA subunits are separated by at least one-third of the molecule (20). This could either have occurred by transposition of one of the cistrons in the 25-μm molecule or more likely we contend, by dimerization followed by devolution of redundant cistrons.

If indeed the mitochondrial DNA of *S. cerevisiae* has been formed from two rounds of dimerization then, in addition to having sites of homology in noncoding AT-rich sequences, it could well have retained some sequence homology in other regions of the genome. Therefore the high frequency of spontaneously arising petites in *S. cerevisiae* may be the summation of two processes of excision, one occurring at sites in AT-rich regions and the second at homologous sites in other regions of the genome. The second process may well not occur in *T. glabrata* because of the uniqueness of the genome, and this could possibly be a further contributing factor to the low frequency of spontaneous mutants in this yeast.

The present results are also crucial to an understanding of petite negativity. It is now apparent that the smaller mitochondrial genome sizes in petite-negative yeasts, in comparison with *S. cerevisiae*, are not simply due to the absence of noncoding AT-rich sequences. Furthermore we have shown that one petite-negative yeast *Hansenula wingei*, with a circular mitochondrial DNA of 17.3 × 10^6 daltons, has the same buoyant density as in *T. glabrata* (17). This suggests, at least in the case of *H. wingei*, that failure to form viable respiratory-deficient mutants may not be a consequence of its DNA structure. Other explanations need to be considered, such as the presence of viability genes in the mitochondrial genome (8) or the absence of appropriate enzymes to catalyze the breakdown of the DNA.

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