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

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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.

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The sequence homology of the mtDNA of each of the four petite subclones to grande mtDNA, and of the mtDNA of other petites, is also examined in detail in this paper. In an earlier publication, we presented a detailed examination of mitochondrial DNA–DNA filter hybridization, together with a validation of the procedure (Gordon and Rabinowitz, 1973). We found that reciprocal DNA–DNA hybridization could be used to detect changes in the mtDNA of a particular petite subclone (R1-6/1). For example, isohybridization (grande–grande or petite–petite mtDNA hybrid formation) was greater than heterohybridization, and we used this evidence to suggest that R1-6/1 petite mtDNA was derived from the grande by both deletion and localized sequence change. In this paper, we have extended these hybridization experiments to examine one petite from each of the four groups described by Cohen *et al.* (1972). In addition, DNA–DNA hybridization in solution, followed by analysis of the hybrid products by thermal elution chromatography on hydroxylapatite, has been used to confirm and extend results obtained from the filter assay procedure. This work has been presented, in part, in preliminary form (Gordon *et al.*, 1973; Rabinowitz *et al.*, 1974).

Methods

Strains and Culture Conditions. The grande and petite strains 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 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 investigation, mtDNA from four of these petite subclones, namely, R1-6/1, R1-6/5, R1-6/6, and R1-6/8, was studied. The growth of the various strains, and the preparation of cells with labeled mtDNA, have been described previously (Gordon and Rabinowitz, 1973).

Mitochondrial Isolation, DNase Treatment, and DNA Extraction. Mitochondria were isolated from both grande and petite strains of yeast after preparation of protoplasts by snail enzyme digestion of the cell walls. Contaminating nuclear DNA was removed from the crude mitochondrial fraction by DNase digestion. The washed mitochondrial pellet was lysed and deproteinated with detergent, and the mtDNA was purified by chromatography on hydroxylapatite. These procedures have been described in detail previously (Rabinowitz *et al.*, 1969; Fauman and Rabinowitz, 1972; Casey *et al.*, 1972a,b; Gordon and Rabinowitz, 1973).

Filter Hybridization Procedures. Nitrocellulose filters, 24-mm diameter (Selectron, Schleicher and Schuell, Inc., Keene, N. H.), were loaded with DNA by the method of Gillespie and Spiegelman (1965). After loading and air-drying, the filters were preincubated for 1–2 hr in Denhardt's solution (Denhardt, 1966), then dried at 80° *in vacuo*. For each single-filter hybridization unit, 0.067 ml of incubation solution, containing sheared [^{3}H]mtDNA in 0.3 M NaCl, 0.03 M sodium citrate, and 36% formamide, was added and the reaction was carried out at 35°. The hybridization procedure and its experimental validation have been described in detail previously (Gordon and Rabinowitz, 1973).

DNA–DNA Hybridization in Solution. The procedure is based on that described by Britten and Kohne (1968), Hoyer and van de Uelde (1971), and Kieff *et al.* (1972). Essentially, a large excess of denatured unlabeled mtDNA is incubated with a small amount of homologous [^{3}H]mtDNA and a small amount of [^{32}P]mtDNA from another strain. At the end of the incubation period, single-stranded DNA is separated from the duplex DNA by hydroxylapatite chromatography, and the relative amounts of homologous and heterologous hybrids are determined by radioassay.

For driving the reaction and minimizing the self-renaturation of the labeled DNA species, a large excess of unlabeled DNA is necessary. We have chosen a 50-fold excess. The grande and petite mtDNAs that we wish to compare, however, have different renaturation rates (Casey *et al.*, 1972b, 1974; Fauman and Rabinowitz, 1972): the petite mtDNAs renature 1.5–3.5 times faster than the grande mtDNA. To compensate for the decrease in the complexities of the petite mtDNAs in reactions driven by grande mtDNA, we increased the excess of grande mtDNA in proportion to the relative renaturation rates, and thus added it in 75- to 175-fold excess.

To measure the homology of petite mtDNA to grande mtDNA, we mixed unlabeled grande mtDNA (75–175 μg) with 2 μg of grande [^{3}H]mtDNA (8500 cpm/μg) and 1 μg of petite [^{32}P]mtDNA (6000 cpm/μg) in 1 ml of 0.1 M sodium phosphate buffer (pH 6.8). Usually, 3.0 ml of this solution was prepared. The DNA was then sheared by sonication (5 × 1 min pulses at maximum output of the standard microprobe, Branson Heat Systems, New York, N. Y.). Alkaline band sedimentation (Studier, 1965) showed an s value of 5.9 S indicating a fragment size of about 500 nucleotide pairs. Next, the DNA was heat denatured (100° for 5 min) and incubated at 52° (T_m = −22°) for 3–6 hr. The incubation time required to obtain 80% reassociation of grande mtDNA and homologous petite DNA was calculated from the theoretical second-order equation of Britten and Kohne (1968): $C/C_0 = 1/(1 + KC_0t)$. Under these conditions, less than 8% self-renaturation of ^{32}P-labeled petite mtDNA would be expected, and this was confirmed by control experiments.

To assess the homology of grande mtDNA to petite mtDNA, we mixed 50 μg of unlabeled petite mtDNA, 1–2 μg of petite [^{32}P]mtDNA (6000 cpm/μg), and 1 μg of grande [^{3}H]-mtDNA (8500 cpm/μg) in 1 ml of 0.1 M sodium phosphate buffer (pH 6.8). The procedure described above was then repeated. Again, less than 8% self-renaturation of ^{3}H-labeled grande mtDNA would be predicted.

Hydroxylapatite Thermal Elution Chromatography. The fidelity of base pairing of the iso- and heterohybrids generated in the experiments described above was evaluated by thermal elution chromatography on hydroxylapatite columns. The "reassociated" DNA solution was poured into a jacketed column containing 4 g of hydroxylapatite (Bio-Rad Corp., Richmond, Calif., lot 10849). Thermal elution chromatography was then carried out as described in the preceding paper (Casey *et al.*, 1974).

Results

Homology of Petite mtDNAs to Grande mtDNA. Typical saturation-type filter hybridization experiments, with grande [^{3}H]mtDNA in solution, are presented in Figure 1. In these experiments, the initial concentration of labeled mtDNA in solution is kept constant, while the amount of mtDNA on the filter in each single-filter hybridization unit is varied (Gordon and Rabinowitz, 1973). In this way, saturation plateaus can be achieved, in the sense that a maximal amount of solution DNA is bound to filters.

Figure 1 shows that all four petite mtDNAs bind a similar proportion of the grande mtDNA (60–70%). Small differ-

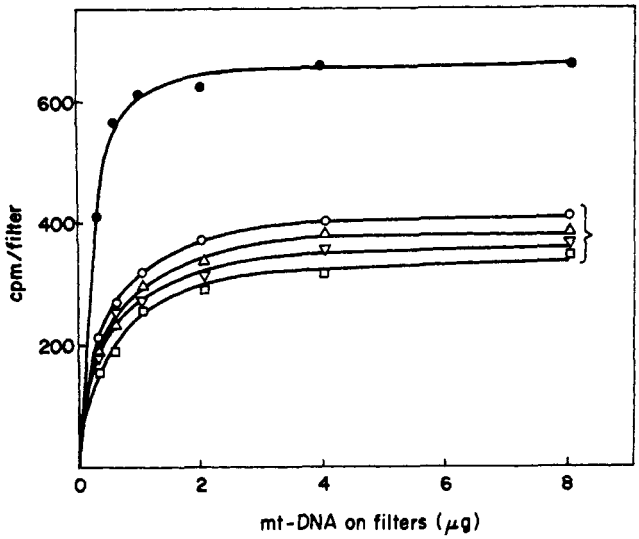

FIGURE 1: Hybridization of ^{3}H-labeled grande (R1) mtDNA to grande and petite mtDNA on filters. Filters containing increasing amounts of grande R1 (●—●), and petite R1-6/1 (○—○), R1-6/5 (□—□), R1-6/6 (△—△), and R1-6/8 (▽—▽) mtDNA were incubated at 35° for 24 hr with grande [^{3}H]mtDNA; single filters were incubated in individual hybridization units with 0.067 ml of grande R1 [^{3}H]mtDNA containing 0.089 μg of DNA. Specific radioactivity was 9450 cpm/μg.

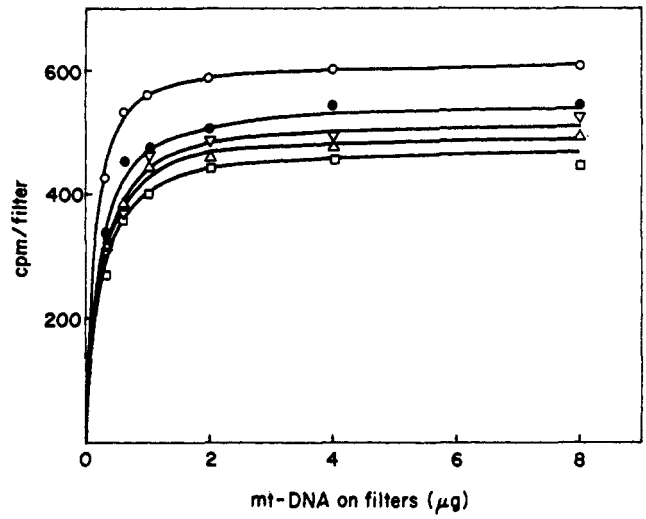

FIGURE 2: Hybridization of ^{3}H-labeled petite R1-6/1 mtDNA to grande and petite mtDNA on filters. Filters containing increasing amounts of grande R1 (●—●) and petite R1-6/1 (○—○), R1-6/5 (□—□), R1-6/6 (△—△), and R1-6/8 (▽—▽) mtDNA were incubated at 35° for 24 hr with petite R1-6/1 [^{3}H]mtDNA. Single filters were incubated in individual hybridization units with 0.067 ml of petite R1-6/1 [^{3}H]mtDNA (0.089 μg of DNA). Specific radioactivity was 3600 cpm/μg).

ences in the binding patterns are evident, however. Table I, part 1, gives the hybridization saturation values relative to grande–grande mtDNA hybridization in a series of independent experiments. The significance of the small differences observed in the binding of grande mtDNA to filter-bound petite mtDNA is difficult to evaluate because of experimental variation. It should be noted that the saturation values given in Table I were obtained from double-reciprocal plots (1/hybridization *vs.* 1/filter DNA content; Bishop *et al.*, 1969).

Homology of Grande and Petite mtDNAs Relative to R1-6/1. The results of hybridization experiments with R1-6/1 petite [^{3}H]mtDNA in solution are shown in Table I (part 2) and Figure 2. In agreement with our previous results (Fauman and Rabinowitz, 1972; Gordon and Rabinowitz, 1973) homologous binding (R1-6/1 to R1-6/1) is greater than binding to filters containing grande mtDNA, again suggesting that this petite is not just a deletion derivative. Binding to filters containing the other petite mtDNAs is substantial, but incomplete (75–85% of homologous binding, Table I). These results suggest that the mtDNA base sequences in all four petites are similar, although the grande and the other three petite mtDNAs exhibit sequence differences of 15–25% relative to the R1-6/1 petite mtDNA.

TABLE I: DNA-DNA Filter Hybridization of mtDNA from Grande and Petite Yeast..

Solution mtDNA	Filter mtDNA				
	Grande (R1)	R1-6/1	R1-6/5	R1-6/6	R1-6/8
1. Grande ^{3}H (R1)					
Binding (cpm ± std dev)	638 ± 26	447 ± 51	375 ± 10	420 ± 81	399 ± 12
(*n*)	(9)	(10)	(3)	(3)	(3)
Relative Binding	100	70	59	64	62.5
2. Petite ^{3}H (R1-6/1)					
Binding (cpm ± std dev)	517 ± 23	615 ± 42	504 ± 11	482 ± 52	538 ± 45
(*n*)	(7)	(8)	(3)	(2)	(3)
Relative Binding	84	100	82	78.5	87.5
3. Petite ^{3}H (R1-6/5)					
Binding (cpm ± std dev)	357 ± 16	309 ± 15	343 ± 12	351 ± 18	354 ± 12
(*n*)	(4)	(4)	(4)	(4)	(4)
Relative Binding	104	90	100	102	103
4. Petite ^{3}H (R1-6/6)					
Binding (cpm ± std dev)	525 ± 23	446 ± 35	477 ± 31	537 ± 37	550 ± 28
(*n*)	(5)	(5)	(2)	(5)	(2)
Relative Binding	98	83	89	100	102
5. Petite ^{3}H (R1-6/8)					
Binding (cpm ± std dev)	477 ± 13	392 ± 15	426 ± 11	478 ± 13	481 ± 8
(*n*)	(5)	(4)	(5)	(5)	(5)
Relative Binding	99	82	89	99	100

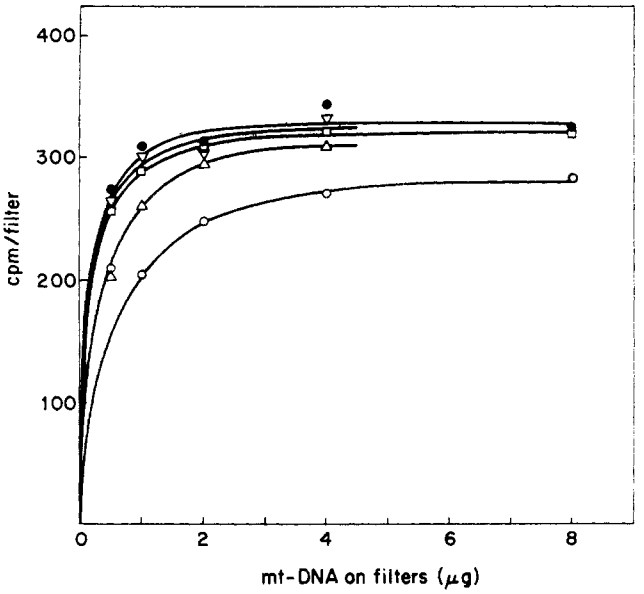

FIGURE 3: Hybridization of petite R1-6/5 [^{3}H]mtDNA with grande and petite mtDNAs on filters. Filters containing increasing amounts of grande R1 (●—●) or petite R1-6/1 (○—○), R1-6/5 (□—□), R1-6/6 (△—△), and R1-6/8 (▽—▽) mtDNA were incubated at 35° 24 hr with ^{3}H-labeled petite R1-6/5 mtDNA. Single filters were incubated with individual hybridization units containing filters charged with mtDNA of each strain and 0.067 ml of petite R1-6/5 [^{3}H]mtDNA containing 0.089 μg/DNA per filter: specific radioactivity was 3660 cpm/μg.

Homology of Grande and Petite mtDNA Relative to R1-6/5. It appears that R1-6/5 mtDNA is changed most, relative to grande mtDNA (Figure 1; Table I, part 1). When the hybridization reactions are carried out with R1-6/5 [^{3}H]mtDNA in solution (Figure 3), the hybridization plateaus obtained with three of the petite mtDNAs and with grande mtDNA are similar, but there appears to be about 10% nonhomology with R1-6/1 mtDNA. A survey of the results of four experiments is given (Table I, part 3). The identical binding to filters containing relatively high concentrations of grande mtDNA or R1-6/5 petite mtDNA (Figure 2) suggests that R1-6/5 mtDNA is predominantly a deletion derivative of the grande mtDNA.

Homology of Grande and Petite mtDNA Relative to R1-6/6. mtDNA from the R1-6/6 petite was examined in more detail by testing its binding to filters containing grande or petite mtDNA. These results are presented in Table I (part 4) which show that R1-6/6 mtDNA is completely homologous to grande mtDNA. This result suggests that R1-6/6 mtDNA is also predominantly a simple deletion derivative of grande mtDNA. On the other hand, homology of R1-6/6 to R1-6/1 mtDNA is only about 80%.

It is interesting to note that R1-6/6 mtDNA binds equally well to filters charged with R1-6/6 or R1-6/8 mtDNA (Table I, part 4).

Homology Relative to R1-6/8 mtDNA. Hybridization of R1-6/8 [^{3}H]mtDNA to grande filters (Table I, part 5), together with results from the hybridization of grande [^{3}H]-mtDNA to filters containing R1-6/8 mtDNA (Figure 1 and Table I, part 1), suggest that this petite mtDNA is also derived from the grande parent mtDNA by deletion. Tritiated R1-6/8 mtDNA hybridized equally well to filters containing R1-6/6 and R1-6/8 mtDNA, whereas binding to R1-6/1 or R1-6/5 mtDNA is significantly less ($p < 0.01$; Table I, part 5) by 20 and 10%, respectively.

Solution DNA–DNA Hybridization of Grande and Petite mtDNA. In hybridization reactions carried out in solution, a small amount of ^{32}P-labeled mtDNA of one strain is mixed with a large excess (>50-fold) of ^{3}H-labeled mtDNA of another strain, and the DNA species is sheared and heat denatured. Renaturation was allowed to proceed at 52° for 4–6 hr, so that about 80% of the mtDNA present in excess had renatured, whereas less than 8% of the labeled DNA present in low amounts had self-renatured (Table II). At the same time, the large excess of one of the DNAs provides the driving force for any heteroduplex reaction. Single-strand and hybrid mtDNA species are then separated on hydroxylapatite, and the relative homologies are calculated from the ratios of the ^{32}P and ^{3}H counts in the two fractions.

TABLE II: Self-Annealing of mtDNAs Present in Low Concentration in DNA-Driven Hybridization Reactions.[a]

Strain	Concn of mtDNA (μg/ml)	Double Stranded[b] (%)
R1	1	6.9
R1-6/1	0.5	7.8
R1-6/5	0.25	6.6
R1-6/6	0.25	6.3
R1-6/8	0.25	6.7

[a] The concentrations of DNA and the incubation conditions were identical with those used to generate petite–grande heterohybrids (Figures 6, 7, 8A, and Table III) except that only the DNA in low concentration was present. The percentage of double-stranded DNA represents the extent of self-annealing that would occur if there were *no* homology with DNA present in excess. The self-annealing that would occur when there is substantial homology with DNA added in excess is probably considerably lower than these figures. [b] Zero time DNA (3–5%) has not been subtracted from the values.

The results of such experiments using small amounts of grande mtDNA driven to form heterohybrids with excess amounts of petite mtDNA are summarized in Table III. The homologies of the grande mtDNA to petite mtDNAs (54–74%), as measured by this method, agree with the filter assay procedure (*cf.* Tables I and III).

In order to test whether these petite mtDNAs resulted from deletions of grande mtDNA sequences, and to determine whether these petite mtDNAs contain sequences which no longer hybridize to grande mtDNA, we performed reciprocal experiments in which grande mtDNA provided the driving force for heterohybrids. The experiments show that three of the petite mtDNAs, namely, R1-6/5, R1-6/6, and R1-6/8, were 93–102% homologous to grande mtDNA. In contrast, a significant fraction (20–30%) of the sequences in petite R1-6/1 mtDNA was found to be nonhomologous to grande mtDNA. This result confirms our previous observation (Gordon and Rabinowitz, 1973), obtained by the filter hybridization procedure, that this petite contains sequences that are unable to form hybrids with grande mtDNA under generally permissive conditions for base pairing ($T_m = -25$).

Thermal Stability of Petite–Grande mtDNA Duplexes. The iso- and heterohybrid products formed when grande and petite mtDNAs are co-renatured in solution have been characterized by their thermal stability when they are bound to hydroxylapatite columns. To test the effect of radioactive label on the melting profile of the mtDNA, ^{32}P-labeled and [^{3}H]adenine-labeled grande mtDNA were co-renatured and analyzed by hy-

TABLE III: Hybridization of Grande and Petite Mitochondrial DNAs in Solution.[a]

Cold DNA Added in Excess	Labeled DNA	Reassociated Fraction A Hetero-hybrid	Reassociated Fraction B Iso-hybrid	% Homology Rel to DNA in Excess A/B × 100
R1[b]	R1 ^{32}P R1 ^{3}H	0.86	0.88	98
R1	R1 ^{3}H R1-6/1 ^{32}P	0.66	0.85	78
R1-6/1	R1 ^{3}H R1-6/1 ^{32}P	0.57	0.77	73
R1	R1 ^{3}H R1-6/5 ^{32}P	0.82	0.88	93
R1-6/5	R1 ^{3}H R1-6/5 ^{32}P	0.47	0.88	54
R1	R1 ^{3}H R1-6/6 ^{32}P	0.82	0.86	96
R1-6/6	R1 ^{3}H R1-6/6 ^{32}P	0.65	0.88	74
R1	R1 ^{3}H R1-6/8 ^{32}P	0.86	0.84	102
R1-6/8	R1 ^{3}H R1-6/8 ^{32}P	0.52	0.85	61

[a] The total number of counts per minute analyzed in these experiments ranged from 5000 to 31,000. [b] Test control of grande to grande renaturation.

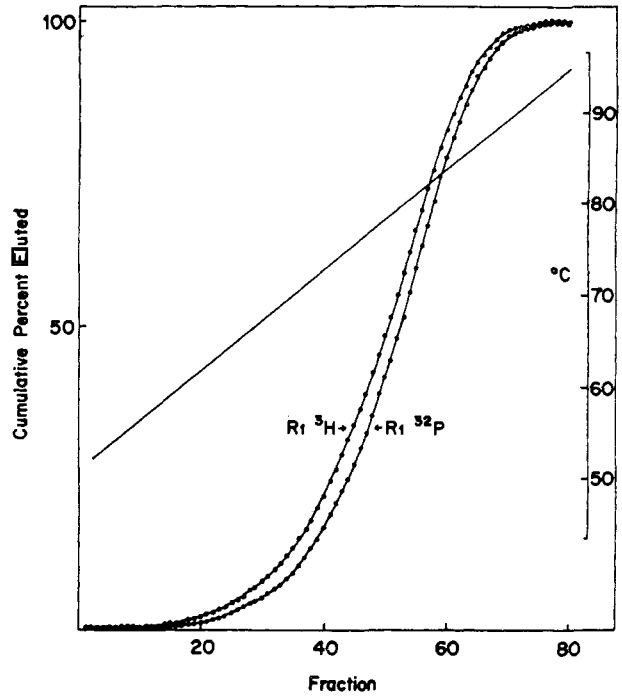

FIGURE 4: Cumulative hydroxylapatite melting profiles of [^{3}H]-adenine labeled and ^{32}P-labeled grande R1 mitochondrial DNA. [^{3}H]Adenine-labeled grande R1 mitochondrial DNA (5 μg; specific activity 8.4 × 10^{3} cpm/μg), ^{32}P-labeled grande R1 mtDNA (5 μg; specific activity 7.8 × 10^{3} cpm/μg), and unlabeled grande R1 (200 μg) were mixed, sheared, denatured, renatured, and chromatographed by thermal elution on hydroxylapatite columns as described in Materials and Methods. The cumulative percentage of total [^{3}H]adenine-labeled (○—○) and ^{32}P-labeled (●—●) grande mitochondrial DNA which eluted at each temperature is shown.

droxylapatite thermal chromatography. It is clear (Figure 4) that the [^{3}H]adenine-labeled DNA melts at a lower temperature than the uniform ^{32}P-labeled DNA. This result is consistent with the current view that the high (A + T) content of yeast mtDNA has a very heterogeneous distribution (Bernardi *et al.*, 1970, 1972; Casey *et al.*, 1974), and indicates that the low-melting region is adenine rich. This melting profile (Figure 4) has been used in the following experiments to correct for the effect of the labeled precursor on the observed profiles.

The melting profiles are highly reproducible; for example, the stability of the four independently measured grande mtDNA isohybrid profiles presented (Figures 5–8) is 78.1 ± 0.3 (T_e(50) ± std dev). Consequently, small differences are significant. Also, the grande or petite homoduplex melting profiles act as internal standards for each set of experiments, so that the different experiments shown in Figures 4–8 can be readily compared.

The petite isohybrids have T_e(50) values on hydroxylapatite which are 1–3° lower than that of the grande mtDNA isohybrid (Figures 5–8, Table IV). Overall, the petite–grande heteroduplex DNAs show thermal stabilities very similar to the corresponding petite homoduplex, regardless of whether the heterohybrid reaction had been driven by an excess of petite or grande mtDNA. However, some small, but significant differences are evident in the low-melting region (<20% melted, Figures 5–8). In this region, the heterohybrids are relatively less stable than the petite isohybrids, indicating some mismatching of bases. This is true for all petite–grande heterohybrid DNAs. Also, the degree of stability of the petite–grande heteroduplex DNAs in the early melting region differs from one petite to another. Thus, approximately 20% of these petite mtDNAs appear to have a 1–4% base sequence change on the basis of a 1° depression representing 1.4% base sequence mismatch (Ullman and McCarthy, 1973).

Discussion

Even though the four petites examined have been isolated as subclones of the same petite strain, it has been shown in previous work from this laboratory that mtDNAs isolated from these subclones exhibit some striking differences. Most significantly, leucyl-tRNA hybridized with mtDNA with an effectiveness 2.5, 4, 1, and 6 times, respectively, that with grande mtDNA from strains R1-6/1, R1-6/5, R1-6/6, and R1-6/8. In the present study, an attempt has been made to provide some basis for these differences by an analysis of DNA base sequence homologies by DNA–DNA hybridization. The hybridization properties of mtDNA from the four petite subclones and the grande can be summarized thus:

Petite–Grande Homologies. Grande [^{3}H]mtDNA hybridization to all petite mtDNAs is approximately the same (60–70% relative to grande isohybridization), indicating that all petite mtDNAs are changed to a similar *extent*. Reciprocal hybridizations, carried out with petite [^{3}H]mtDNA in solution, showed that the petites R1-6/5, R1-6/6, and R1-6/8 behave predominantly as deletion mutants; they appear to be completely homologous to grande mtDNA on filters, *i.e.*, almost all of the sequences of these petite mtDNAs are still represented in the grande mtDNA, as measured by the filter assay. In a previous report (Gordon and Rabinowitz, 1973), we discussed in detail that the filter hybridization results with R1-6/1

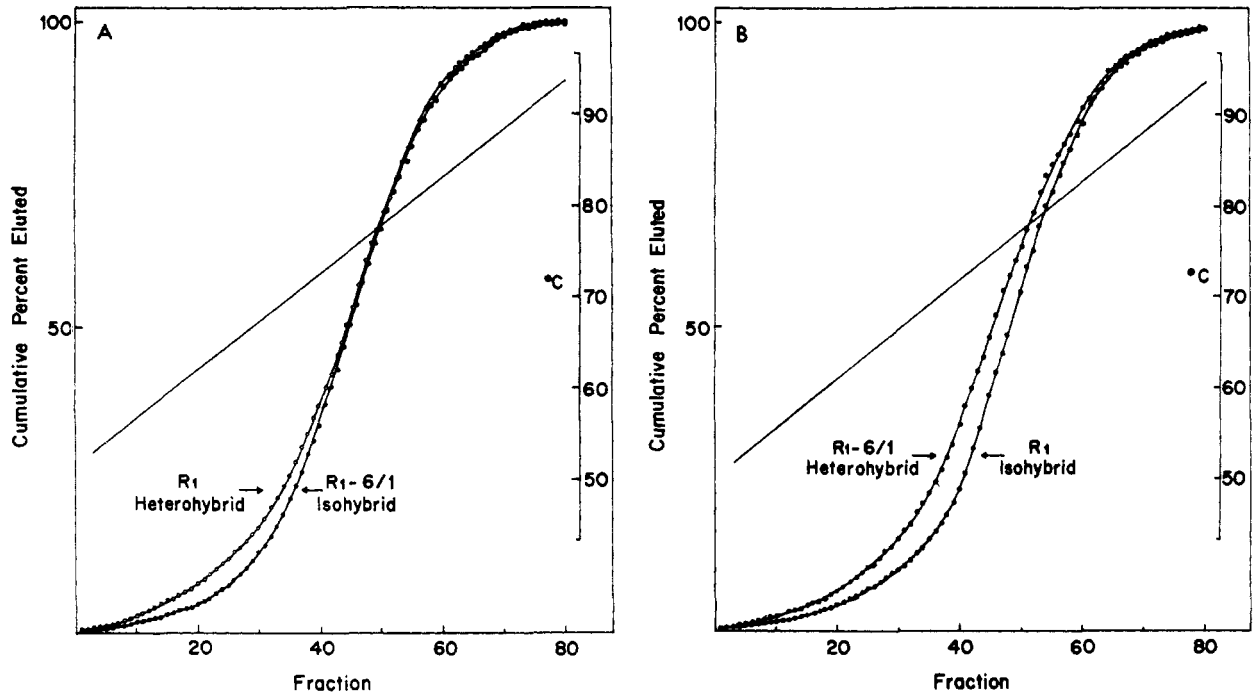

FIGURE 5: Thermal elution chromatography of co-renatured grande R1 and petite R1-6/1 mtDNAs. (A) [^{3}H]Adenine-labeled grande R1 (3 μg; specific activity 8.4 $\times$ 10^3 cpm/μg), ^{32}P-labeled petite R1-6/1 (3 μg; specific activity 5.2 $\times$ 10^3 cpm/μg), and unlabeled petite R1-6/1 (130 μg) were mixed, sheared, denatured, renatured, and chromatographed as described in Materials and Methods. The cumulative percentages of [^{3}H]adenine-labeled grande R1 (O—O) and ^{32}P-labeled petite R1-6/1 (●—●) mtDNA which eluted at each temperature is shown. (B) ^{3}H-labeled grande R1 (2 μg; specific activity 8.4 $\times$ 10^3 cpm/μg), ^{32}P-labeled petite R1-6/1 (2 μg specific activity 5.2 $\times$ 10^3 cpm/μg), and unlabeled grande R1 (200 μg) were mixed, sheared, denatured, renatured, and chromatographed as described in Materials and Methods. The cumulative percentage of ^{3}H-labeled grande R1 (O—O) and ^{32}P-labeled petite R1-6/1 (●—●) mtDNA which eluted at each temperature is shown.

petite [^{3}H]mtDNA in solution indicate that the mtDNA of this petite is changed relative to grande mtDNA by both deletion and base-sequence alteration. Results presented in this paper are consistent with this interpretation. Renaturation of R1-6/1 [^{3}H]mtDNA driven by a large excess of grande mtDNA, analyzed by hydroxylapatite chromatography, confirms that

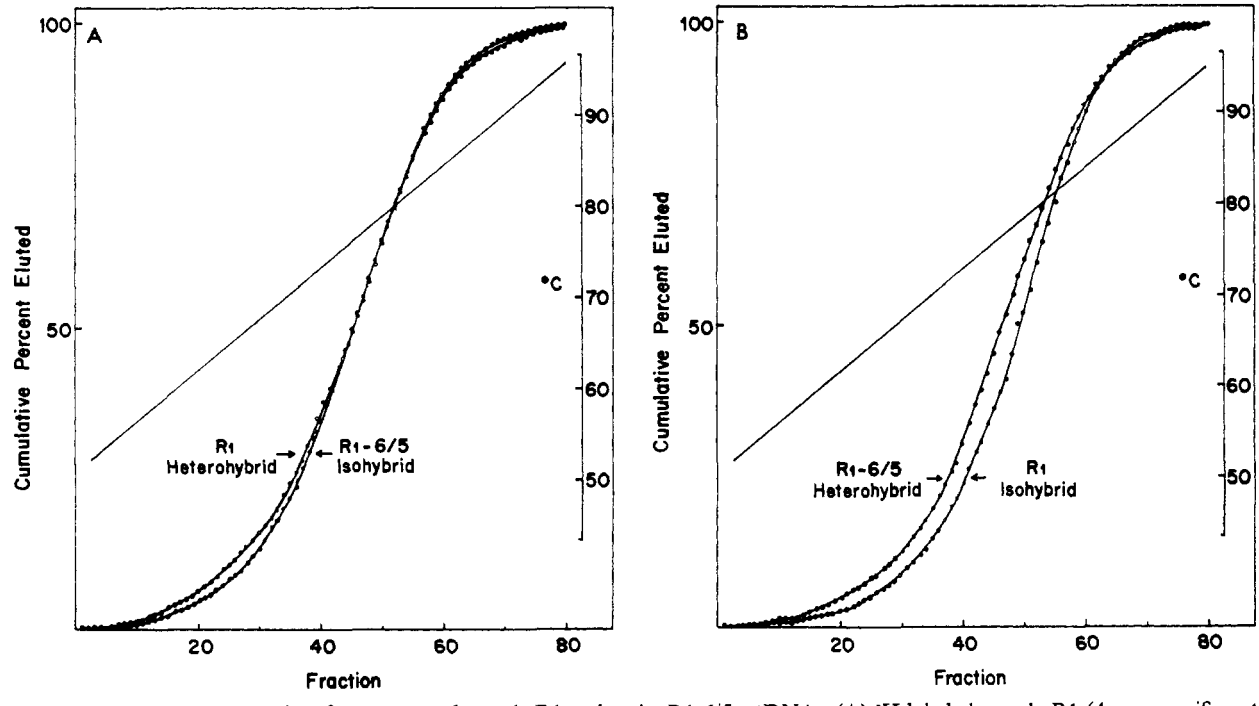

FIGURE 6: Thermal chromatography of co-renatured grande R1 and petite R1-6/5 mtDNAs. (A) ^{3}H-labeled grande R1 (4 μg; specific activity 8.4 $\times$ 10^3 cpm/μg) and unlabeled petite R1-6/5 (100 μg) were mixed, sheared, denatured, renatured, and chromatographed as described in Materials and Methods. The cumulative percentage of ^{3}H-labeled grande R1 (O—O) and ^{32}P-labeled petite R1-6/5 (●—●) mtDNA which eluted at each temperature is shown. (B) ^{3}H-labeled grande R1 (2 μg; specific activity 8.4 $\times$ 10^3 cpm/μg), ^{32}P-labeled petite R1-6/5 (1 μg; specific activity 6 $\times$ 10^3 cpm/μg), and unlabeled grande R1 (185 μg) were mixed, sheared, denatured, renatured, and chromatographed as described in Materials and Methods. The cumulative percentage of ^{3}H-labeled grande R1 (O—O) and ^{32}P-labeled petite R1-6/5 (●—●) mtDNA which eluted at each temperature is shown.

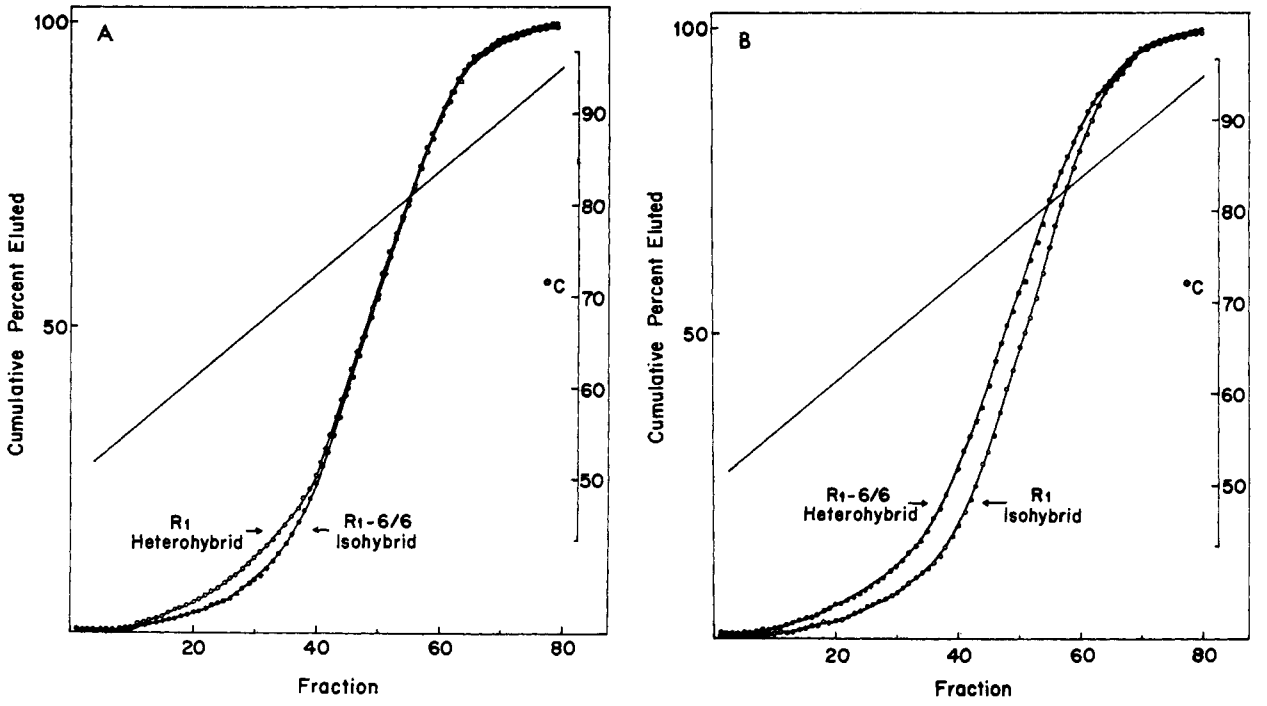

FIGURE 7: Thermal chromatography of co-renatured grande R1 and petite R1-6/6 mtDNAs. (A) ^{3}H-Labeled grande R1 (2 μg; specific activity 8.4 $\times$ 10^{3} cpm/μg), ^{32}P-labeled petite R1-6/6 (4 μg; specific activity 6.4 $\times$ 10^{3} cpm/μg), and unlabeled petite R1-6/6 (90 μg) were mixed, sheared, denatured, renatured, and chromatographed as described in Materials and Methods. The cumulative percentage of ^{3}H-labeled grande R1 (O—O) and ^{32}P-labeled petite R1-6/6 (●—●) mtDNA which eluted at each temperature is shown. (B) ^{3}H-Labeled grande R1 (2 μg; specific activity 8.4 $\times$ 10^{3} cpm/g), ^{32}P-labeled petite R1-6/6 (1 μg; specific activity 6.5 $\times$ 10^{3} cpm/μg), and unlabeled grande R1 (205 μg) were mixed, sheared, denatured, renatured, and chromatographed as described in Materials and Methods. The cumulative percentage of ^{3}H-labeled grande R1 (O—O) and ^{32}P-labeled petite R1-6/6 (●—● mtDNA which eluted at each temperature is shown.

this petite mtDNA contains sequences that do not hybridize with grande mtDNA. The depressed thermal stabilities of most of the petite–grande heterohybrids in the low (G + C) part of the melting curves also indicate that base sequence

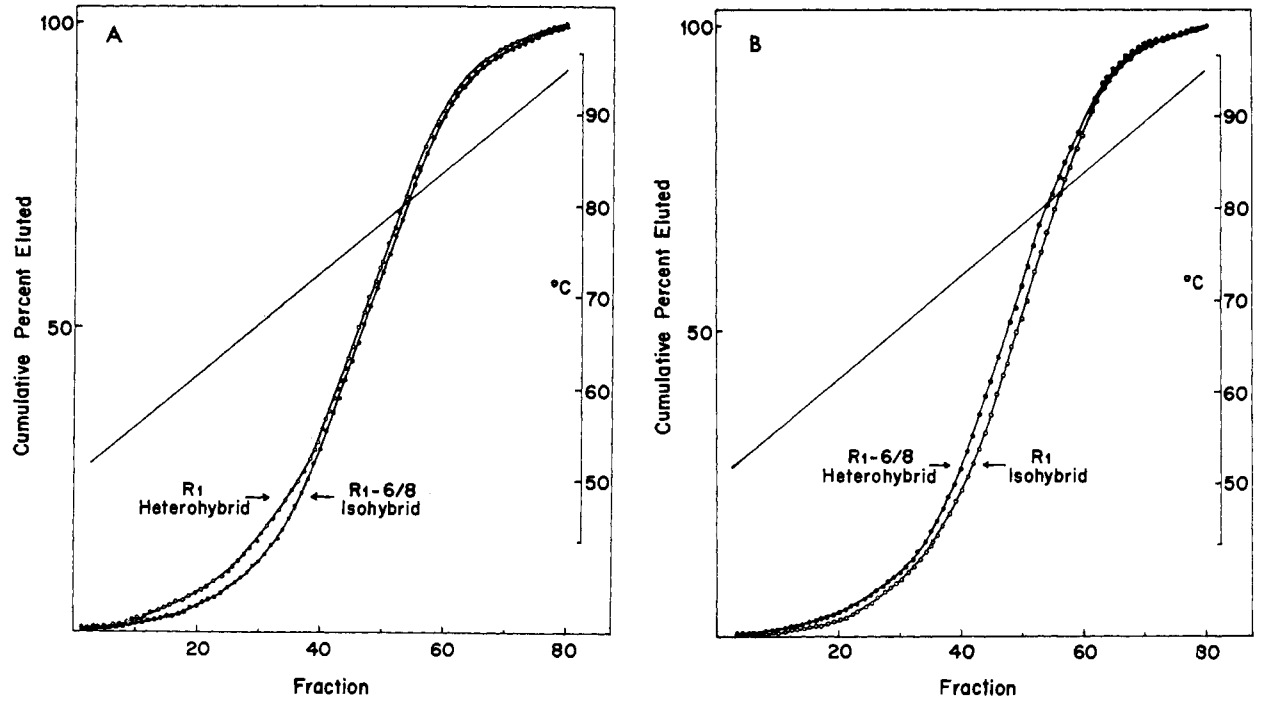

FIGURE 8: Thermal chromatography of co-renatured grande R1 and petite R1-6/8 mtDNAs. (A) ^{3}H-Labeled grande R1 (4 μg; specific activity 8.4 $\times$ 10^{3} cpm/μg), ^{32}P-labeled petite R1-6/8 (4 μg; specific activity 6.3 $\times$ 10^{3} cpm/μg), and unlabeled petite R1-6/8 (210 μg) were mixed, sheared, denatured, renatured, and chromatographed as described in Materials and Methods. The cumulative percentage of ^{3}H-labeled grande R1 (O—O) and ^{32}P-labeled petite R1-6/8 (●—●) mtDNA which eluted at each temperature is shown. (B) ^{3}H-Labeled grande R1 (2 μg; specific activity 8.4 $\times$ 10^{3} cpm/μg), petite R1-6/8 (1 μg; specific activity 6.3 $\times$ 10^{3} cpm/μg), and unlabeled grande R1 (200 μg) were mixed, sheared, denatured, renatured, and chromatographed as described in Materials and Methods. The cumulative percentage of ^{3}H-labeled grande R1 (O—O) and ^{32}P-labeled petite R1-6/8 (●—●) which eluted at each temperature is shown.

TABLE IV: Thermal Stability of DNA Hybrids on Hydroxylapatite.[a]

DNA in Excess	T_e(50) (°C) Isohybrid	T_e(50) (°C) Heterohybrid	ΔT_e(50) (°C) Grande Heterohybrid and Petite Isohybrid
R1	78	75	0.3
R1-6/1	75	75.3	
R1	78	76	0
R1-6/5	76	76	
R1	78.5	77	0.2
R1-6/6	77	77.2	
R1	78	76.7	0.1
R1-6/8	76.6	76.8	

[a] Values taken from Figures 5 to 8.

changes of much smaller magnitude are present in all the petite mtDNAs examined.

Petite–Petite Homologies. The petites R1-6/5, R1-6/6, and R1-6/8 show approximately the same hybridization levels (78–87%) to R1-6/1 petite [^{3}H]mtDNA in solution. This decreased hybridization could be due to the presence of "changed sequences," and/or deletions in different or incompletely overlapping positions. Hybridizations with R1-6/5, R1-6/6, or R1-6/8 petite [^{3}H]mtDNA in solution to filters containing R1-6/1 petite mtDNA also are lower than the respective isohybridization levels, namely, 82–90%. These binding patterns indicate that the R1-6/1 petite mtDNA contains sequences not present in the other petites.

From section "petite–grande homologies" above, it appears that deletions of about 35% of the grande genome are the major lesions in three of the petites studied. The mtDNA sequences of R1-6/6 and R1-6/8 cannot be distinguished by the DNA–DNA hybridization procedures used. Thus, in reciprocal hybridization experiments, tritiated R1-6/5 or R1-6/1 mtDNA hybridizes to filters containing R1-6/5 or R1-6/1 to approximately to the same extent. The only differences between the two petites that we have detected in the present study are small shifts in the low-melting region of thermal stability profiles of the heterohybrids. Consequently, the 6-fold differences in the leucyl-tRNA hybridization of R1-6/6 and R1-6/8 mtDNA (Cohen *et al.*, 1974) cannot be explained on the basis of large-scale differential deletion. Although somewhat higher levels of homology than that actually present may be obtained with the DNA–DNA hybridization system used, because of the presence of single-stranded tails in the hybrids, this effect could not be of sufficient magnitude to account for the very large differences in tRNA hybridization levels. These results provide evidence, in addition to that presented in the accompanying paper (Casey *et al.*, 1974), for reiteration of segments of mtDNA containing the leucyl-tRNA cistron, or, alternatively, for heterogeneity of mtDNA populations in these petite strains.

The deletion (or deletions) effected during the formation of R1-6/5 petite mtDNA seems to be greater than that resulting in the production of R1-6/8 and R1-6/6, but is still in approximately the same region of the genome. The hybridization levels and petite R1-6/5 [^{3}H]mtDNA to R1-6/6 or R1-6/8 mtDNA were similar to the isohybridization level, but in the reciprocal reactions (R1-6/6 or R1-6/8 [^{3}H]mtDNA in solution), the hybridizations were only 90% of the isohybridization levels. It appears that R1-6/6 and R1-6/8 mtDNA contain sequences not present in R1-6/5, but that almost all R1-6/5 sequences are represented in the R1-6/6 and R1-6/8 petite mtDNA.

The results summarized in the two sections above suggest that the major portions of (a) the deletion and "changed sequences" resulting in the formation of R1-6/1 mtDNA, (b) the deletion whose consequence is the formation of R1-6/6 and R1-6/8 mtDNA, and (c) the main part of the deletion whose loss accounts for the production of R1-6/5 mtDNA have occurred in approximately the same general region of the grande mitochondrial genome. This series of petite subclones therefore differs greatly from a series of ethidium bromide induced petites which have much more extensive deletions of different parts of their mitochondrial genome (Faye *et al.*, 1973; Fauman and Rabinowitz, 1974; Laskowsky *et al.*, 1974).

A possible interpretation of the data is that the original mutagenic event resulted in the deletion of about 30% of a grande mtDNA molecule, which then segregated in subsequent division cycles, giving rise to the petite R1-6 population. Secondary events may then have modified this petite mtDNA population further, possibly by small deletions and/or localized base sequence changes and reiteration of small segments of the genome. Subcloning of the parent petite population yielded several petite clones, the mtDNA of which differed in several aspects.

Alternatively, the different petites of the R1-6 series may have arisen by independent deletion events in different mtDNA molecules of the same grande cell. In this case, however, these events must have occurred largely in the same region of the different mtDNA molecules.

The above hypotheses assume that deletion was the outcome of the primary mutagenic event. It is also possible to assume that a base-sequence change was the primary event, and that the deletions were generated subsequently (in some cases possibly deleting the original base-sequence change).

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Studies on the Structure of Deoxyribonucleoproteins. Spectroscopic Characterization of the Ethidium Bromide Binding Sites†

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ABSTRACT: In order to understand the chemical nature and the specificity of the interactions between ethidium bromide and its binding sites in deoxyribonucleoproteins, we have measured the quantum yield, fluorescence lifetime, and polarization of fluorescence of bound dye as a function of the DNA-phosphate/dye ratio (P/D). These studies have enabled us to interpret more accurately the basis for the heterogeneity of ethidium bromide binding sites in deoxyribonucleoproteins (DNP), which we have found to be even greater than previously reported (Angerer, L. M., and Moudrianakis, E. N. (1972), *J. Mol. Biol. 63*, 505). Although no differences in the nature of the intercalation sites between DNP and DNA are apparent from measurements of the quantum yield and fluorescence lifetime of bound dye, Scatchard plots of titration data indicate that there are at least two classes of highly fluorescent intercalation sites in DNP compared to only one class in DNA. In addition, polarization studies show that the rotational relaxation time for dye bound to DNP is about 470 nsec compared to only about 200 nsec for dye bound to DNA, suggesting that the internal structure of the DNP fiber is more rigid than that of DNA. Changes in polarization values as a function of the P/D ratio also raise the possibility that the sites of intercalation are more clustered in DNP than those in DNA. By preparing partial DNP's deproteinized by various procedures which control the type and order of removal of different classes of histones, we have demonstrated that the restriction of the dye-binding capacity of DNP is dependent upon the kind as well as the amount of its complexed protein. On a weight basis, the lysine-rich histones appear to be the most effective inhibitors of dye binding in DNP.

A number of physical and biochemical studies have recently been made on deoxyribonucleoproteins[1] (DNP) to probe the organization of proteins along the DNA. These studies have examined the sensitivity of whole and partially deproteinized DNP's to various nucleases and the capacity of these DNP preparations to bind various small molecules and synthetic polypeptides (Mirsky, 1971; Mirsky and Silverman, 1972; Clark and Felsenfeld, 1971; Itzhaki, 1971; Kleiman and Huang, 1971; Minyat *et al.*, 1970) or to serve as templates for RNA and DNA synthesis (Murray, 1969; Georgiev *et al.*, 1966; Spelsberg and Hnilica, 1971; Smart and Bonner, 1971; Schwimmer and Bonner, 1965). In general, it has been found that a portion of the DNA in DNP is masked through its association with bound protein, although the amount of DNA estimated as masked depends on the probe used and the conditions of the measurement. Recently, the interaction of the intercalative drug, ethidium bromide (EtBr), with whole and partial DNP's and DNA has been characterized by both absorption and fluorescence spectroscopy in order to determine the relative effectiveness of various chromosomal proteins in altering the dye-binding capacity of DNP and to compare the nature of the dye-binding sites in DNP and DNA (Angerer and Moudrianakis, 1972; Lurquin and Seligy, 1972; Williams *et al.*, 1972). These studies have shown that the optical properties of EtBr bound to either DNP or to DNA are identical and that both primary (highly fluorescent) and secondary binding sites are present in both DNP and DNA. However, the number of

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[1] Abbreviations used are: DNP, deoxyribonucleoproteins; EtBr, ethidium bromide.