Biochemistry

Volume 13, Number 6 March 12, 1974

Characterization of Mitochondrial Deoxyribonucleic Acid from Grande and Petite Yeasts by Renaturation and Denaturation Analysis and by Transfer Ribonucleic Acid Hybridization; Evidence for Internal Repetition or Heterogeneity in Mitochondrial Deoxyribonucleic Acid Populations†

James Casey, Paul Gordon, and Murray Rabinowitz*

ABSTRACT: Mitochondrial DNA (mtDNA) prepared from four spontaneous cytoplasmic petite yeast strains and the parental grande (wild type) strain has been examined by thermal denaturation, hybridization with tRNA, and renaturation kinetic analysis. The T_m values and first derivative plots obtained from the DNA denaturation profiles of three of the petites were quite similar to those of the grande. However, one petite, R1-6/1, had a T_m of 1.5° lower than that of the grande. Increased saturation levels of mitochondrial leucyl-tRNA hybridization were obtained with petites R1-6/1, R1-6/5, and R1-6/8. Leucyl-tRNA hybridized with petite strain R1-6/6 mtDNA to the same extent as with the grande mtDNA. Renaturation kinetic analysis showed that the four petite mtDNAs renatured at a faster rate than grande mtDNA. Moreover, the petite mtDNAs have multiple renaturation components that comprise a variable amount of their respective genomes. The leucyl-tRNA cistrons were shown to be localized in the rapidly renaturing components of the mtDNA in these strains. The muticomponent renaturation profiles and the variably elevated contents of leucyl-tRNA cannot be explained entirely on the basis of differential deletion of segments of petite mtDNA. The results indicate either a variable repetition of petite mtDNA segments, or heterogeneity of mtDNA populations in these petite strains.

One of the many experimental advantages of yeast for the study of mitochondrial biogenesis is the availability of cytoplasmic petite mutants which show considerable structural changes in their mtDNA. Although petite mutants are isolated on the basis of their common respiration-deficient phenotype, it is now clear that the mitochondrial genome may differ greatly from one of these mutants to another in genetic and biochemical properties (Rabinowitz and Swift, 1970; Borst, 1972; Faye *et al.*, 1973). Genetic analysis has shown that specific antibiotic resistance markers, localized on mtDNA, may be either lost or retained in different cytoplasmic petites (Coen *et al.*, 1970; Nagley and Linnane, 1972; Faye *et al.*, 1973). In addition, there may be retention or loss of cistrons for mitochondrial tRNA (Casey *et al.*, 1972a; Cohen *et al.*, 1972; Cohen and Rabinowitz, 1972) and mitochondrial rRNA (Wintersberger and Viehauser, 1968; Fukuhara *et al.*, 1969; Fauman *et al.*, 1973) in various petite strains. In those petite strains where the mtDNA retains the cistron for leucyl-tRNA, this tRNA is transcribed and appears to be functional in that it can be aminoacylated *in vitro* and has the same hybridization properties as the parent wild type (Cohen *et al.*, 1972; Faye *et al.*, 1973).

Several laboratories have shown that mtDNAs from some petite mutants display large changes in buoyant density and thermal stability, presumably a consequence of a radical alteration in base composition (Mounolou *et al.*, 1966; Bernardi *et al.*, 1970; Borst, 1972). These changes have generally been attributed to extensive deletion of grande (wild-type) mtDNA sequences. Selective deletion could also account for the selective loss of antibiotic resistance markers, or of specific mitochondrial tRNA and mitochondrial tRNA cistrons,

† From the Departments of Medicine and Biochemistry, University of Chicago and the Franklin McLean Memorial Research Institute, Chicago, Illinois 60637. *Received August 22, 1973.* This work was supported in part by Grants HL04442, HL09172, HL05673, and HD00174 from the National Institutes of Health of the U. S. Public Health Service. Franklin McLean Memorial Research Institute is operated by the University of Chicago for the U. S. Atomic Energy Commission.

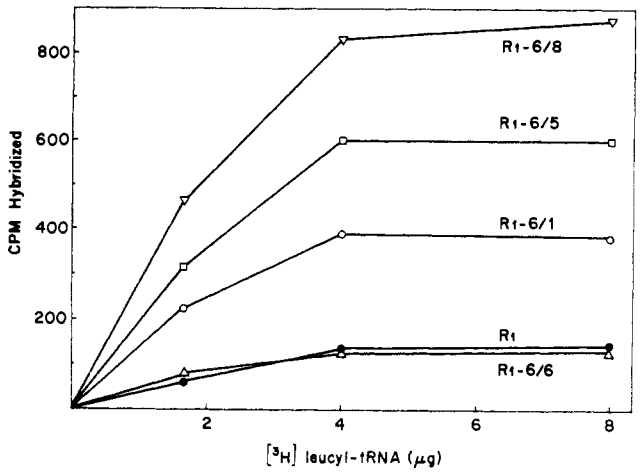

FIGURE 1: Hybridization of grande R1 mitochondrial leucyl-tRNA with mtDNA of subclones R1-6/1, R1-6/5, R1-6/6, and R1-6/8. R1 mitochondrial [^{3}H]leucyl-tRNA (specific activity 5.8 × 10^3 cpm/μg) was hybridized to filters containing 10 μg of mtDNA from the following strains: R1 (●—●), R1-6/1 (○—○), R1-6/5 (□—□), R1-6/6 (△—△), R1-6/8 (▽—▽). Blank values of 32–60 cpm were subtracted from each point.

as summarized above. It is consistent with this hypothesis that mtDNAs from several petite strains have decreased kinetic complexities (Casey *et al.*, 1972b; Fauman and Rabinowitz, 1972; Hollenberg *et al.*, 1972; Faye *et al.*, 1973; Michel *et al.*, 1974; Rabinowitz *et al.*, 1974). Besides, DNA–DNA hybridization studies have demonstrated that deletions can occur (Gordon and Rabinowitz, 1973; Gordon *et al.*, 1973). Recent evidence suggests, however, that the petite mutation can be considerably more complex. Hybridization plateaus of leucyl-tRNA to some petite mtDNAs are increased severalfold relative to the grande mtDNA (Casey *et al.*, 1969; Cohen *et al.*, 1972) suggesting repetition of some sequences of mtDNA. Also, in at least one petite there is a localized base-sequence alteration of approximately 15% of the mtDNA (Gordon and Rabinowitz, 1973). This alteration is extreme enough to prevent complete hybridization to grande mtDNA under moderately stringent conditions.

In this paper we report experiments designed to characterize grande and petite mtDNA in more detail. The parameters examined include renaturation kinetic analysis, thermal stability, and tRNA–mtDNA hybridization. The petite mtDNAs examined are kinetically less complex than grande mtDNA, and contain fast-renaturing fractions; specific mitochondrial tRNA cistrons are localized in separated "fast-" and "slow-renaturing" fractions. In the following paper (Gordon *et al.*, 1974), we present DNA–DNA hybridization data indicating that deletion of grande mtDNA sequences cannot be the sole explanation for the decrease in the kinetic complexity of these petite mtDNAs, or for the increased mitochondrial tRNA–mtDNA hybridization plateaus observed. It is concluded that petite mtDNAs may contain repetition of segments of the DNA, or there may be stable heterogeneous populations of mtDNA in these petite strains. Part of this work has been presented in preliminary form (Casey *et al.*, 1972b; Rabinowitz *et al.*, 1974).

Methods

Strains and Culture Conditions. The grande and petite strains 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 original petite strain, D243-2B-R1-6 (denoted R1-6), was isolated as a spontaneously mutated cytoplasmic petite derived from the grande strain, D243-2B-R1 (denoted R1). As described previously (Cohen *et al.*, 1972), the petite strain was subcloned to yield colonies denoted R1-6/1, R1-6/2, R1-6/3, etc. In the present paper, studies on mtDNA from four of these petite subclones, namely, R1-6/1, R1-6/5, R1-6/6, and R1-6/8, are discussed.

The growth of petite and grande strains has been described previously (Gordon and Rabinowitz, 1973). For the preparation of labeled DNA, 5 mCi/l. of ^{32}P (carrier free) were added to the growth medium which contained a reduced yeast extract concentration (0.25%). The lower concentration of yeast extract facilitated the labeling of cells, but did not reduce the exponential growth rate of the cultures, or the final cell yield.

Isolation of Mitochondria, mtDNA, tRNA, and Aminoacylating Enzymes. Mitochondria were isolated from both grande and petite strains of yeast after preparation of protoplasts with snail digestive juice (Glusulase Endo Laboratories) as previously described (Rabinowitz *et al.*, 1969). Mitochondria from grande and petite strains used for DNA isolation were digested with DNase to remove contaminating nuclear DNA (Casey *et al.*, 1972). DNA was isolated by lysis of the mitochondria with 1% sodium dodecyl sulfate and by deproteination with chloroform–octanol (9:1), and purified by hydroxylapatite chromatography as described earlier (Fauman and Rabinowitz, 1972; Gordon and Rabinowitz, 1973). The purity of the mtDNA was verified by CsCl isopycnic centrifugation in the analytic ultracentrifuge. Only samples which contained no detectable nuclear DNA when 5–7 μg of DNA were analyzed were used in the experiments.

Aminoacyl-tRNA and mitochondrial aminoacyl-activating enzymes were isolated from mitochondria as previously described (Halbreich and Rabinowitz, 1971; Casey *et al.*, 1972a). Grande (R1) mitochondrial tRNA was aminoacylated *in vitro* with [^{3}H]leucine or [^{3}H]valine of high specific activity as previously described (Casey *et al.*, 1972a).

tRNA–DNA Hybridization. Tritiated mitochondrial aminoacyl-tRNA was hybridized to filter-bound grande and petite mtDNA using a modification (Casey *et al.*, 1972a) of the procedure originally described by Weiss *et al.* (1968). Hybridization reactions were carried out under conditions shown to minimize deacylation, *i.e.*, at low temperature, 33°, in 50% formamide–2 × SSC (pH 5.0) for 4.5 hr. These stringent hybridization conditions (T_m = −15°) result in hybrids which melt sharply and have appropriate thermal stability (Casey *et al.*, 1972a; Cohen *et al.*, 1972).

DNA Renaturation Kinetic and Optical Denaturation Analysis. Mitochondrial DNA samples in 0.1 M sodium phosphate (pH 6.8)–1 mM EDTA were sheared at 50,000 psi in a Sorvall Ribi cell fractionator at 7–10°. The average molecular size of the DNA fragments was 5.4 S, as determined by alkaline band sedimentation analysis according to Studier (1965). By electron microscopy (samples mounted from formamide), the fragment size was 300–400 nucleotides. After shearing, the DNA was applied to a 2 × 12 cm column containing 5 g of hydroxylapatite equilibrated with 0.1 M sodium phosphate (pH 6.8), and the column was washed with 200 ml of the same buffer. The sheared DNA was then eluted with 0.4 M sodium phosphate (pH 6.8) and dialyzed extensively against 0.1 M sodium phosphate (pH 6.8).

For renaturation studies, the DNA samples at 0.4–0.6 A_{260nm}/ml in 0.1 M sodium phosphate (pH 6.8) were flushed with helium gas and placed in Teflon-stoppered cuvettes. The

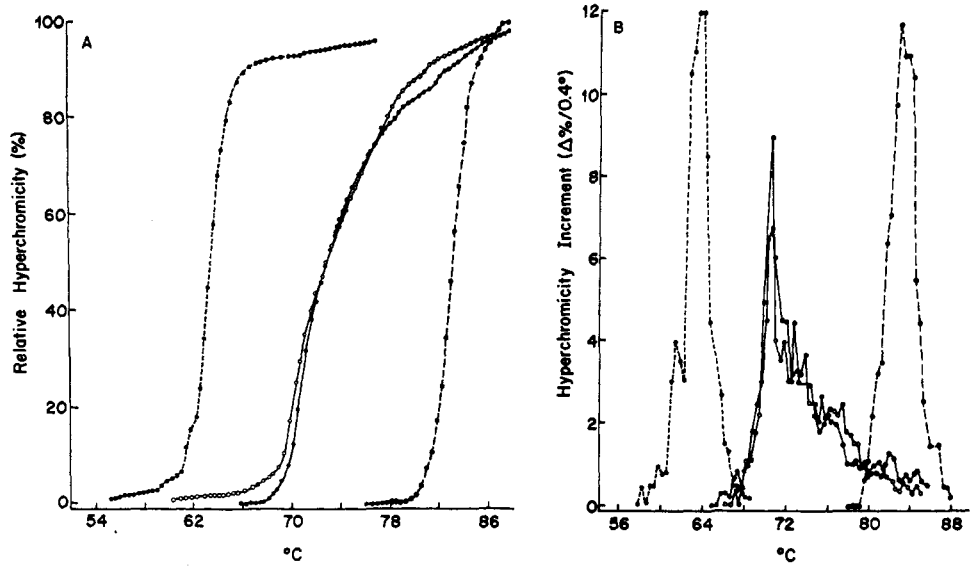

FIGURE 2: Optical melting profiles of grande and petite mtDNAs. (A) Cumulative optical melting profiles of grande R1 (○—○), petite R1-6/8 (●—●), T4 (●--●) and poly[d(A-T)] (●···●) DNA. DNA samples (25 μg/ml) were melted in 0.1 M phosphate buffer. Hyperchromicities were normalized to 100% and plotted as a function of temperature. (B) First derivative plot of optical melting profiles of grande R1 (○—○), petite R1-6/8 (●—●), T4 (●---●), and poly[d(A-T)] (●···●) DNA. Conditions were the same as above.

samples were heated to 100° for 5 min and then cooled to $T_m = -25°$ (52°). The cuvettes were placed in the constant temperature chamber (52°) of a Gilford spectrophotometer Model 2000 and the absorbance at 260 nm was recorded as a function of time. A melting curve (see below) was obtained after every renaturation reaction to determine the maximum absorbance of denatured DNA (A_0). The initial A_0 and the A_0 recorded at the end of the renaturation reactions were in close agreement. Second-order renaturation constants (K_2) were calculated by the procedure of Wetmur and Davidson (1968), and C_0t curves were plotted according to the method of Britten and Kohne (1968).

For optical denaturation analysis, mitochondrial DNA samples were flushed with helium gas, sealed in Teflon-stoppered cuvettes, and placed in a Gilford spectrophotometer Model 2400 at a temperature of 50°. The A_{260nm} was measured every 2 min as the temperature was raised at a rate of 12°/hr with a Haake PG11 linear temperature programmer coupled to a Haake temperature circulator.

Hydroxylapatite Chromatography. For thermal elution chromatographic analysis, samples of renatured ^{32}P-labeled grande and ^{3}H-labeled petite mtDNAs were applied to a jacketed column (at 52°) containing 4 g of hydroxylapatite (Bio-Rad Corp., Richmond, Calif., lot 10849) equilibrated in 0.14 M sodium phosphate (pH 6.8). The renatured DNA, which was derived from controls used in DNA–DNA hybridization experiments reported in the accompanying paper (Gordon *et al.*, 1974), yielded melting profiles which were identical with those obtained with native DNA. The DNA for these experiments was sheared using a Branson sonicator (Gordon *et al.*, 1974) which produced fragments having an alkaline *s* value of 5.9 S, equivalent to about 500 nucleotide pairs (Gordon *et al.*, 1974). Buffer was then pumped through the column at 2.8 ml/min, while the column temperature was raised 12°/hr by a Haake F425 circulator coupled to a Haake PG11 linear temperature programmer. Column fractions (8 ml) were mixed with an equal volume of Instagel (Packard Instrument Corp., Downers Grove, Ill.) and assayed for radioactivity. Solutions used for chromatography were extensively deaerated prior to use.

The separation of fast and slow renaturing fractions of mitochondrial DNA was accomplished using hydroxylapatite chromatography after renaturing heat-denatured mitochondrial DNA to 30–50% completion. The DNA was applied to a 0.5 × 12 cm jacketed column (maintained at 52°) containing 1 g of hydroxylapatite in 0.14 M sodium phosphate (pH 6.8). Single-stranded DNA was eluted with 0.14 M sodium phosphate. Following the elution, the double-stranded DNA was eluted with 0.4 M sodium phosphate. The enriched fast- and slow-renaturing DNA fractions were then dialyzed against 0.1 × standard sodium citrate (0.15 M NaCl–0.015 M sodium citrate). The per cent cross-contamination of fast- and slow-renaturing fractions after one hydroxylapatite cycle are calculated from the theoretical second-order rate equation (Britten and Kohne, 1968): $C/C_0 = 1/(1 + KC_0t)$.

Results

Hybridization of Mitochondrial [^{3}H]Leucyl-tRNA to Grande and Petite mtDNA. Our recent observation (Cohen *et al.*, 1972) that the mtDNAs of several related cytoplasmic petite strains showed substantial increases in their hybridization saturation levels with mitochondrial [^{3}H]leucyl-tRNA can be interpreted in several ways. One explanation is a deletion of a considerable fraction of the mtDNA with retention of the leucyl-tRNA cistron. Another possibility is a repetition of the segment of mtDNA specifying leucyl-tRNA. We have chosen four of the petite strains previously studied to try to characterize further the structural alterations in their mtDNA that may account for these observations.

Figure 1 shows the hybridization of mitochondrial [^{3}H]-leucyl-tRNA (derived from grande yeast) to mtDNA of petite subclones R1-6/1, R1-6/5, R1-6/6, and R1-6/8, and to parental grande (R1) mtDNA. Hybridization saturation levels with the four petite mtDNAs were, respectively, about 2, 4, 1, and 6 times that with grande mtDNAs. These results have been observed repeatedly after many subcultures of the original subclones, indicating that the mtDNAs of these petite strains are quite stable, at least with respect to the genetic information content for leucyl-tRNA.

Further analysis of the petite and grande mtDNA was carried out by thermal denaturation and renaturation kinetic analysis.

TABLE I: Optical Thermal Stability of Mitochondrial DNAs.[a]

Source	T_m (°C)	% Hyperchromicity
Grande R1	73.0	42.0
Petite R1-6/1	71.2	41.0
Petite R1-6/5	72.8	39.0
Petite R1-6/6	72.8	38.5
Petite R1-6/8	73.0	43.5
T4	83.1	45.5
Poly[d(A-T)]	63.4	58.0

[a] DNAs were melted in 0.1 M phosphate buffer at a concentration of 25 μg/ml.

Thermal Denaturation Analysis. Yeast mtDNA characteristically shows a very broad and heterogeneous melting curve, indicating considerable heterogeneity of base distribution (Bernardi *et al.*, 1970). Large deletions might result in specific elimination of some segments of the melting profile.

Results of thermal stability analysis of grande and petite mitochondrial DNAs, monitored by optical hyperchromicity at 260 nm, are presented in Figure 2A,B and Table I. The melting profiles of the four petite mitochondrial DNAs were similar to the grande mitochondrial DNA. The melting profiles for R1-6/8 mitochondrial DNA shown in Figure 2A,B are typical for this group. The T_m's are listed in Table I. One petite mtDNA (R1-6/1) has a slightly lower T_m as previously reported by Gordon and Rabinowitz (1973), but the form and distribution of its melting profile were similar to the others.

The melting profile of T4 DNA and the synthetic copolymers of alternating dA-T, poly[d(A-T)], are also presented for comparison. These DNAs denature over a narrow range of temperatures (Figure 2A) and show symmetrical peaks in their differential melting curve (Figure 2B), as expected for DNAs with a relatively uniform distribution of bases. In contrast, the melting profiles of grande and petite mitochondrial DNAs are asymmetrical (Figure 2B). In both grande and petite strains, a sharp major peak comprising approximately 40% of the mitochondrial DNA melts at about 70°, and thus represents (A + T)-rich segments. The remainder of the mitochondrial DNA is heterogeneous, melting over a range of 12–15° (from 71 to 84°).

The distribution of the (A + T)-rich segment in grande and petite mtDNA was examined by comparing the optical melting curves, with the thermal hydroxylapatite chromatographic profile of DNA sheared to an average size of 500 nucleotide pairs. Results with grande and petite mtDNA were identical (Figure 3A,B). As in the case of the optical measurements, the hydroxylapatite thermal elution patterns are also broad. Melting takes place over about 20°, again indicating a considerable heterogeneity of base distribution. However, the large early melting peak at about 70° observed optically is absent in the hydroxylapatite elution profile. The $T_e(50)$ (temperature at which 50% of DNA eluted from the column) of the hydroxylapatite melting profile is 4–5° higher than the optically determined T_m. The differences in optical and thermal elution melting profiles indicate that sequences containing runs of high (A + T) content are heterogeneously dispersed throughout the grande and petite mitochondrial genome in lengths smaller than the shear size eluting from the hydroxylapatite column (500 nucleotide pairs). Similar conclusions regarding the dispersion of (A + T)-rich sequences in grande mitochondrial DNA have been reported by Bernardi *et al.* (1972), who used ionic gradient elution of sheared DNA from hydroxylapatite columns.

The thermal elution patterns (Figure 3A,B) of grande and petite yeast were very similar, indicating that no gross differences in base compositional distribution were present in these petite mtDNAs.

Renaturation Kinetic Analysis. Renaturation kinetic analysis provides information about the kinetic complexity of a genome, and also about the presence of repeated sequences (Britten and Kohne, 1968; Wetmur and Davidson, 1968). In our renaturation kinetic analyses of grande and petite mtDNA, we have expressed the data in terms relative to those for grande mtDNA rather than in absolute terms, because of several kinetic features that may make the direct calculation of kinetic complexity very difficult (see Discussion). All renaturation reactions were carried out under identical experimental conditions (salt concentration, shear size, and temperature), and monitored to approximately 85% completion.

The renaturation data are presented as second-order rate plots according to the procedure of Wetmur and Davidson

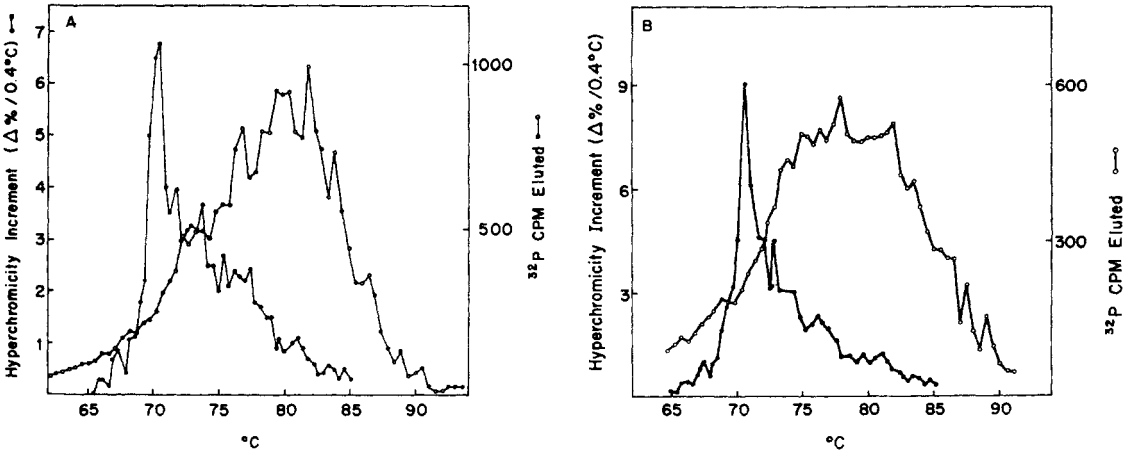

FIGURE 3: (A) First derivative plot of the optical and hydroxylapatite melting profiles of grande and petite mtDNAs. Conditions: Grande R1 mtDNA (25 μg/ml) was monitored optically at 260 nm in 0.1 M phosphate buffer (●—●). Grande R1 ^{32}P-labeled renatured DNA (6 μg; specific activity 6 × 10^3 cpm/μg) was eluted from a hydroxylapatite column as a function of temperature (○—○) as described in the Methods. (B) First derivative plot of the optical and hydroxylapatite melting profiles of petite R1-6/8 mtDNA. Conditions: petite R1-6/8 mtDNA (25 μg/ml) was monitored optically at 260 nm in 0.1 M phosphate buffer (●—●). Petite R1-6/8 ^{32}P-labeled renatured mtDNA (4 μg; specific activity 6.3 × 10^3 cpm/μg) was eluted from the column as a function of temperature (○—○).

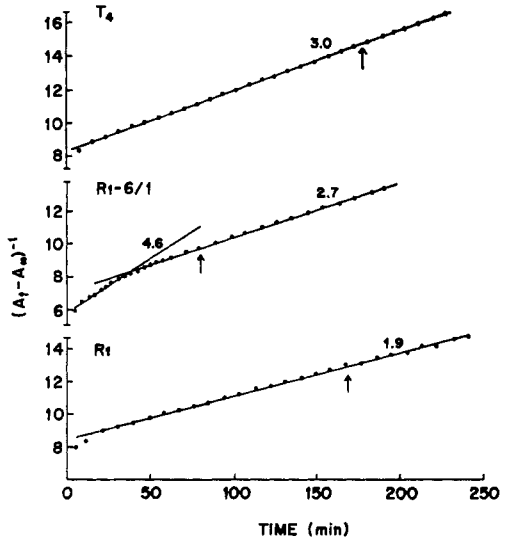

FIGURE 4: Renaturation kinetic analysis of R1, R1-6/1 mtDNA, and T4 DNA. Conditions: R1, 27 μg/ml; R1-6/1, 27 μg/ml; and T4, 26 μg/ml were renatured at 0.1 M phosphate buffer (pH 6.8) at 52°. The data are presented according to Wetmur and Davidson (1968). The arrows indicate the point at which 50% renaturation was observed.

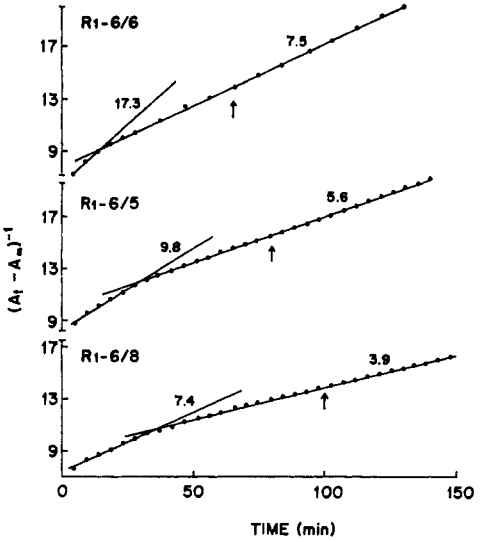

FIGURE 5: Renaturation kinetic analysis of R1-6/6, R1-6/5, and R1-6/8 mtDNA. Conditions: R1-6/8, 18 μg/ml; R1-6/5, 17 μg/ml; and R1-6/6, 20 μg/ml were renatured in 0.1 M phosphate buffer (pH 6.8) at 52°. The data are presented according to Wetmur and Davidson (1968). The arrows indicate the point at which 50% renaturation was observed.

(1968). Second-order rate plots for T4 DNA and grande (R1) and petite R1-6/1 mtDNA are shown in Figure 4. Arrows indicate the point at which 50% renaturation has occurred. T4 DNA generally follows the second-order kinetics for unique or single copy DNAs, but the curve for grande mtDNA is somewhat curvilinear throughout. C_0t curves, plotted according to Britten and Kohne (1968) (data not shown), indicate that both grande and petite mtDNAs renature over a wider range of C_0t values than T4 DNA. The range is considerably larger than expected for an ideal second-order reaction involving a unique population of DNA. The initial 5% decrease in hyperchromicity observed in Figure 4 for all three DNAs is probably due to the time required to attain temperature equilibration. The renaturation profile of petite R1-6/1 mtDNA differs strikingly from grande mtDNA in that it contains at least two renaturing fractions. The fast renaturing fraction of R1-6/1 mtDNA comprises approximately 40% of the petite genome, and renatures about twice as fast as the slower renaturing fraction.

Similar results were obtained with the mtDNA of the three other petite strains (Figure 5). When renaturation was followed to approximately 60% completion, these petite mitochondrial DNAs also had fractions that renatured at approximately twice the rate of the slow-renaturing fraction. From inspection of the second-order renaturation plots, we estimate that the fast-renaturing fractions comprise about 30, 23, and 35% of the genomes of petite strains R1-6/5, R1-6/6, and R1-6/8, respectively. It is assumed that renaturation proceeds to completion at the rate displayed by the slow-renaturing fraction.

The possibility that fast- and slow-renaturing fractions of petite mtDNA might be due to heterogeneity of cell types within each petite strain was evaluated by further subcloning of petite strains R1-6/1 and R1-6/5. Renaturation kinetic analyses of mtDNA isolated from these subclones of strains R1-6/5 (Figure 6) and R1-6/1 (not shown) gave essentially the same results as had been obtained from the original strain. Thus, cellular heterogeneity is not the explanation for the presence of two mtDNA fractions having different renaturation rates in each of these petite strains.

Fast-renaturing fractions in the petite mtDNAs indicate that some segments of the mtDNA are present in increased numbers. We tested this conclusion by trying to enrich for fast and slow components by means of differential renaturation and separation of single- and double-stranded DNA on hydroxylapatite columns (see Methods). Conditions were selected which would allow the renaturation of approximately 70% of the fast fractions and less than 30% of the slow-renaturing fraction. Renaturation kinetic analysis of the "separated" fast and slow fractions of R1-6/8 petite mtDNA is shown in Figure 7. It is apparent that a substantial enrichment for early- and late-renaturing components has been achieved. The enriched fast fraction still shows a broader C_0t plot than predicted for an ideal and second-order reaction, indicative perhaps of the presence of more than one renaturing class.

The second-order rate constants (K_2) of the fast- and slow-renaturing components of the petite mtDNAs compared to the rate constant for grande mtDNA taken as unity are summarized in Table II. For these estimates, the fast- and slow-

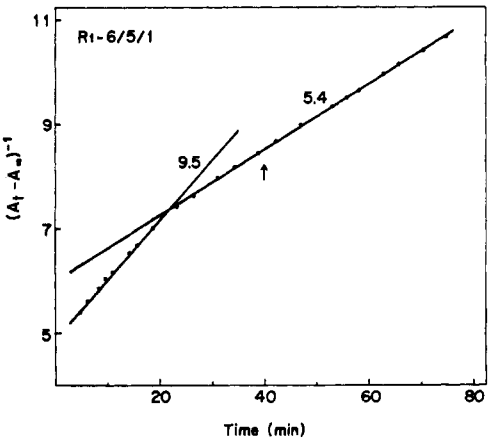

FIGURE 6: Renaturation kinetic analysis of petite subclone R1-6/5/1 mtDNA. Conditions: R1-6/5/1 mtDNA (27 μg/ml) was renatured in 0.1 M phosphate buffer (pH 6.8) at 52°. The data are presented according to Wetmur and Davidson (1968). The arrow indicates the point at which 50% renaturation was observed.

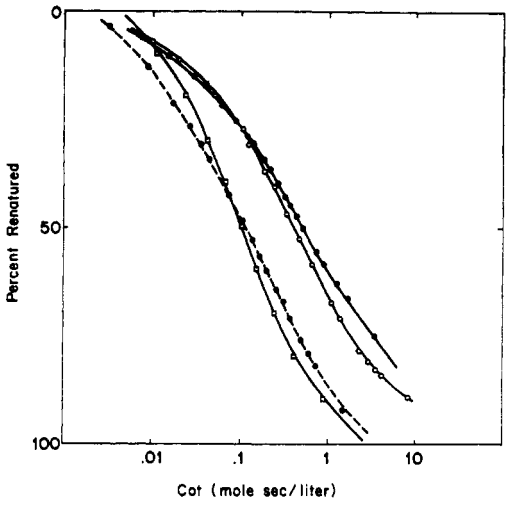

FIGURE 7: Renaturation kinetic analysis of isolated enriched fast- and slow-renaturing fractions of peptide R1-6/8 mtDNA. Petite R1-6/8 mtDNA (25 μg/ml) was renatured to a C_0t of 0.3 (45% renatured) and separated into fast- and slow-renaturing fractions by hydroxylapatite chromatography as described in Materials and Methods. Conditions: R1-6/8 fast-renaturing mtDNA fraction, 21 μg/ml, ●--●; R1-6/8 slow-renaturing mtDNA fraction, 18 μg/ml, ●—●; R1-6/8 unfractionated mtDNA, 21 μg/ml, ○—○; all renatured at 52° in 0.1 M phosphate buffer (pH 6.8). The theoretical curve for an ideal second-order reaction is shown (□—□). Data are presented according to Britten and Kohne (1968).

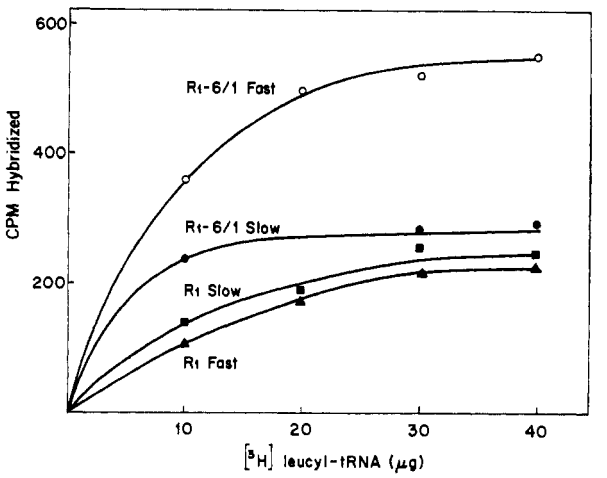

FIGURE 8: Hybridization of grande R1 mitochondrial leucyl-tRNA with fast- and slow-renaturing mtDNA fractions from grande R1 and petite R1-6/1 strains. Grande R1 mtDNA (25 μg/ml) was denatured and renatured to a C_0t of 0.6 (40% renatured) and separated into fast- (▲—▲) and slow- (■—■) renaturing fractions by hydroxylapatite chromatography. Petite R1-6/1 mtDNA (25 μg/ml) was denatured and renatured to a C_0t of 0.402 (50% renatured) and separated into fast- (○—○) and slow- (●—●) renaturing fractions by hydroxylapatite chromatography. The fast- and slow-renaturing fractions were then fixed to nitrocellulose membrane filters (10 μg/filter) and hybridized with mitochondrial [^{3}H]-leucyl-tRNA (specific activity 1.3 × 10^4 cpm/μg). Hybridization was carried out in a 33% formamide system. Reaction volume was 1.5 ml.

renaturing components are assumed to be uniform populations. It is apparent that, if the leucyl-tRNA cistron is located exclusively in the fast-renaturing fraction, and if the relative K_2 provides a reasonable estimate of the relative number of copies of that segment of the DNA, the enhanced hybridization saturation levels observed with [^{3}H]leucyl-tRNA in some strains could in part be explained on the basis of multiple repetition of some mtDNA segments. The correlation between enhanced hybridization and K_2 is not exact however.

Hybridization of Mitochondrial Leucyl-tRNA with Enriched Fast- and Slow-Renaturing Fractions of Petite Mitochondrial DNAs. To localize the leucyl-tRNA cistron, enriched fast- and slow-renaturing fractions of the petite mitochondrial DNAs were prepared on the basis of their different rates of renaturation, as described in Methods. The DNAs were renatured under conditions which resulted in an overall renaturation of 30–50%. As a control, the grande mitochondrial DNA, which lacks a substantial fast renaturing fraction, was similarly separated into early- and late-renaturing components. Mitochondrial [^{3}H]leucyl-tRNA was then hybridized with these fractions.

Hybridization saturation levels of mitochondrial leucyl-tRNA with the enriched fast- and slow-renaturing fractions of grande (R1) and petite R1-6/1 mitochondrial DNA are shown in Figure 8. Both fractions of grande (R1) mitochondrial DNA saturated at approximately the same level. In contrast, the saturation level of the fast fraction of petite (R1-6/1) mtDNA was approximately twice that of the slow-renaturing fraction, indicating that substantial enrichment for the leucyl-tRNA cistron had been attained.

The results of the hybridization of mitochondrial leucyl-tRNA with the enriched fast- and slow-renaturing DNA fractions of the four petite strains are shown in Table III. When corrections are made for cross-contamination of fast- and slow-renaturing DNA fractions, estimated from their second-order rate constants (K_2), the cistron for leucyl-tRNA appears to be localized only in the fast-renaturing components of all petite strains tested.

Hybridization of Mitochondrial [^{3}H]Valyl-tRNA with Enriched Fast- and Slow-Renaturing Mitochondrial DNA Fractions of Petite R1-6/1. Fast- and slow-renaturing mitochondrial DNA fractions of petite R1-6/1 were further characterized by hybridization with mitochondrial [^{3}H]valyl-tRNA (Figure 9). The hybridization level of valyl-tRNA with the unfractionated mtDNA of this strain is about 30% lower than with grande mtDNA (Cohen *et al.*, 1972). It is apparent that valyl-tRNA hybridized to approximately the same extent with both renaturing fractions of the grande mtDNA. In contrast, hybridization with valyl-tRNA is much greater with the slow renaturing component of petite R1-6/1 mtDNA than with the fast-renaturing fraction. Thus, the cistrons for valyl-tRNA

TABLE II: Comparison of Leucyl-tRNA Hybridization Levels, Second-Order Renaturation Rates, and Sequence Homology Estimated by DNA–DNA Hybridization.

Strain	Leucyl-tRNA Hybridization (Rel to Grande)	Renaturation Rate (K_2 Rel to Grande)		% mtDNA in Fast Fraction	% Homology to Grande mtDNA by DNA–DNA Hybridization in Solution[a]
		Fast Fraction	Slow Fraction		
R1 (grande)	1	1	1		100
R1-6/1	2.5	2.3	1.3	40	70
R1-6/5	4	4.9	2.8	30	54
R1-6/6	1	8.7	3.7	23	74
R1-6/8	6	3.7	1.9	35	64

[a] Values taken from Gordon *et al.* (1974).

TABLE III: Hybridization of Leucyl-tRNA with Fast- and Slow-Renaturing DNA Fractions.[a]

Strain	DNA Fraction	$C_0t_{1/2}$	C_0t Incubated	Calcd % Enrichment	Obsd Hybridization (cpm)	Cor Hybridization (cpm)
R1	Fast	1	0.6		240	240
	Slow	1			230	230
R1-6/1	Fast	0.435	0.402	62	530	858
	Slow	0.740		60	320	0[b]
R1-6/5	Fast	0.204	0.127	64	630	985
	Slow	0.357		55	350	0[b]
R1-6/6	Fast	0.115	0.074	70	320	460
	Slow	0.266		56	200	0[b]
R1-6/8	Fast	0.256	0.259	66	800	1210
	Slow	0.5		60	460	0[b]

[a] Fast- and slow-renaturing mtDNA fractions were prepared by hydroxylapatite chromatography (see Materials and Methods). Each mtDNA fraction (10 μg/filter) was hybridized with mitochondrial [^{3}H]leucyl-tRNA (specific activity 1.3 × 10^4 cpm/μg) in a 33% formamide system. Reaction volume was 1.5 ml. [b] ±5%.

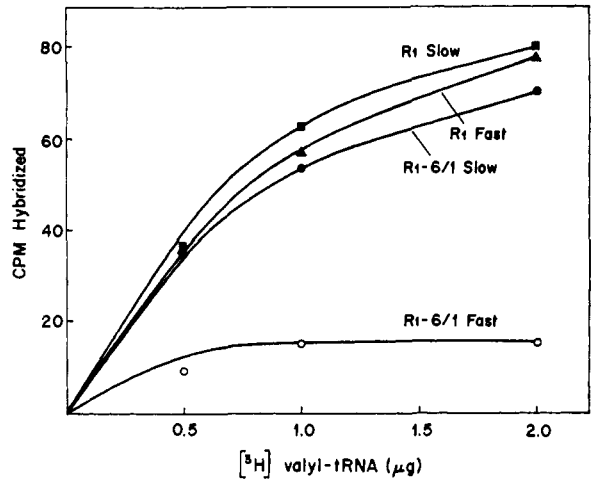

FIGURE 9: Hybridization of grande R1 mitrochondrial valyl-tRNA with fast- and slow-renaturing fractions from grande R1 and petite R1-6/1 mtDNA. Ten micrograms of grande fast- (▲—▲) and slow- (■—■) renaturing fractions, and petite fast- (○—○) and slow- (●—●) renaturing fraction were then hybridized with mitochondrial [^{3}H]valyl-tRNA (specific activity 3.4 × 10^3 cpm/μg). Hybridization was carried out in a 33% formamide system. Reaction volume was 1.5 ml.

appear to be localized largely, if not exclusively, in the slow renaturing component of this strain. Very low valyl-tRNA hybridization saturation levels were previously found with petite strains R1-6/5, R1-6/6, and R1-6/8 (Cohen *et al.*, 1972); therefore, separated fractions of these mtDNAs were not tested for valyl-tRNA hybridization.

Discussion

It has become increasingly clear that the changes produced in grande mtDNA during the formation of petite mtDNA can be both extensive and complex. In the present study we have applied renaturation kinetic analysis, thermal stability measurements, and tRNA–DNA hybridization to better define these changes, in an attempt to understand the basic structure of mtDNA.

Role of Deletion in Genesis of Petite mtDNA. The distribution of base sequences in yeast mtDNA is remarkably heterogeneous. Consistent with this view, both grande and petite mtDNA have broad optical melting profiles (Bernardi *et al.*, 1970) and hydroxylapatite thermal elution patterns (this paper). Also, (A + T)-rich or (A + T)-poor fragments 400–500 nucleotides in length have been isolated from grande mtDNA (Bernardi *et al.*, 1972). Deletion of (G + C)- or (A + T)-rich segments probably accounts for the buoyant density shifts observed for some petite mtDNAs (Borst, 1972).

More direct evidence for the involvement of a deletion mechanism (at least in part) has been obtained from DNA–DNA hybridization studies (Gordon and Rabinowitz, 1973; Gordon *et al.*, 1974), and from renaturation kinetic analysis (Casey *et al.*, 1972b; Fauman and Rabinowitz, 1972; Hollenberg *et al.*, 1972; Faye *et al.*, 1973; Michel *et al.*, 1974). The decreased kinetic complexity of all petite mtDNAs (as reflected in increased renaturation rates relative to the parent grande mtDNA) could be explained in part by deletion of grande mtDNA sequences.

Complex Renaturation Kinetics of Grande and Petite mtDNA. In contrast to T4 or bacterial DNA, the renaturation kinetics of yeast mtDNA were complex. The C_0t plots for both grande and petite mtDNA were unusual in that they were much broader than that expected for an ideal second-order reaction, such as T4 DNA renaturation where uniform reacting species are involved. Furthermore, the second-order renaturation plot of grande mtDNA (Figure 5) is slightly curvilinear, as is also apparent from previously published data (Hollenberg *et al.*, 1970). This effect may be due largely to the heterogeneous base distribution in the DNA fragments used in the renaturation reactions, as base composition has a significant effect on renaturation rate (Wetmur and Davidson, 1968). Unfortunately, it is not possible at present to evaluate quantitatively the effects of base clustering and extremes of base composition on the mtDNA renaturation rate because model studies with DNA species of such low (G + C) content (18%, Bernardi *et al.*, 1970) or such heterogeneous distribution are not available. Other difficulties with regard to the interpretation renaturation kinetic studies of fungal mtDNA have also been reported (Wood and Luck, 1969; Christiansen *et al.*, 1971).

Petite mtDNA renaturation kinetics were significantly different from those of grande mtDNA. When renaturation was carried out to greater than 85% completion, the reactions were not only more rapid than for grande mtDNA, but at least two classes of sequences could also be distinguished. In each petite strain, one class of sequences constitutes a reproducible and characteristic proportion of the DNA and renatures more rapidly than the other class. Differential melting plots of either optically monitored denaturation or thermal chromatography on hydroxylapatite revealed no major differences between grande and petite mtDNAs. Thus, the differences in mtDNA renaturation between grande and the group of petites reported on in this paper cannot be explained on the basis of different base distribution or content. Given this conclusion, it is difficult to account for the generation of increased heterogeneity of sequence distribution on the basis of a simple deletion mechanism; nor can deletion account for the extent of change observed (Gordon *et al.*, 1974). Therefore, it may be concluded that the prominent early renaturation fractions in the petite mtDNAs represent, at least in part, multiple copies of some DNA segments.

Isolation and Preparation of Enriched Rapidly Renaturing Fractions of Petite mtDNA. To verify that the complex renaturation kinetics of the petite mtDNA represent the behavior of individual and independent classes of DNA sequences, enriched fast- and slow-renaturing fractions were separated. The enriched fast and slow components were kinetically distinct, even though renaturation appeared to be still somewhat heterogeneous, even in the fast fraction, suggesting the presence of further renaturing classes. This heterogeneity may have been partly due to effects of distribution of base composition, as discussed above. Mitochondrial leucyl-tRNA hybridized selectively with petite fast-renaturing fractions, while mitochondrial valyl-tRNA hybridized selectively to the petite slow-renaturing fraction. In contrast, no selective hybridization was observed when grande mtDNA was similarly fractionated. These experiments suggest that the fast and slow fractions are functionally as well as kinetically distinct, even though each fraction may contain more than one renaturing component.

Attempted Correlation of Renaturation Kinetic Data and tRNA–DNA Hybridization. Hybridization saturation levels of [^{3}H]leucyl-tRNA to petite mtDNAs were up to sixfold higher than that to grande mtDNA. If these increased saturation levels were due to deletion, with selective retention of the leucyl-tRNA cistron, there would be two experimentally verifiable consequences. First, in some strains the deletions would have to be massive (up to 85% of grande mtDNA sequences), but this was not observed (Gordon *et al.*, 1974). Second, there should be a correlation of the hybridization saturation level with kinetic complexity. Even though the kinetic complexities of yeast mtDNA are difficult to calculate accurately, it is still possible to compare the grande and petite mtDNAs, as discussed above, to obtain an estimate of relative kinetic complexity. When the relative K_2 values of the slow renaturing DNA fractions are compared (Table II), there is no apparent correlation with the level of leucyl-tRNA hybridization. For example, strain R1-6/8, with a sixfold increment in leucyl-tRNA hybridization, only shows a twofold increase in K_2 of the bulk DNA, and strain R1-6/6 mtDNA has the most rapidly renaturing bulk DNA of the strains tested; yet its hybridization plateau is the same as that for grande mtDNA. The finding that the leucyl-tRNA cistron is located exclusively in the fast-renaturing component of petite mtDNA provides a rational basis on which much of the preceding information can be explained. It is also evident from Table II, however, that there is no strict correlation between the K_2 of the fast renaturing component and the hybridization level. The presence of subpopulations within the fast-renaturing components would render the establishment of such a correlation difficult. Perhaps the reiterated segment containing the leucyl-tRNA cistron constitutes only a minor component of a heterogeneous "fast" fraction.

All of these observations indicate that petite mtDNA is not simply a deleted portion of grande mtDNA, even though deletion is involved. Strong evidence has been presented for the repetition of certain segments of the petite mtDNA. These repetitions may be intramolecular, *i.e.*, they may be tandem repeats on each mtDNA molecule. Alternatively, they may represent the presence of stable heterogeneous populations of mtDNA within a single petite cell. If there were overlaps of some segments of the mtDNA in such a heterogeneous population, or if the subpopulation contained differentially repeated segments, these would produce the complex kinetics observed. Whatever the molecular explanation for the presence of repeated DNA segments, it is evident that these represent intracellular and not cellular heterogeneity, since repeated subcloning does not alter the patterns observed. Moreover, the heterogeneity is stable in that it persists unchanged over many hundreds of cell generations.

In summary, this paper provides evidence for the presence of repeated DNA segments and/or heterogeneity in the mtDNA populations of spontaneous petite cells. This conclusion is based on the following results: (1) the detection of two or more renaturing classes in petite mtDNA; (2) the demonstration, by selective hybridization with leucyl- or valyl-tRNA, that these classes are functionally distinct; (3) the elevated leucyl-tRNA hybridization levels observed with some of the petite mtDNAs cannot be explained by deletion alone, as indicated both by DNA–DNA hybridization data (Gordon *et al.*, 1974) and by the lack of correlation with the relative increase in K_2 values of bulk DNA. On the other hand, localization of the leucyl-tRNA cistron in the rapidly renaturing fraction can account for the elevated tRNA hybridizations. The imperfect correlation between the values of the fast-renaturing fraction and tRNA hybridization indicates further microheterogeneity within the fast-renaturing fractions.

Acknowledgments

We are greatly indebted to Dr. Godfrey S. Getz for his innumerable stimulating discussions, and critical appraisal of this work.

References

Bernardi, G., Faures, M., Piperno, G., and Slonimski, P. P. (1970), *J. Mol. Biol. 48*, 23.

Bernardi, G., Piperno, G., and Fonte, G. (1972), *J. Mol. Biol. 64*, 1.

Borst, P. (1972), *Annu. Rev. Biochem. 41*, 333.

Britten, R. J., and Kohne, D. E. (1968), *Science 161*, 529.

Casey, J., Cohen, M., Rabinowitz, M., Fukuhara, H., and Getz, G. S. (1972a), *J. Mol. Biol. 63*, 431.

Casey, J., Fukuhara, H., Getz, G. S., and Rabinowitz, M. (1969), *J. Cell Biol. 43*, 18a.

Casey, J., Gordon, P., and Rabinowitz, M. (1972b), *J. Cell Biol. 55*, 69a.

Christiansen, C., Leth Bak, A., Stenderup, A., and Christiansen, L. (1971), *Nature (London), New Biol. 231*, 176.

Coen, D., Deutsch, J., Netter, P., Petrochilo, E., and Slonimski, P. P. (1970), *Symp. Soc. Exp. Biol. 24*, 449.

Cohen, M., Casey, J., Rabinowitz, M., and Getz, G. S. (1972), *J. Mol. Biol. 63*, 441.

Cohen, M., and Rabinowitz, M. (1972), *Biochim. Biophys. Acta 281*, 192.

Fauman, M., and Rabinowitz, M. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 28*, 317.

Fauman, M., Rabinowitz, M., and Swift, H. M. (1973), *Biochemistry 12*, 124.

Faye, G., Fukuhara, H., Grandchamp, C., Lazowska, J., Michel, F., Casey, J., Getz, G. S., Locker, J., Rabinowitz, M., Bolotin-Fukuhara, M., Coen, D., Deutsch, J., Dujon, B., Netter, P., and Slonimski, P. P. (1973), *Biochimie 55*, 779.

Fukuhara, H., Faures, M., and Genin, C. (1969), *Mol. Gen. Genet. 104*, 264.

Gordon, P., Casey, J., and Rabinowitz, M. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32*, 663 Abstr.

Gordon, P., Casey, J., and Rabinowitz, M. (1974), *Biochemistry 13*, 1067.

Gordon, P., and Rabinowitz, M. (1973), *Biochemistry 12*, 116.
Halbreich, A., and Rabinowitz, M. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 294.
Hollenberg, C. P., Borst, P., Flavell, R. A., van Kreijl, C. F., Van Bruggen, E. F. J., and Arnberg, A. C. (1972), *Biochim. Biophys. Acta 277*, 44.
Hollenberg, C. P., Borst, P., and Van Bruggen, E. F. J. (1970), *Biochim. Biophys. Acta 209*, 1.
Michel, F., Lazowska, J., Faye, G., Fukuhara, H., and Slonimski, P. P. (1974), *J. Mol. Biol.* (in press).
Mounolou, J. C., Jakob, H., and Slonimski, P. P. (1966), *Biochem. Biophys. Res. Commun. 24*, 218.
Nagley, P., and Linnane, A. W. (1972), *J. Mol. Biol. 66*, 181.
Rabinowitz, M., Casey, J., Gordon, P., Locker, J., and Hsu, H. J. (1974), *in* Symposium on Mitochondrial Biogenesis, Kroon, A. M., and Saccone, C., Ed., New York, N. Y., Academic Press (in press).
Rabinowitz, M., Getz, G. S., Casey, J., and Swift, H. (1969), *J. Mol. Biol. 41*, 381.
Rabinowitz, M., and Swift, H. (1970), *Physiol. Rev. 50*, 376.
Studier, F. W. (1965), *J. Mol. Biol. 11*, 373.
Weiss, S. B., Hsu, W.-T., Foft, J. W., and Scherberg, N. H. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 114.
Wetmur, J. G., and Davidson, N. (1968), *J. Mol. Biol. 21*, 34.
Wintersberger, E., and Viehauser, G. (1968), *Nature (London) 220*, 699.
Wood, D. D., and Luck, D. J. L. (1969), *J. Mol. Biol. 41*, 211.

Characterization of Mitochondrial Deoxyribonucleic Acid from a Series of Petite Yeast Strains by Deoxyribonucleic Acid–Deoxyribonucleic Acid Hybridization†

Paul Gordon, James Casey, and Murray Rabinowitz*

ABSTRACT: Filter DNA–DNA hybridization techniques and DNA–DNA hybridization in solution with analysis on hydroxylapatite columns (DNA-driven reactions) have been used in a study of the base-sequence homology of mtDNA in four spontaneously mutated cytoplasmic petite strains with grande mtDNA and with the mtDNA of the other petites. One of the petite strains (R1-6/1) appears to contain sequences (15–20%) which are sufficiently changed so as not to hybridize with mtDNA of the grande strain, or with the other three petites. These others, namely, R1-6/5, R1-6/6, R1-6/8, appear to be predominantly deletion mutants, however, and contain no detectable sequences which do not hybridize to grande mtDNA. Thermal elution chromatography of the grande–petite heteroduplexes shows a small, but consistent decrease in the thermal stability of the early melting region[S] compared with petite–petite homoduplexes. Therefore, there seem to be small scattered base sequence changes in the (A + T)-rich segments of the petite mtDNAs. The loss of wild-type sequences in the petite mtDNAs is estimated to be between 30 and 50%, and is not of sufficient magnitude to explain on a deletion basis alone the 2.5- to 6-fold increase in saturation levels of leucyl-tRNA hybridization observed in these strains. We therefore suggest that, in addition to deletion, and in some cases base change, these petite mtDNAs contain reiteration of the segments of DNA containing the leucyl-tRNA cistron. Alternatively, microheterogeneity of mtDNA populations may be present.

In this and the preceding paper (Casey *et al.*, 1974), we have examined the physical and molecular hybridization properties of mtDNA of a series of stable petite subclones of *Saccharomyces cerevisiae*, in order to obtain information regarding either the amplication of segments of petite mtDNA or heterogeneity of mtDNA populations within the petite cell.

It has previously been shown in our laboratory (Casey *et al.*, 1969; Cohen *et al.*, 1972; Cohen and Rabinowitz, 1972) that tRNA hybridization saturation levels may be greatly elevated and may vary considerably with the mtDNA of a series of spontaneously mutated petite subclones. On the basis of the levels of the hybridization plateau with [^{3}H]leucyl mitochondrial tRNA, the petite subclones were tentatively divided into four groups (Cohen *et al.*, 1972), in which hybridization levels were 1–6 times that with grande mtDNA. In the preceding paper (Casey *et al.*, 1974), it has been shown, by means of thermal stability and renaturation kinetic analyses, that simple deletion could not account for the elevated tRNA hybridization levels. Rapidly renaturing fractions, which contained the leucyl-tRNA cistron, were present in the mtDNA of all four petite subclones studied. Furthermore, the direct correlation between the renaturation rate of the mtDNA and the level of tRNA hybridization to be expected from simple deletion was not found. This paper presents further evidence, using filter DNA–DNA hybridization, that deletion alone cannot explain the hybridization properties of the petite mtDNAs. Only relatively small differences in sequence homologies of the mtDNAs of the petite subclones examined were evident. We suggest that repetition of small segments of the mitochondrial genome, or possibly microheterogeneity of mtDNA populations within each cell type, may account for the observed differences in tRNA hybridization.

† From the Departments of Medicine and Biochemistry, University of Chicago and the Franklin McLean Memorial Research Institute, Chicago, Illinois 60637. *Received August 22, 1973.* This work was supported in part by U. S. Public Health Service Grants HL04442, HL09172, and HL05673 from the National Heart and Lung Institute, National Institutes of Health. Franklin McLean Memorial Research Institute is operated by the University of Chicago for the U. S. Atomic Energy Commission.