Brandhorst, B. P., and Humphreys, T. (1972), *J. Cell Biol.* (in press).
Chamberlain, J. (1968), *J. Cell Biol. 39*, 23a.
Colby, C., and Duesberg, P. H. (1969), *Nature (London) 222*, 940.
Colby, C., Jurale, C., and Kates, J. R. (1971), *J. Virol. 7*, 71.
Craig, S. P. (1970), *J. Mol. Biol. 47*, 615.
Darnell, J. E., Wall, R., and Tushinski, R. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1321.
Edmonds, M., Vaughan, M. H., Jr., and Nakazato, H. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1336.
Emerson, C. P., Jr., Humphreys, T. (1970), *Develop. Biol. 23*, 86.
Emerson, C. P., Jr., and Humphreys, T. (1971), *Science 171*, 898.
Franklin, R. M. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 1504.
Hyatt, E. A. (1967), *Biochim. Biophys. Acta 142*, 254.
Jurale, C., Kates, J. R., and Colby, C. (1970), *Nature (London) 226*, 1027.
Lee, S. Y., Mendecki, J., and Brawerman, G. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1331.
Lim, L., and Canellakis, E. S. (1970), *Nature (London) 227*, 710.
Montagnier, L. (1968), *C. R. Acad. Sci., Ser. D 267*, 1417.
Montagnier, L., and Harel, L. (1971), *Nature (London), New Biol. 229*, 106.
Nygaard, A. P., and Hall, B. D. (1964), *J. Mol. Biol. 9*, 125.
Penman, S., Vesco, C., and Penman, M. (1968), *J. Mol. Biol. 34*, 49.
Philipson, L., Wall, R., and Glickman, R., and Darnell, J. E. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2806.
Spiegelman, S., Pace, N. R., Mills, D. R., Levisohn, R., Eikhom, T. S., Tayler, M. M., Peterson, R. L., and Bishop, D. H. L. (1968), *Cold Spring Harbor Symp. Quant. Biol. 33*, 101.
Stern, R., and Friedman, R. (1970), *Nature (London) 226*, 612.
Stern, R., and Friedman, R. (1971), *Biochemistry 10*, 3635.
Stollar, V., and Stollar, B. D. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 993.
Twu, J. S., and Bretthauer, R. K. (1971), *Biochemistry 10*, 1576.
Weissmann, C., Feix, G., and Slor, P. (1968), *Cold Spring Harbor Symp. Quant. Biol. 33*, 83.

In Vitro Transcription of Mitochondrial Deoxyribonucleic Acid from Yeast†

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ABSTRACT: Mitochondrial and nuclear DNAs from various wild-type and petite yeast strains have been highly purified by hydroxylapatite chromatography. Yeast mitochondrial, nuclear, and *Escherichia coli* DNA-dependent RNA polymerases were used to transcribe mitochondrial DNA of yeast into RNA. The RNA produced by these enzymes was used to study both the nature of the enzyme products and the properties of the template DNAs. The *E. coli* enzyme and mitochondrial enzyme transcribed the ribosomal genes of mitochondrial DNA as shown by competition with cold mitochondrial ribosomal RNA. The labeled *in vitro* synthesized RNAs were hybridized to various DNAs in an attempt to compare the extent of homology among these DNAs. Very little homology was indicated between nuclear and mitochondrial DNA of yeast. Total cell DNA from two petite mutants lacking the mitochondrial satellite band showed the same low degree of homology to mitochondrial DNA. Wild-type mitochondrial DNAs differed in their homologies to each other and appeared to contain different amounts of repetitive DNA. Mitochondrial DNAs from two petite mutants showed reduced homology to wild-type mitochondrial DNA. However, no additional sequences which differed from those of wild-type mitochondrial DNA could be detected in the mitochondrial DNA of one petite mutant studied, thus indicating a simple deletion mechanism for the origin of this strain. This petite mutant lacks 50–60% of the wild-type mitochondrial genome transcribed *in vitro*.

The interest in the genetic function of mitochondrial DNA has initiated during the last few years a large number of investigations on this subject (Borst and Kroon, 1969; Ashwell and Work, 1970). Yeast mitochondrial DNA is particularly interesting as mutations in this DNA can block mitochondrial functions but are not lethal to the cell. Very often mutations cause a change in the buoyant density of mitochondrial DNA (Mounolou *et al.*, 1966; Bernardi *et al.*, 1968; Mehrotra and Mahler, 1968) and even a complete loss of mitochondrial DNA in this organism has been reported (Goldring *et al.*, 1970; Nagley and Linnane, 1970; Michaelis *et al.*, 1971). Relatively large amounts of yeast mitochondrial DNA can be isolated by chromatography on hydroxylapatite (Bernardi *et al.*, 1970). This enabled us to set up and characterize an *in vitro* transcription system using yeast mitochondrial DNA as template. We have used this system for studying the question of sequence homology be-

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tween mitochondrial and nuclear DNA, the presence of mitochondrial DNA of nuclear buoyant density in neutral petite mutants, the variability of wild-type mitochondrial DNA, and the nature of the petite mutation.

Materials and Methods

Saccharomyces cereviseae Strains. The following yeast strains were used in these experiments: S288C: wild type: α, gal_2, mal, mel; obtained from R. Snow, S288C/11: petite; acriflavin induced; no mitochondrial DNA: Tsai *et al.* (1971); D243-4A: wild type; a, ade_1, lys_2 trp_1; obtained from F. Sherman, D243-4A/8: neutral petite; ethidium bromide induced; no mitochondrial DNA: Michaelis *et al.* (1971); D243-4C: wild type; α, ade_1; from F. Sherman; D-243-2B-R_1; wild type: α, ade_1, *P* from P. P. Slonimiski; Bernardi *et al.* (1970); D243-2B-R_1-6: low suppressive (13%) petite; spontaneous mutation; a, ade_1 from P. P. Slonimiski; derived from D-243-2B-R_1; D243-2B-13: suppressive (95%) petite; spontaneous mutation; a, P_7, ade_1, lys_2; from P. P. Slonimiski: Bernardi *et al.* (1970).

Isolation of Yeast DNA. Mitochondrial DNA was purified as described by Bernardi *et al.* (1970). Six to eight hydroxylapatite chromatography steps were necessary to obtain mitochondrial DNA free of nuclear DNA, as measured by CsCl density gradient centrifugation. Based on this method all but one of the mitochondrial DNAs used in this study, contained less than 1% nuclear contamination. Only preparation II of strain D243-4C showed observable traces of nuclear DNA. The nuclear DNA of strain D243-4A appeared pure after three hydroxylapatite chromatography steps and the total DNAs of the two neutral petite strains S288C/11 and D243-4A/8 were passed through only one hydroxylapatite column to remove protein and RNA. The following DNA preparations were used in this study: mitochondrial DNA from strain S288C, preparation I; mitochondrial DNA from strain S288C, preparation II; mitochondrial DNA from strain D243-4A, mitochondrial DNA from strain D243-4C, preparation I; mitochondrial DNA from strain D243-4C, preparation II, traces of (<5%) nuclear DNA; mitochondrial DNA from strain D243-2B-R_1, preparation I; mitochondrial DNA from strain D243-2B-R_1-6, preparation I; mitochondrial DNA from strain D243-2B-R_1-6, preparation II; mitochondrial DNA from strain D243-2B-13, preparation I; nuclear DNA, D243-4A, whole cell DNA, S288C/11, whole cell DNA, D243-4A/8; considered as pure nuclear DNAs as these petites lack mitochondrial DNA. The buoyant densities of the mitochondrial DNAs from all wild-type and petite strains used are close to 1.684 g/cm^3. Density gradient centrifugation was carried out as described previously, Michaelis *et al.* (1971).

Transcription of DNA to Prepare RNA and Isolation of RNA Products. Transcription was carried out as described by Cohen *et al.* (1967) except that the reaction mixture of Tsai *et al.* (1971) was used. The reaction mixtures were incubated for 120 min at 30° when the yeast RNA polymerases were used and at 37° when *Escherichia coli* enzyme was used.

Mitochondrial Ribosomal RNA. RNA was isolated from purified ribosomes according to the procedure of Kuntzel (1969). RNA from whole mitochondria was isolated using the procedure of Aloni and Attardi (1971).

Preparation of DNA Containing Filters and Hybridization. Hybridization of RNA to denatured DNA was carried out using the filter technique of Gillespie and Spiegelman (1965) as modified by Church and McCarthy (1968). Membrane filters (50 or 25 mm in diameter) were loaded with sufficient heat-denatured DNA, that 6-mm filters, containing 5 μg of DNA each, could be punched from the large filter. The retention of DNA on each filter was monitored by the method of Burton (1968) after each hybridization study. In a typical hybridization experiment a small vial contained: 0.20 ml of formamide, 0.10 ml of 20 × SSC,[1] 0.09 ml of H_2O, 0.010 ml of [^{3}H]RNA (20,000 cpm), and up to six 6-mm filters. The mixture was covered with 0.2 ml of paraffin oil and was incubated 24 hr at 37°. After the hybridization, filters were washed twice in 5 ml of 50% formamide–5 × SSC (at 37°) and two times in 5 ml of 5 × SSC (at 37°). After drying *in vacuo* each filter was counted in 10 ml of toluene scintillation fluid. Filters were washed twice with toluene and dried *in vacuo*. Each filter was treated with 4 ml of 2 × SSC and 0.020 ml of pancreatic RNase solution (4 mg/ml) for 1 hr at room temperature, washed twice with 5 ml of 5 × SSC, dried *in vacuo*, and counted.

DNA-Dependent RNA Polymerases. Escherichia coli RNA polymerase was prepared by the method of Burgess (1969), yeast nuclear RNA polymerase was isolated by the method of Frederick *et al.* (1969), and the yeast mitochondrial enzymes by the method of Tsai *et al.* (1971).

RNase Resistance of in Vitro RNAs. Preparations of *in vitro*[2] RNA were diluted to provide the correct range of radioactivity. Aliquots (10 ml) were then treated with 10 μg/ml of pancreatic RNase for various times and precipitated with 10% trichloroacetic acid. Samples were collected on Millipore filters, dried, and counted in a scintillation counter. Alternatively, RNA samples were subjected to boiling for 10 min or incubation at 37° in formamide using the conditions for RNA–DNA hybridization and then rapidly diluted 25-fold with ice-cold 5 × SSC prior to RNase treatment.

RNA melting curves were run on preparations of *in vitro* RNA in the presence of 50% formamide with buffer conditions identical with those used for the RNA–DNA hybridization studies. The change in optical density with temperature was observed in a Gilford spectrophotometer.

Chemicals. [^{3}H]Uridine triphosphate (0.5 mCi/ml; 25 Ci/mmole was purchased from Schwartz/Mann Company and foramide from Mallinckrodt Chemical Company. DNase (electrophoretically purified) was obtained from Worthington Biochemical Corporation. RNase was obtained from Calbiochem Co. and was heated to 80° for 10 min before use. Bac-T-flex membrane filters (B-6; 50 mm) from Schleicher and Schuell and Millipore Corporation filters (25 mm) were used for the hybridization experiments.

Results

Nature of the RNA Transcription Products of Mitochondrial DNAs. To gain information concerning which genes and how much of the isolated mitochondrial genome is transcribed *in vitro* by RNA polymerases, labeled RNA was prepared and hybridized to denatured wild-type mitochondrial DNA both in the absence and presence of excess cold ribosomal RNA extracted from purified mitochondrial ribosomes. These experiments were carried out using RNAs

[1] SSC is 0.15 M NaCl plus 0.015 M sodium citrate buffer. 20 × SSC or 5 × SSC, etc., refer to the citrate–saline buffer at 20 or 5 times the above concentrations.

[2] *In vivo* RNA: RNA isolated from mitochondria; *in vitro* RNA: RNA synthesized by enzymatic transcriptions of DNA using RNA polymerase.

TABLE I: Hybridization of *in Vitro* RNAs with Mitochondrial DNA in the Presence and Absence of Unlabeled Mitochondrial Ribosomal RNA.[a]

DNA-Dependent RNA Polymerase	DNA Template	DNA on Filter	Without rRNA (cpm, %)	With rRNA (cpm, %)
Yeast nuclear	Mit DNA D243-4A	Mit DNA D243-4A	1861 (100)	1801 (97)
Yeast mitochondrial I	Mit DNA D243-4A	Mit DNA D243-4A	2130 (100)	1529 (72)
Yeast mitochondrial III	Mit DNA D243-4A	Mit DNA D243-4A	1951 (100)	1471 (75)
E. coli	Mit DNA D243-4A	Mit DNA D243-4A	1480 (100)	1234 (83)
E. coli	Mit DNA D243-4A	Mit DNA D243-2B-R_1-6 I	754 (100)	646 (86)
E. coli	Mit DNA D243-4A	Mit DNA D243-2B-R_1-6 II	748 (100)	641 (86)
E. coli	Mit DNA D243-4A	None	14	16
E. coli	Mit DNA D243-4A	*E. coli* DNA	28	21
E. coli	Mit DNA D243-4A	Mit DNA D243-4A[b]	1806 (100)	1354 (75)
E. coli	Mit DNA D243-4A	Mit DNA D243-4C[b]	2169 (100)	1297 (59.8)

[a] The final concentration of the hybridization mixture was 50% formamide and 5 × SSC. Each vial contained in a final volume of 1 ml; 20,000 cpm of *in vitro* [^{3}H]RNA (~ 24 ng of RNA), two 6-mm DNA filters with 5 μg of mitochondrial DNA per filter and a blank filter without DNA. Mitochondrial ribosomal RNA (20 μg) (assuming an OD_{260} 24 = 1 mg of RNA) were added to a vial for competition experiments. The vials were incubated at 37° for 24 hr. The radioactivity retained on the two DNA filters of a vial after digestion with RNase is given in the table. The values are corrected for a blank of 30 cpm per filter. [b] Run in separate experiment.

TABLE II: Hybridization of *in Vitro* RNAs from Four Different DNA-Dependent RNA Polymerases with Mitochondrial and Nuclear DNA.[a]

DNA-Dependent RNA Polymerase	DNA Template	DNA on Filter	
		Mit DNA D243-4A (cpm, %)	Nuclear DNA Petite D243-4A/8 (cpm, %)
Yeast nuclear	Mit DNA D243-4A	1599 (100)	19 (1.2)
Yeast mitochondrial I	Mit DNA D243-4A	1929 (100)	20 (1.0)
Yeast mitochondrial III	Mit DNA D243-4A	1631 (100)	14 (0.9)
E. coli	Mit DNA D243-4A	1979 (100)	13 (0.7)

[a] The hybridization mixture was as described in Table I. Each of the four vials contained in a total volume of 1 ml 20,000 cpm of *in vitro* [^{3}H]RNA (~ 24 ng of RNA), two filters with mitochondrial DNA (5 μg of DNA per filter), two filters with nuclear DNA (5 μg of DNA per filter), and a blank filter without DNA. The radioactivity retained on the two mitochondrial DNA filters after digestion with RNase is considered as 100%. The values are corrected for a blank of 27 cpm per filter.

produced *in vitro* by the yeast nuclear and two mitochondrial RNA polymerases and also by *E. coli* RNA polymerase in order to compare the products of these four transcription systems. The experimental details and results of these hybridizations are shown in Table I.

As only a limited amount of cold ribosomal RNA isolated from purified mitochondrial ribosomes was available, saturation curves were not run in each case. Therefore, the results may be discussed only in a semiquantitative manner. The numerical values obtained for the amount of competition may be somewhat smaller than the true values. From the hybridization values it is seen that marked competition occurs in the presence of cold ribosomal RNA. It seems that at least the mitochondrial and the *E. coli* RNA polymerases transcribe the ribosomal genes of mitochondrial DNA. It should be noted that the competition values of 15 to 25% are larger than expected from a random transcription of the whole mitochondrial genome. As also shown in Table I, competition experiments were run using filters with mitochondrial DNA isolated from the petite strain D243-2B-R_1-6. It can be seen that this altered DNA (see later) maintains all or some parts of the ribosomal genes.

In a further attempt to compare products of the four transcription systems, the possibility that the yeast nuclear RNA polymerase might preferentially transcribe "nuclear-like" regions on the mitochondrial DNA (Wintersberger and Viehauser, 1968) was checked. The *in vitro* RNAs were hybridized to purified, denatured mitochondrial wild-type DNA and nuclear DNA of yeast. The details of this experiment and the results are given in Table II. All four RNAs hybridized very well to mitochondrial DNA and to only a very low extent to nuclear DNA.

RNase Resistance Tests of in Vitro RNA and of Possible RNA–RNA Hybrid Formation during Competition Experiments. Double strandedness of the *in vitro* RNA preparations was estimated from a kinetic analysis of RNA hydrolysis by RNase. The RNase resistances of the transcripts of mitochondrial DNA (D243-4A) using the various RNA

TABLE III: RNase Resistance of *in Vitro* RNAs.[a]

Template DNA	RNA Polymerase Source	Ribonuclease Resistance (% of total cpm)			
		Original Preparation	After Heat and Rapid Cool	After Incubation in DNA–RNA Hybridization Conditions	After Annealing with Unlabeled RNA[b]
Mitochondrial (D243-4A) wt	*E. coli*	34	6.4	18	24
	Yeast nuclear	28	4.4		
	Yeast mitochondrial I	19	4.9		
	Yeast mitochondrial III	16	4.9		
Mitochondrial petite 13	*E. coli*	20		14	
Mitochondrial petite(243-2B-R_1-6)	*E. coli*	39	3.32	37	
Nuclear	*E. coli*	12		6.4	

[a] [^{3}H]RNA samples (~10,000 cpm) from each of the above preparations were rapidly diluted (25-fold) into ice-cold buffer. RNase was then added and incubated at 28° for 15 min. The solution was made 5% in trichloroacetic acid and insoluble polynucleotides were collected on Millipore filters, washed, dried, and counted in toluene scintillation fluid. [b] ~16 μg of RNA (10–35 S).

polymerases and of several other DNAs are shown in Table III. Heating the samples to boiling, followed by rapid (25-fold) dilution, and cooling to 0° resulted in virtually complete digestion of the *in vitro* RNA by added RNase. Exposure of the *in vitro* RNA to conditions employed for RNA–DNA hybridization on filters (50% formamide, 5 × SSC, 37°) followed by dilution and rapid cooling generally reduced the amount of RNase resistant sequences to near 0.5 their original amounts. It therefore appears that some competition between RNA–DNA hybridization and RNA–RNA hybridization could occur during these studies.

The relatively large amounts of RNase resistant sequences in the *in vitro* RNA preparations apparent from the digestion studies were analyzed by thermal denaturation measurements in the formamide annealing solution. The midpoint of the thermal transition was found to be 34.2°. The hyperchromicity observed was completely and very rapidly reversible upon cooling, with a half-time of less than 3 min with the conditions employed. Thus, the nucleotides involved in this reaction behave as though they contain repeated segments. Due to the high percentages of A:T base pairs in yeast mitochondrial DNAs it may be suggested that repeated A:U sequences in the RNA transcripts account for a major portion of the double-stranded RNA segments. If this is the case, it should be noted that RNase insensitivity determinations would indicate a higher degree of double strandedness than truly representative since [^{3}H]uridine was used as the only source of radioactive label in preparation of *in vitro* RNAs.

Annealing *in vitro* RNA with mitochondrial DNA containing filters in the presence of unlabeled *in vivo* (10–35 S) RNA isolated from whole mitochondria, followed by dilution and rapid cooling yields a small increase in the amount of RNase resistance of the preparation. This increase (Table III) indicates that limited competition between DNA–RNA and RNA–RNA hybridization occurs under these conditions, resulting in some additional double-stranded RNA. It was not possible, however, to detect sedimentation of *in vitro* [H^3]RNA (~5 S) with the more rapidly sedimenting unlabeled RNAs following the incubation.

Effects of RNA–RNA Hybridization on the Competition between in Vitro and in Vivo RNA Binding to Mitochondrial DNA. Mitochondrial RNA isolated from whole mitochondria and separated by sedimentation on a sucrose gradient gave the distribution shown in Figure 1. The larger components were tested separately or as pooled fractions for their ability to inhibit [^{3}H]RNA (*in vitro*) binding to mitochondrial DNA. As may be seen from Figure 2, the extent of competition is large and depends somewhat upon the RNA species added, and saturation (by competitor) is obtained at 25–30 μg of added RNA. This corresponds to ratios of unlabeled RNA:[^{3}H]RNA near 60:1 and unlabeled RNA:DNA near 6:1. Note that even in the presence of a great excess of unlabeled RNA, no further decrease in [^{3}H]RNA binding is observed indicating that no appreciable nonspecific (or low specificity) RNA–RNA hybridization takes place. Peak I RNA inhibits hybridization to a somewhat greater extent than does either of the other components. Preliminary competition experiments using mixtures of peak I with peak II and III RNA suggest sequence overlap among the components of this preparation and thus no meaning can be applied to addive values of competition by each fraction. Hybridization competition studies with the total RNA preparation show that the *in vitro* RNA contains more sequences of the entire genome than are present in isolated mitochondrial *in vivo* RNA and might therefore be more suitable for homology studies.

DNA Homology Studies. In vitro RNA was used to study possible sequence homologies between nuclear and mitochondrial DNA, among three different wild-type mitochondrial DNAs, and between mitochondrial wild-type and petite DNA of yeast. Labeled *in vitro* RNA, transcribed using the *E. coli* RNA polymerase and mitochondrial DNA from the wild-type strain D243-4A as template, was hybridized to the various highly purified DNAs listed in Table IV. The RNA transcribed by the *E. coli* RNA polymerase was chosen for these experiments mainly because the high specific activity of the *E. coli* enzyme allowed larger amounts of *in vitro* RNA to be isolated. RNA transcribed from heat-denatured mitochondrial DNA template was also used but no changes

TABLE IV: Hybridization of *in Vitro* RNA with Various DNAs.[a]

DNA on Filter	Exp I: Cpm before RNase	Exp I: Cpm after RNase	Exp I: μg of DNA/2 Filters	Exp I: Cpm/μg of DNA	Relative Hybridization (%): Exp I	Relative Hybridization (%): Exp II	Relative Hybridization (%): Exp III
Mit DNA D243-4A	3658	1691					
	3288	1336	9.5	312	100	100	100
Blank	36	30					
Total DNA D243-4A/8	44	35					
	46	41	5.7	2.8	0.9		2
Blank	30	31					
Total DNA S288C/11	47	38					
	48	34	9.0	1.3	0.4		0.9
Blank	32	28					
Nuclear DNA D243-4A	64	41					
	75	47	8.5	3.3	1.1	2.5	
Blank	31	31					
I. Mit DNA D243-2B-R_1-6	2304	752					
	2236	760	10.1	144	46	68	66
Blank	40	29					
II. Mit DNA D243-2B-R_1-6	1469	608					
	1375	452	9.8	102	33	49	58
Blank	28	32					
Mit DNA D243-2B-13	423	107					
	499	111	9.9	16	5.1	6	8
Blank	31	26					
I. Mit DNA S288C	2804	1118					
	2297	908	8.8	223	71	71	78
Blank	39	30					
II. Mit DNA S288C						63	86
Blank							
I. Mit DNA D243-4C							
						138	133
Blank							
II. Mit DNA D243-4C							
						186	190
Blank							

[a] Each vial contained 20,000 cpm of *in vitro* [^{3}H]RNA, transcribed with the *E. coli* RNA polymerase from the mitochondrial DNA of strain D243-4A. In a final volume of 0.4 ml two DNA filters (5 μg of DNA per filter) and one blank filter were suspended. Experiment III was done with RNA transcribed with the *E. coli* RNA polymerase from heat-denatured mitochondrial DNA of strain D243-4A. The hybridization of the *in vitro* RNA to its own template is considered as 100%.

of the hybridization values compared to those obtained using native template could be observed. The high purity of the mitochondrial template DNA was demonstrated by the very low affinity of the mitochondrial *in vitro* RNA to purified nuclear DNA. Thus, very low sequence homology between nuclear and mitochondrial DNA was found, confirming the data of Table II. The reverse experiment in which pure nuclear DNA was used as template for transcription by RNA polymerase to form product which could be tested for homology with mitochondrial DNA also indicated a complete lack of homology. Whole cell DNAs from two petite mutants, lacking the mitochondrial satellite band (Michaelis *et al.*, 1971), showed even less hybridization to mitochondrial *in vitro* RNA, thus providing an independent method illustrating a lack of mitochondrial DNA in these mutants.

The different wild-type mitochondrial DNAs hybridized to quite different extents with D243-4A *in vitro* mitochondrial RNA. RNA transcribed from mitochondrial DNA of strain D243-4A was hybridized less to the DNA of strain S288C and better to the DNA of strain D243-4C as compared to its own template DNA. This result indicates that the transcription of mitochondrial DNA with the *E. coli* RNA polymerase was not completely random and the DNA regions transcribed by this enzyme must occur with lower frequency in S288C mitochondrial DNA preparations and with greater frequency in D243-4C mitochondrial DNA than in the template. Thus it would appear that the mitochondrial wild-type DNAs contain some regions or genes in different numbers. The last two lines of Table I support this conclusion even further as it is shown by competition experiments that ribosomal RNA sequences appear with greater frequency on the D243-4C wild-type DNA than on the D243-4A wild-type DNA preparations. It should be noted that the difference between D243-4A and S288C wild-type DNA was also

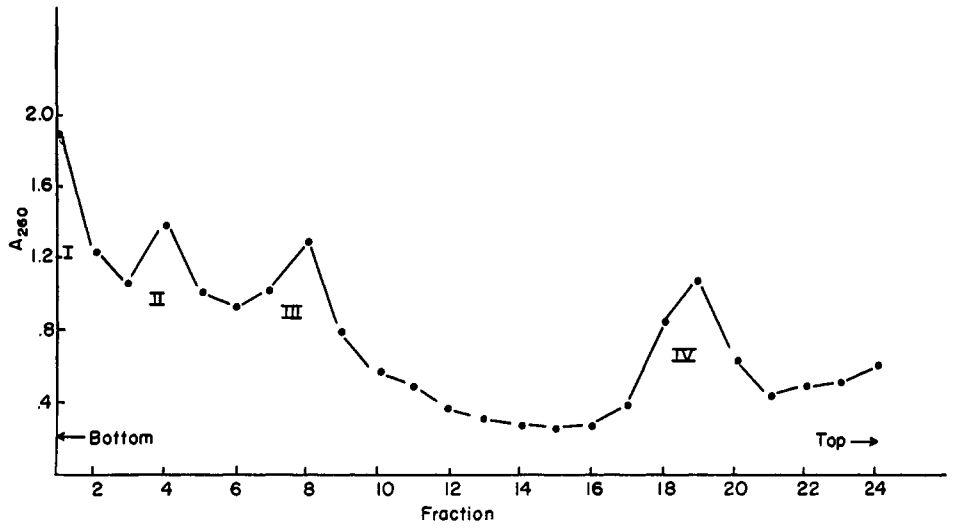

FIGURE 1: Sedimentation pattern of mitochondrial RNA from isolated yeast mitochondria in a linear 5–20% sucrose gradient containing 0.02 M phosphate, pH 7.4, 0.1 M KCl, and 1% HCHO at 4°. A Spinco SW40 rotor was used and samples centrifuged for 12 hr at 40,000 rpm. Fractions were collected by puncturing the tube at the bottom and collecting alliquots.

obtained (Table V) when *in vitro* RNA transcribed from the mitochondrial DNA of the petite mutant D243-2B-R_1-6 was used.

A decreased amount of hybridization was observed when wild-type *in vitro* RNA was annealed with the mitochondrial DNA of the low suppressive petite mutant D243-2B-R_1-6. This hybridization value becomes very low in the case of the highly suppressive petite mutant D243-2B-13. The low homology between wild-type mitochondrial DNA and the mitochondrial DNA of this petite mutant indicates a large alteration of the mutated DNA.

An exhaustive hybridization study was carried out to determine if petite D243-2B-R_1-6 mitochondrial DNA contains sequences not present in the wild-type D243-4A mitochondrial DNA (Table VI, Figure 3). *In vitro* RNA, transcribed from the mitochondrial DNA of the petite strain, was exhaustively hybridized with wild-type mitochondrial DNA on cellulose filters to remove all or most of the wild-type-like RNA sequences from solution. The remaining RNA was tested for its affinity to wild-type mitochondrial and petite mitochondrial DNA (and nuclear DNA which defines a background level). The results indicate that the petite mitochondrial DNA contains no new sequences. As a control, wild-type RNA was exhaustively hybridized both to wild type and to petite mitochondrial DNA on filters. When wild-type DNA was used to remove sequences, the residual RNA in solution still hybridizes somewhat better to its own template, but roughly with the same ratio as at the start of exhaustive hybridization. However, when petite DNA on filters was used to remove all "petite sequences," a large amount of RNA with sequences homologous to wild-type

TABLE V: Hybridization of Nuclear and Mitochondrial DNAs to D243-2B-R_1-6 *in Vitro* [^{3}H]RNA.[a]

DNA on Filter	Cpm/2 Filters	μg of DNA/2 Filters	Cpm/μg of DNA	Rel Hybridization (%)
Mitochondrial D243-2B-R_1-6	1202	9.6		
	1336	9.6	262	100
	1235	9.5		
Mitochondrial D243-4A	1030	8.8		
			233	89
	1067	9.2		
Mitochondrial S288C	901	8.3		
			206	79
	798	8.2		
Nuclear D243-4A/8	11	6.1	3.6	1.4

[a] The hybridization mixture was as described in Table IV. Each vial contained 26,000 cpm of *in vitro* [^{3}H]RNA, transcribed from D243-2B-R_1-6 mitochondrial DNA with *E. coli* RNA polymerase. Each vial contained two 6-mm filters with ~5 μg of DNA and one blank filter.

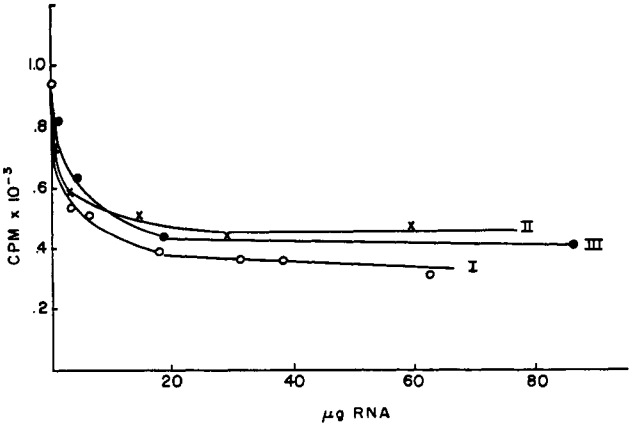

FIGURE 2: Inhibition of binding of *in vitro* [^{3}H]RNA to mitochondrial DNA on Millipore filters by the addition of unlabeled *in vivo* RNA isolated from whole mitochondria. The larger molecular weight components from gradient fractionation of RNAs (Figure 1) were used individually to test inhibition of binding. Hybridization conditions were as described in materials and methods except that varying amounts of *in vivo* RNA were added to the incubation mixtures.

TABLE VI: Exhaustive Hybridization of RNA Transcribed from Mitochondrial DNA of Strain D243-4A and D243-2B-R_1-6.[a]

Mitochondrial DNA as Template, DNA on Filters Used to Exhaustively Hybridize *in Vitro* RNA			D243-4A				D243-2B-R_1-6	
			D243-4A		D243-2B-R_1-6		D243-4A	
Hybridization Step	Number of 5-μg DNA Filters	DNA on Filter	Cpm of All Filters	Relative Hybridization (%)	Cpm of All Filters	Relative Hybridization (%)	Cpm of All Filters	Relative Hybridization (%)
1	4	As indicated for exhaustive hybridization	20706		8571		16471	
2	4	As indicated for exhaustive hybridization	7222		3454		6709	
3	4	As indicated for exhaustive hybridization	4596		3170		2923	
4	4	As indicated for exhaustive hybridization	1974		1458		1508	
5	4	As indicated for exhaustive hybridization	907		706		779	
6	4	As indicated for exhaustive hybridization	831		640		639	
7	4	As indicated for exhaustive hybridization	545		511		602	
8	4	As indicated for exhaustive hybridization	369		538		292	
9a	2	Nuclear DNA	31	14			26	7
	2	D243-2B-R_1-6	143	64	105	3.5	357	100
	2	D243-4A	225	100	3012	100	150	42
9b	2	Nuclear DNA	32	24			10	3
	2	D243-2B-R_1-6	117	87			288	100
	2	D243-4A	135	100			122	42

[a] Each of the three vials contained in a final volume of 1 ml approximately 2×10^5 cpm of *in vitro* [^{3}H]RNA ($\sim$240 ng of RNA). The two RNA preparations used were transcribed by the *E. coli* RNA polymerase from mitochondrial DNA of strains D243-4A and D243-2B-R_1-6. The three mixtures were repeatedly incubated each time with four filters which were loaded with 5 μg of denatured DNA. The incubations were done at 37° for approximately 24 hr. The radioactivity retained on the four filters after digestion with RNase is given. The values are corrected for a blank of 30 cpm per filter. After the eighth incubation two of the RNA samples were divided into two parts and each part, containing 0.3 ml, was incubated with two nuclear DNA filters (D243-4A/8), two filters with mitochondrial petite DNA (D243-2B-R_1-6), two filters with mitochondrial wild-type DNA (D243-4A), and one blank filter without DNA. The third RNA sample, run at a later time, was not divided and only incubated with two filters with mitochondrial petite DNA, two filters with mitochondrial wild-type DNA, and one blank filter without DNA. The radioactivity retained on the two homologous filters after digestion with RNase is given. The values are corrected for a blank of 30 cpm per filter.

DNA was left. Therefore, it may be concluded while that mitochondrial DNA of the petite strain D243-2B-R_1-6 does not contain measurable sequences which are not also present in the wild-type DNA, the mitochondrial DNA of this petite mutant has lost sequences present in the wild-type.

The fact that it was possible to make only a limited amount of *in vitro* [^{3}H]RNA from any one DNA precluded the possibility of doing saturation studies to determine the exact size of the deletion. In all cases tested, however, the petite DNA containing filters from D243-2B-R_1-6 bound only 50–60% as many counts as the wild-type DNA filters when these two were incubated with wild-type *in vitro* RNA. The relative hybridization observed remained nearly constant even when the amount of added RNA was varied over a 20-fold concentration range (note data of Table VII or of Table VI at the various stages in the exhaustive hybridization study).

TABLE VII: Relative Hybridization of D243-4A and D243-2B-R_1-6 DNAs to D243-4A *in Vitro* [^{3}H]RNA.

Concentration *in Vitro* RNA (cpm/ml)	D243-2B-R_1-6 (cpm/μg)	D243-4A (cpm/μg)	Relative Hybridization of D243-2B-R_1-6 (%)
2.1×10^5	424	1120	38
1.05×10^5	216	500	43
5.25×10^4	144	312	46
Very low (end of exhaustive hybridization study)	26	39	68

TABLE VIII: Relative Hybridization of D243-4A *in Vitro* RNA with DNAs from Petite D243-2B-R_1-6 Its Wild-Type Parent, D243-2B-R_1, and the Wild-Type (w.t.) D243-4A.[a]

DNA Preparation	Cpm on Filters	Cpm/μg of DNA	Relative Hybridization %
I. D243-4A (w.t.)	2524 1886	277	100
II. D243-2B-R_1 (w.t.)	3276	271	98
III. D243-2B-R_1-6 (petite)	1281 1191	134	48

[a] Hybridization was as described in Table IV. The above counts have been corrected for background (30 cpm for blank filter).

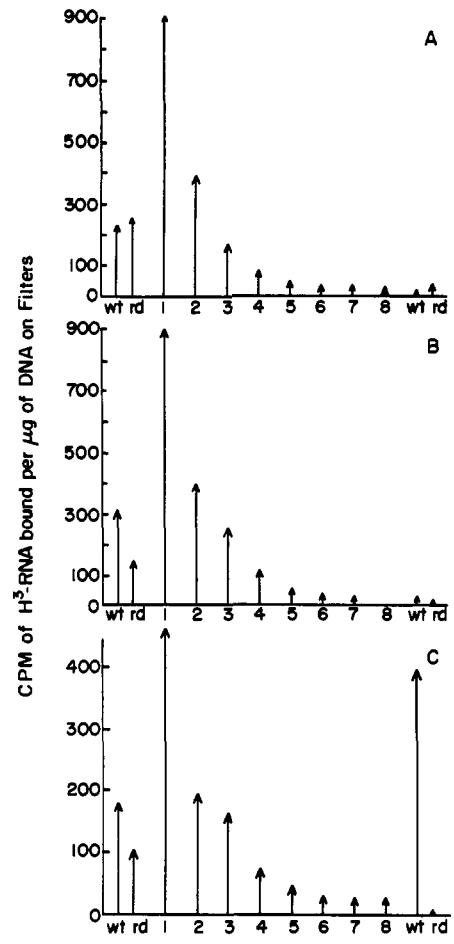

FIGURE 3: Exhaustive hybridization studies of *in vitro* RNAs binding to mitochondrial DNA containing filters. The vertical axis represents amount of [^{3}H]RNA binding to DNA filters and the horizontal axis represents the filter type and the hybridization step for each experiment. The left two bars in each graph represent the relative amounts of [^{3}H]RNA bound by wild-type and respiratory-deficient mutant DNAs at the start of the exhaustive hybridization study. The right two bars in each graph give this value at the termination of the exhaustive hybridization. A. Hybridization of D243-2B-R_1-6 mitochondrial *in vitro* [^{3}H]RNA with D243-4A mitochondrial DNA filters. The relative hybridization values are: wild-type 89% before, and 42% after; respiratory deficient 100% before and 100% after. B. The exhaustive hybridization of D243-4A mitochondrial *in vitro* [^{3}H]RNA with D243-4A mitochondrial DNA filters. The relative hybridization values are: wild-type 100% before, and 100% after; respiratory deficient 40% before, and 74% after. C. The exhaustive hybridization of D243-4A mitochondrial *in vitro* [^{3}H]RNA with D243-2B-R_1-6 mitochondrial DNA filters. The relative hybridization values are: wild-type 100% before, and 100% after; respiratory deficient 59% before, and 3% after.

These data all support the conclusion that a deletion of approximately 50–60% of the DNA sequences of the wild-type has occurred in formation of this petite.

The wild-type DNA used for preparation of filters for the exhaustive hybridization studies is closely related to, but not the immediate parent of, petite D243-2B-R_1-6. Thus, a secondary comparison of the parent strain D243-2B-R_1 with the wild-type used in our studies was required. As shown in Table VIII, the relative hybridization of D243-4A [^{3}H]RNA to both of the wild-type DNAs and the petite DNA is consistent with the conclusion that both these wild-type DNAs are very similar in base sequences and that petite D243-2B-R_1-6 is a deletion mutation with respect to its wild-type parent.

Discussion

RNA polymerase preparations from several sources have been used to transcribe mitochondrial DNA and the *in vitro* RNA produced was used to study the properties of normal and mutant mitochondrial DNAs. As a relatively large amount of *in vitro* RNA could best be prepared using the *E. coli* enzyme, RNA products from reaction using this enzyme were used in most of our experiments. However, some interesting results have been obtained from a comparison of the RNA products of four different polymerases. If it is assumed that yeast mitochondrial DNA contains only one gene for each ribosomal RNA, these two genes would represent about 5–10% of the yeast mitochondrial genome, based on the following molecular weights: 5×10^7 for yeast mitochondrial DNA (Hollenberg *et al.*, 1970), 1.6×10^6 for the 21S ribosomal RNA, and 0.87×10^6 for the 14S ribosomal RNA (Borst and Grivell, 1971). A more direct measurement of this value comes from hybridization experiments with *in vivo* ribosomal RNA which suggest a value of 3–5% (Cohen *et al.*, 1970). With these values in mind it is interesting to compare the amounts of ribosomal RNA synthesized *in vitro* with four RNA polymerases using mitochondrial wild-type DNA as template. These amounts (15–25%), as measured by competition with cold mitochondrial ribosomal RNA, are of course dependent on the purity of the

ribosomal RNA preparation. However, due to the gradient centrifugation step used in the preparative procedures for obtaining this RNA we consider a contamination with mitochondrial nonribosomal RNA, which would interfere with such a competition experiment, rather unlikely. This conclusion is supported also by the fact, that nearly no competition is found using RNA produced by transcription with yeast nuclear RNA polymerase. Although we cannot exclude the presence of some nuclear ribosomal RNA in our mitochondrial preparation, this RNA would not have a very important effect on the final results due to its lack of homology with mitochondrial DNA (see Ashwell and Work, 1970).

The existence of ribonuclease resistant sequences in the *in vitro* RNA preparations is consistent with the results in other systems (Aloni and Attardi, 1971; Tabak and Borst, 1970; Schäfer *et al.*, 1971) and suggests another uncertainty in the interpretation of these results. RNase sensitivity studies suggest that near 30% of the [^{3}H]uridine label is associated with nonhydrolyzable segments of the RNA. This value is in good agreement with values reported by Schäfer *et al.* (1971). The actual amount of double-stranded RNA appears to be somewhat lower than this based on hyperchromicity studies. These segments contain a very high proportion of [^{3}H]uridine relative to the remainder of the *in vitro* RNA. As renaturation following heat treatment is very fast and 100% complete, it is suggested that such double-stranded sequences may represent regions of high poly(U) poly(A) or poly(A-U) content. It is difficult to determine just what effect such double-stranded segments may have on the quantitative determination of DNA–RNA hybridization in these studies. Three factors rule against it having a very large effect, however. First, only 10–30% of the RNA is involved. Second, the temperature for RNA–DNA hybridization is greater than the melting temperature for the RNA–RNA strands so that free exchange is possible. Third, in our experiments which include DNA present in excess, RNA–DNA hybridization should be favored and would at least minimize the measured effect of any RNA–RNA hybridization.

Some increase in RNase resistant sequences is consistantly noted upon incubation of [^{3}H]RNA with excess unlabeled mitochondrial RNA. This increase, however, again plateaus at high levels of added RNA and therefore again indicates specific hybridization rather than a nonspecific absorption at the high RNA concentrations.

With the above controls and using subsaturating amounts of unlabeled RNA isolated from purified ribosomes for competition experiments, it is possible to draw the following conclusions. The RNA mixtures prepared from transcription of mitochondrial DNA with yeast nuclear, yeast mitochondrial, and *E. coli* RNA polymerases are not identical. Using both the *E. coli* and the mitochondrial RNA polymerases, at least the ribosomal RNA genes are transcribed *in vitro* as well as other portions of the DNA. The RNA products of the mitochondrial RNA polymerases contained much more ribosomal RNA than expected from a random transcription of mitochondrial DNA containing one gene for each ribosomal RNA, suggesting that preferential transcription of ribosomal RNA regions takes place and/or there exists some repetitive sequences for this RNA on the genome (see below).

Competition studies at saturating levels of RNA isolated from whole mitochondria and purified on sucrose density gradients indicate that nearly 60% of the binding of *in vitro* RNA prepared using *E. coli* enzyme can be blocked by high molecular weight RNA. The question of whether some of this competition is only apparent, resulting from RNA–RNA hybridization, is answered by the saturation studies. Formation of specific RNA–RNA hybrids between the [^{3}H]-RNA and competing RNA will not effect the interpretations regarding the amount of competition. Nonspecific RNA–RNA hybridization must be minimal as evidenced by the horizontal terminal slopes of the competition curves. The general conclusion to be reached from these studies is that nearly 60% of the hybridizing sequences synthesized *in vitro* are homologous to hybridizing sequences of RNA isolated from intact mitochondria. The *in vitro* RNA contains more mitochondrial DNA sequences than *in vivo* RNA isolated using our procedures.

The high values obtained in competition studies using subsaturating levels of purified mitochondrial ribosomal RNA can not be interpreted to indicate the fraction of the mitochondrial DNA specifying ribosomal RNAs. Rather, this is most probably an indication of preferential transcription by RNA polymerase of those regions homologous to the ribosomal RNAs.

Homology studies comparing RNA binding by different mitochondrial wild-type DNAs provided further information on this question. We have observed that *in vitro* RNA transcribed from mitochondrial wild-type DNA of strain D243-4A hybridized to a lesser extent with the mitochondrial DNA of strain S288C and to a larger extent with the mitochondrial DNA of strain D243-4C as compared with its own template. One explanation for this result would be that the DNA regions which are transcribed from D243-4A mitochondrial DNA occur less frequently in S288C mitochondrial DNA and more frequently in D243-4C mitochondrial DNA. Thus, the mitochondrial wild-type DNAs do appear to contain some regions or genes in different amounts. Competition studies with mitochondrial ribosomal RNA indicate a possible increase in the amount of these sequences in the D243-4C to partially account for these differences and therefore, suggest some repetition of this region on the DNA. It would be very interesting if this is in fact the *in vivo* situation. At the moment we cannot completely exclude the possibility that these differences between the mitochondrial wild-type DNAs are due to the DNA isolation procedure. The three wild-type strains might differ in nuclease content and activity, or certain mitochondrial DNA fragments might be enriched or eliminated during the hydroxylapatite chromatography. One would however, have to propose that by some means nearly 50% of some "low-homology" sequences of D243-4C DNA were specifically lost during isolation to account for the observed increase in homology. This seems less likely than a true difference in the existing wild-type DNAs. It has recently been noted that two types of wild-type mitochondria, distinguished by their recombination behavior, can be demonstrated in yeast (Coen *et al.*, 1969). Therefore, it may be quite interesting to look further for differences in the DNA of these various types of mitochondria. The mitochondria of the three yeast wild-type strains used in this study have not yet been characterized with respect to their recombination behavior.

Several workers have suggested a structural relationship between mitochondrial DNA and nuclear DNA in yeast (for literature, see Ashwell and Work, 1970). The question of a sequence homology between nuclear DNA and mitochondrial DNA is a very important one, as mitochondrial RNA is usually characterized by its hybridization with mitochondrial DNA. Evidence against a high degree of homology between nuclear DNA and mitochondrial DNA has come

from recent studies in which yeast mitochondrial *in vivo* RNA has been purified by hybridization with mitochondrial DNA. This RNA hybridized very poorly with yeast nuclear DNA (Fukuhara, 1970; Cohen *et al.*, 1970). These analyses are limited of course to the DNA regions, which are transcribed *in vivo* under the respective growth conditions and also to the RNA sequences which are extracted using the isolation procedures employed. We have used in this study the *in vitro* transcripts of four polymerases which contain mitochondrial sequences, as shown by competition with cold mitochondrial ribosomal RNA. With all four *in vitro* RNAs we found very low specific binding to yeast nuclear DNA, thus again demonstrating low, if any, homology between nuclear and mitochondrial DNA.

Our studies benefited from the fact that we could isolate pure nuclear DNA from neutral petite mutants lacking mitochondrial DNA as shown by CsCl density gradient centrifugation (Goldring *et al.*, 1970; Nagley and Linnane, 1970; Michaelis *et al.*, 1971). It is of course very difficult or nearly impossible by biochemical methods to demonstrate the complete absence of a compound. Nevertheless, the low hybridization values of mitochondrial *in vitro* RNA with the total DNA of the neutral petite mutants substantiate this conclusion. Thus, no mitochondrial DNA can be detected in these mutants by a technique quite different from those previously employed. The possibility that in this class of mutants a mitochondrial DNA species exists with a buoyant density similar to that of nuclear DNA (and has therefore avoided detection in previous centrifugal analyses) seems unlikely as no common sequences are found.

The cytoplasmic petite mutation of yeast has some peculiar characteristics. The frequency of the spontaneous mutation is very high, no reversion to the wild-type occurs and no recombination or complementation between two cytoplasmic petite mutants to wild-type has been found. The petite mutation is often accompanied by a change in the buoyant density of mitochondrial DNA, usually reflecting an increase in adenine and thymine content. A complete loss of mitochondrial DNA seems to be correlated with the occurrence of neutral petites (Michaelis *et al.*, 1971). The change in buoyant density of mitochondrial petite DNA has variously been discussed as a result of major deletions, selection of mitochondrial minority DNA, errors in the replication of mitochondrial DNA or addition of random poly(A,T) (Borst and Kroon, 1969; Carnevali *et al.*, 1969; Bernardi *et al.*, 1970). We found a reduced hybridization of wild-type *in vitro* RNA with the mitochondrial DNA of the low suppressive petite mutant D243-2B-R_1-6. The hybridization values suggest that about 50% of the transcribed mitochondrial wild-type sequences are preserved in the mitochondrial DNA of mutant D243-2B-R_1-6 and a much lower per cent (~6%) in D243-2B-13. The value for mutant D243-2B-R_1-6 is in very good agreement with the results of Fukuhara and coworkers (Fukuhara *et al.*, 1969; Fukuhara, 1970) obtained with mitochondrial *in vivo* RNA. Also, since the same value of 50% homology of D243-4A RNA with D243-2B-R_1-6 DNA was obtained even in the presence of a tenfold increase in RNA concentration (Table VI), this value must be quite representative of the true extent of homology. Such a reduced homology between mitochondrial petite and wild-type DNA can either be the result of a partial loss of wild-type sequences in the case of the petite DNA or be the result of replacing wild-type sequences by erroneous DNA, poly(A-T), or pieces of repetitive DNA. To decide between these two main possibilities we looked for sequences present in the petite DNA and absent in the wild-type DNA by exhaustive hybridization. It should be mentioned that this method was sensitive enough to characterize an insertion into phage λ dg of about 450 nucleotide pairs (Michaelis *et al.*, 1969). As the size of λ-DNA is 25×10^6 compared to 50×10^6 for yeast mitochondrial DNA, comparable sensitivity should be obtained. No sequences different from the wild-type mitochondrial DNA could be detected in the RNA transcribed from mitochondrial DNA of the petite mutant D243-2B-R_1-6, thus supporting a simple deletion mechanism for the origin of this petite mutation.

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References

Aloni, Y., and Attardi, G. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1757.

Ashwell, M., and Work, T. S. (1970), *Annu. Rev. Biochem. 39*, 251.

Bernardi, G., Carnevali, F., Nicolaieff, A., Piperno, G., and Tecce, G. (1968), *J. Mol. Biol. 37*, 493.

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

Borst, P., and Grivell, L. A. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 13*, 73.

Borst, P., and Kroon, A. M. (1969), *Int. Rev. Cytol. 26*, 107.

Burgess, R. R. (1969), *J. Biol. Chem. 244*, 6160.

Burton, K. (1968), *Methods Enzymol. 12*, 163.

Carnevali, F., Morpurgo, G., and Tecce, G. (1969), *Science 163*, 1331.

Church, R. B., and McCarthy, B. J. (1968), *Biochem. Genet. 2*, 55.

Coen, D., Deutsch, J., Netter, P., Petrochilo, E., and Slonimski, P. P. (1969), *Symp. Soc. Exp. Biol. 24*, 499.

Cohen, L. H., Hollenberg, C. P., and Borst, P. (1970), *Biochem. Biophys. Acta 224*, 610.

Cohen, S. N., Maitra, U., and Hurwitz, J. (1967), *J. Mol. Biol. 26*, 19.

Frederick, E. W., Maitra, U., and Hurwitz, J. (1969), *J. Biol. Chem. 244*, 413.

Fukuhara, H. (1970), *Mol. Gen. Genet. 107*, 58.

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

Gillespie, D., and Spiegelman, S. (1965), *J. Mol. Biol. 12*, 829.

Goldring, E. S., Grossman, L. I., Krupnick, D., Cryer, D. R., and Marmur, J. (1970), *J. Mol. Biol. 52*, 323.

Hollenberg, C. P., Borst, P., and Van Bruggen, E. F. J. (1970), *Biochim. Biophys. Acta 209*, 1.

Küntzel, H. (1969), *Nature (London) 222*, 142.

Mehrotra, B. D., and Mahler, H. R. (1968), *Arch. Biochem. Biophys. 128*, 685.

Michaelis, G., Douglass, S., Tsai, M., and Criddle, R. S. (1971), *Biochem. Genet. 5*.

Michaelis, G., Saedler, H., Venkov, P., and Starlinger, P. (1969), *Mol. Gen. Genet. 104*, 371.
Mounolou, J. C., Jakob, H., and Slonimski, P. P. (1966), *Biochem. Biophys. Res. Commun. 24*, 218.
Nagley, P., and Linnane, A. W. (1970), *Biochem. Biophys. Res. Commun. 39*, 989.
Schäfer, K. P., Bugge, G., Grandi, M., and Küntzel, H. (1971), *Eur. J. Biochem. 21*, 478.
Tabak, H. F., and Borst, P. (1970), *Biochim. Biophys. Acta 217*, 356.
Tsai, M., Michaelis, G., and Criddle, R. S. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 473.
Wintersberger, E., and Viehauser, G. (1968), *Nature (London) 220*, 699.

Synthesis and Turnover of *Euglena gracilis* Nuclear and Chloroplast Deoxyribonucleic Acid†

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ABSTRACT: The mode of replication of DNA in the chloroplast and in the nucleus of *Euglena gracilis* was investigated by use of a density transfer experiment. The rate of turnover of these DNAs was simultaneously determined by distribution of the density species of nuclear and chloroplast DNA and by uniformly labeling the DNA with [^{32}P]P_i and [^{3}H]adenine in the presence of the heavy-density label and observing the $^{3}H:^{32}P$ ratio following removal of the [^{3}H]adenine and the heavy-density label. Examination of the density profiles for DNA extracted from whole cells and from isolated chloroplasts showed that the nuclear and chloroplast DNA replicate by a semiconservative mechanism. While the nuclear DNA doubles once per cell generation in an exponential culture, chloroplast DNA replication is approximately 1.5 times as fast. Analysis of the $^{3}H:^{32}P$ ratios for nuclear DNA during this density-transfer experiment showed that this DNA was stable. For example, the specific activities of the ^{15}N strands of the heavy and hybrid DNA were constant prior to the density transfer and following 1.1 and 2.2 cell doublings after the density transfer. Measurement of similar ratios for chloroplast DNA showed decreasing values relative to nuclear DNA during depletion of ^{3}H pools and after the density transfer, indicative of a half-life of approximately 1.6 generations for this DNA. Since the chloroplast DNA:nuclear DNA ratio is constant in a culture in logarithmic growth, the replication patterns for chloroplast DNA and for nuclear DNA are consistent with the turnover data.

Deoxyribonucleic acid has been clearly demonstrated in chloroplasts and in mitochondria of various organisms. Density-transfer experiments by Chiang and Sueoka (1967) demonstrate semiconservative replication of the chloroplast DNA in *Chlamydomonas reinhardi*. Similar experiments for mitochondrial DNA by Reich and Luck (1966) in *Neurospora crassa* and by Gross and Rabinowitz (1969) in rat liver are interpreted to show semiconservative replication. Temporal separation of the synthesis of nuclear and extranuclear DNA has been observed in synchronously dividing cultures of *Euglena* (Cook, 1966), *Chlamydomonas* (Chiang and Sueoka, 1967), human liver cells (Koch and Stokstad, 1967), *Tetrahymena* (Parsons, 1965), *Physarum polycephalum* (Guttes *et al.*, 1967), and yeast cells (Smith *et al.*, 1968).

The synthesis and breakdown of extranuclear DNA has been studied under various conditions. In regenerating rat liver (Nass, 1967) and during oxygen adaptation in yeast (Rabinowitz *et al.*, 1969), the ratio of mitochondrial to nuclear DNA is greatly increased. In maturing tobacco seedlings (Green and Gordon, 1966) and in normal adult rat liver (Neubert *et al.*, 1968; Schneider and Kuff, 1965; Nass, 1967; Gross *et al.*, 1969), the rate of labeling of extranuclear DNA has been shown to greatly exceed that of nuclear DNA. To maintain a constant ratio between the two classes of DNA, the rapid synthesis of new extranuclear DNA must be balanced by loss of this type of DNA. Such loss has been observed in wheat cells (Hotta *et al.*, 1965) and in various rat tissues (Gross *et al.*, 1969; Neubert *et al.*, 1968). This apparent DNA turnover could be due to mitochondria having a shorter lifetime than the cells within which they exist as "symbionts" (Borst and Kroon, 1969); however, loss and resynthesis of DNA within a persisting mitochondrion has not been excluded.

This paper reports an investigation of the replication and turnover of nuclear and chloroplast DNA in *Euglena gracilis*. Our results indicate that both the nuclear and chloroplast DNA in *E. gracilis* replicate by a semiconservative mechanism. The nuclear DNA is stable while the chloroplast DNA shows turnover equivalent to a half-life of about two cell doublings under our growth conditions.

Materials and Methods

Culture Conditions. Euglena gracilis Klebs, strain Z, was maintained in pure culture at 25° by modification of the autotrophic medium described by Eisenstadt and Brawerman (1967). The KH_2PO_4 and NH_4Cl concentrations (either [^{15}N]-

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