*INFORMATIONAL ROLE OF MITOCHONDRIAL DNA STUDIED BY HYBRIDIZATION WITH DIFFERENT CLASSES OF RNA IN YEAST**

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The existence of mitochondrial DNA in yeast has been described by several groups of workers.[1-8] The informational role of this DNA in the biogenesis of the yeast respiratory system was strongly suggested by the work of Mounolou, Jakob, and Slonimski,[6] who demonstrated that respiratory-deficient mutants, lacking a cytoplasmic determinant $\rho+$, contain mitochondrial DNA with a modified buoyant density. An obvious way of studying specific functions of the mitochondrial DNA is to analyze its transcription products by DNA-RNA hybridization.

Two types of questions can be asked: (*a*) to what extent do transcription products vary with changes in physiological state in the presence of a constant mitochondrial genome, i.e., a comparison of O_2-induced cells synthesizing a full complement of respiratory enzymes with O_2-noninduced cells,[9-12] and (*b*) to what extent do transcription products differ because of mitochondrial mutations in the presence of constant physiological conditions, i.e., a comparison of various cytoplasmic mutants.

In this paper, we have tried to answer the first question by comparing various classes of RNA for their hybridizability with mitochondrial DNA (mit-DNA) and nuclear DNA (nuc-DNA). The technique used was the nitrocellulose membrane procedure described by Gillespie and Spiegelman.[13] The terms mitochondrial and nuclear DNA have been used operationally to designate the two DNA components described by Mounolou *et al.*[6] for the R_1 strains. (Density: nuc-DNA, 1.701 $\pm$ 0.002 gm/cm^3; mit-DNA, 1.687 $\pm$ 0.002 gm/cm^3.)

Materials and Methods.—Strain of yeast: *Saccharomyces cerevisiae*, D 243-2B-R_1, is haploid *grande* ($\rho+$, ad_1), producing a relatively high proportion of mit-DNA.[6]

Preparation of H^3-labeled mit-DNA and nuc-DNA: Stationary phase cells, grown in a glucose (5%) yeast extract (1%) medium containing H^3-adenine (400 μc/liter), were disrupted in a Braun shaker with glass beads, and the total DNA was extracted by a modification of Marmur's method[14]; the purification procedure included deproteinization by shaking in chloroform isoamylalcohol, RNase/pronase treatments, methylated albumin kieselguhr chromatography, and CsCl density equilibrium centrifugation. Final separation of mit-DNA and nuc-DNA was achieved by Cs_2SO_4 density equilibrium centrifugation in the presence[5, 15] of titrated amounts of Hg^{++} (ratio Hg^{++}/DNA-P = 0.2). Mutual contamination of mitochondrial and nuclear components in the final DNA preparations was less than 2% as judged by isopycnic CsCl centrifugation in the analytical ultracentrifuge. Alkali-labile material in the preparations was less than 4%. The buoyant densities in CsCl were 1.685 gm/cm^3 for mit-DNA, and 1.701 for nuc-DNA, taking as reference *Micrococcus lysodeikticus* DNA, 1.731 gm/cm^3. The purified DNA's were denatured in 0.2 *N* NaOH, and, after neutralization, immobilized on nitrocellulose filters (Membranfilter MF 50, 24-mm disk), in the presence of 6.6 $\times$ SSC.[13] Usually, about 95% of DNA was retained on the filter with both mit-DNA and nuc-DNA. The amount of DNA on the filter was determined by its H^3 content.

Preparation of P^{32}-labeled RNA: Purification of P^{32}-labeled yeast RNA for use in hybridization experiments was complicated by the fact that RNase-resistant radioactive material heavily contaminated the RNA preparations. The difficulty was overcome by discarding low-molecular-weight RNA, including 4*S*-RNA, as described below.

(a) *Steady-state labeled RNA:* Cells were grown aerobically or anaerobically[12] in a yeast extract glucose medium containing $P^{32}O_4$, 20–30 mc/500 ml. Exponential phase cells were poured on crushed ice, washed with cold water, and broken with a Braun shaker in 0.02 *M* Tris (hydroxymethyl) aminomethane, pH 7.4, and 0.002 *M* $MgSO_4$, containing bentonite, 500 μg/ml. Total RNA was extracted by overnight agitation in phenol-Duponol mixture.[11] The RNA was precipitated with ethanol, treated with pancreatic DNase (10 μg/ml, 60 min), and centrifuged in a 4–20% sucrose density gradient.[12] The 8 to 35*S* region, including the ribosomal RNA peaks, was collected. The lighter and heavier regions were excluded because of a heavy contamination by non-RNA radioactive material which could not be eliminated in the later purification steps. The pooled RNA was adsorbed on a methylated albumin kieselguhr column,[16] washed with 0.6 *M* NaCl–0.05 *M* phosphate buffer, pH 7.0, and eluted with 1.2 *M* NaCl, 0.05 *M* phosphate, pH 7.0. At this stage an aliquot of RNA was incubated with a MF 50 filter in 2 × SSC (standard saline-citrate) at 60°C overnight; if large amounts of radioactive material were adsorbed on the filter, they could be eliminated efficiently by a second sucrose gradient centrifugation. The purities of the final preparations used in hybridization experiments were as follows: pancreatic RNase-resistant counts, 1–2%; pancreatic DNase-resistant counts, 100%; alkali-resistant counts, *ca.* 4%; counts adsorbed on a MF 50 filter, 0.003–0.006% (100 μg of P^{32}-RNA was incubated with a filter at 45°C overnight in 2 × SSC, then filter was treated with RNase).

(b) *Metabolically stable P^{32}-RNA (chased with P^{31}):* Cells were grown aerobically in the presence of $P^{32}O_4$ as described above. At a cell density of about $^1/_4$ of the maximum growth, fresh medium containing $P^{31}O_4$ was added; the culture was continued for 3 hr (1.5–2 generations). Cells were harvested and fractionated according to Fukuhara.[12] A subcellular fraction sedimenting between 3,000 × *g* and 24,000 × *g*, and a 24,000 × *g* supernatant fraction were obtained. From the 24,000 × *g* pellet, "membrane-bound RNA" was extracted by phenol-Duponol treatment[11]; from the supernatant, "cytoplasmic ribosomal RNA" was extracted in the same way. The purification procedure was identical to that described in (*a*); low-molecular-weight and 4*S*-RNA were again removed from the preparations. The purity of the *metabolically stable P^{32}-RNA* was always better than that of the *steady-state labeled P^{32}-RNA* preparations.

Hybridization: MF 50 filters charged with 2 to 20 μg of denatured H^3-DNA were incubated with various concentrations of P^{32}-RNA in 5 ml of 2 × SSC. Incubations were carried out in 20-ml vials for 20 hr without agitation, usually at 45°C. In some experiments, two or three filters charged with different DNA's were incubated together in the same vial. After incubation, the filters were washed extensively with 2 × SSC, treated with pancreatic RNase (10 μg/ml, 1 hr), and again washed. The dried filters were assayed for H^3 and P^{32} counts in a liquid scintillation counter.

H^3-adenine (6.8 c/mμmole) and P^{32}-phosphate (carrier-free) are the products of the Commissariat à l'Energie Atomique, Saclay, France.

Results.—When yeast cells are grown in the presence of radioactive phosphate for a sufficient number of generations, all RNA species acquire an equal specific activity. Such an RNA population is called *steady-state labeled RNA*. When cells, homogeneously labeled with P^{32}, are then grown in the presence of nonradioactive phosphate for at least one generation, that RNA which has a high turnover rate loses it radioactivity, and only metabolically stable RNA species retain their radioactive label. RNA extracted from these cells is called *metabolically stable RNA*.

The steady-state labeled RNA's extracted from aerobically grown and from anaerobically grown cells were compared for their capacity to hybridize with mit-DNA and nuc-DNA. When increasing concentrations of RNA were annealed with a fixed amount of mit-DNA or nuc-DNA, the hybrid RNA counts on the filters increased with a progressively decreasing rate without reaching definite saturation. Our interpretation is that there existed several RNA species, each in low concentration. Although the kinetics of hybridization as a function of RNA concentration may have varied somewhat in different preparations, this variability did not obscure a sys-

TABLE 1

HYBRIDIZATION OF STEADY-STATE LABELED RNA WITH MIT-DNA AND NUC-DNA: COMPARISON OF O_2-INDUCED AND NONINDUCED CELLS

Expt. no.	H^3-DNA on filter (μg)	P^{32}-RNA in solution (μg/ml)		Condition	Hybrid (%)	Ratio induced/ noninduced
1	Mit-DNA 5.5	Induced	27	55°C 20 hr	2.53	2.0
		Noninduced	26		1.25	
	Nuc-DNA 5.5	Induced	27		2.36	1.0
		Noninduced	26		2.36	
2	Mit-DNA 5.5	Induced	13.4	50°C 20 hr	2.00	2.1
		Noninduced	13.8		0.93	
	Nuc-DNA 5.5	Induced	13.4		2.07	0.7
		Noninduced	13.8		2.93	
3	Mit-DNA 4.5	Induced	88	45°C 20 hr	3.42	2.1
		Noninduced	93		1.67	
	Total DNA (1) 15.0	Induced	88		1.94	1.2
		Noninduced	93		1.61	
	Total DNA (2) 10.0	Induced	88		3.73	1.0
		Noninduced	93		3.68	

All experiments were carried out at three to six increasing concentrations of P^{32}-RNA. Only the results at the highest concentration of RNA are presented, as no definite saturation plateau was observed for either mit-DNA or nuc-DNA. P^{32}-RNA used were steady-state preparations labeled as described in *Materials and Methods*. In Expts. 1 and 2, the same preparation of mit-DNA was used. Otherwise, all RNA and DNA were independent preparations. The specific radioactivity of H^3-DNA was 200–800 cpm/μg, and that of P^{32}-RNA was 20,000–80,000 cpm/μg. The P^{32} noise on blank filters was less than 0.05% "hybrid" level. Per cent hybrid = (μg P^{32}-RNA found on filter)/(μg H^3-DNA immobilized on filter) × 100.

tematic difference that was observed between steady-state labeled RNA from aerobic cells versus anaerobic cells (Table 1). Only the experimental points at the highest RNA concentration from each hybridization curve are shown. It is clear that the RNA extracted from aerobic cells hybridized with mit-DNA about twice as well as RNA extracted from anaerobic cells, at comparable hybridization conditions. This result suggests that aerobic cells contain a higher concentration of RNA which hybridizes with mit-DNA than anaerobic cells. It may be argued that the difference of kinetics is due to some preparative artifacts such as partial fragmentation of RNA molecules, which may change the rate of RNA-DNA interaction. This seems improbable because no significant difference was observed in the hybridization of aerobic and anaerobic RNA with nuc-DNA.

The results shown in Table 1 were confirmed by competition experiments which are presented in Figure 1. Steady-state labeled RNA extracted from aerobic cells was hybridized with mit-DNA in the presence of increasing concentrations of nonradioactive RNA extracted from either aerobic cells or anaerobic cells. The amount of P^{32}-RNA hybridized with mit-DNA decreased proportionally with the added amount of unlabeled RNA extracted from aerobic cells. However, competition by RNA extracted from anaerobically grown cells was incomplete. This difference was not observed in hybridization with nuc-DNA.

In order to characterize the RNA which hybridizes with mit-DNA, we prepared metabolically stable RNA from two distinct subcellular fractions,[12] one rapidly sedimenting, membrane-rich fraction, and the other mostly free 80*S* ribosomes (25,000 × *g* supernatant). The RNA preparations obtained from aerobic cells were hybridized with either mit-DNA or nuc-DNA. The "membrane-bound RNA" and the "cytoplasmic ribosomal RNA" showed a striking difference as to their hybridizability with mit-DNA (Fig. 2). Only the membrane-bound RNA showed appreciable hybridization with mit-DNA, while the cycloplasmic ribosomal RNA hybridized poorly. With nuc-DNA, in contrast, we found a saturation plateau, which was about the same for both membrane-bound RNA and cyto-

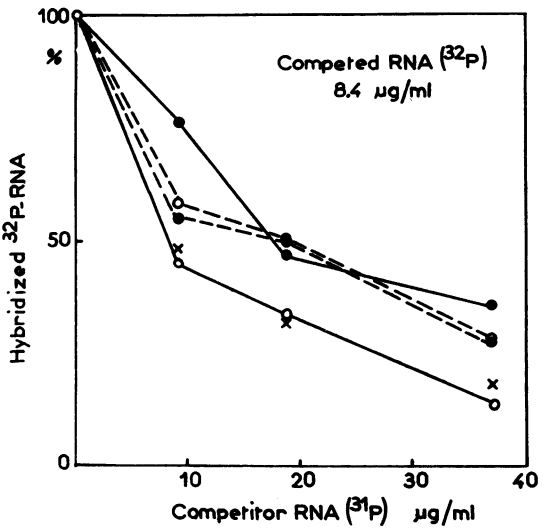

FIG. 1.—Hybridization of steady-state labeled P^{32}-RNA extracted from aerobic cells: competition with nonradioactive RNA extracted from aerobic and anaerobic cells.

Each system contained in a final volume of 5 ml, 2 × SSC: a mit-DNA filter (4.4 µg DNA), a nuc-DNA filter (4.1 µg DNA), a blank filter, steady-state labeled P^{32}-RNA extracted from aerobic cells (8.4 µg/ml, 11,200 cpm/µg), and increasing amounts of nonradioactive total RNA extracted from either aerobic or anaerobic cells.

O———O: Mit-DNA + P^{32}-RNA, competition by aerobic P^{31}-RNA; ●———●: mit-DNA + P^{32}-RNA, competition by anaerobic P^{31}-RNA; O-----O: nuc-DNA + P^{32}-RNA, competition by aerobic P^{31}-RNA; ●-----●: nuc-DNA + P^{32}-RNA, competition by anaerobic P^{31}-RNA. ×, Theoretical values for complete competition.

plasmic ribosomal RNA. This may mean that the membrane-rich fraction contains a large amount of an RNA species identical to cytoplasmic ribosomal RNA.

The low and somewhat variable hybridization between mit-DNA and cytoplasmic ribosomal RNA could be minimized when the RNA was prepared by phenol extraction *without* detergents. This suggests that the cytoplasmic ribosomal RNA extracted with phenol and detergent may contain a small amount of membrane-bound RNA due to contamination of the 25,000 × *g* supernatant fraction with membrane fragments released during cell fractionation.

Cytoplasmic ribosomal RNA, carefully prepared by extraction by phenol without detergent, was hybridized with mit-DNA and nuc-DNA (Fig. 3). At various concentrations of RNA, a mit-RNA filter, a nuc-DNA filter, and a blank filter were incubated together in the same bottle in order to assure identical conditions of incubation for each DNA. The saturation plateau value with nuc-DNA was found to be approximately 2.5 per cent (eight independent experiments gave values ranging between 2.0 and 3.0%), reproducing the result of Figure 2. The mit-DNA showed a hybridization lower than 0.1 per cent, a value not significantly different from that obtained with *E. coli* DNA included as reference.

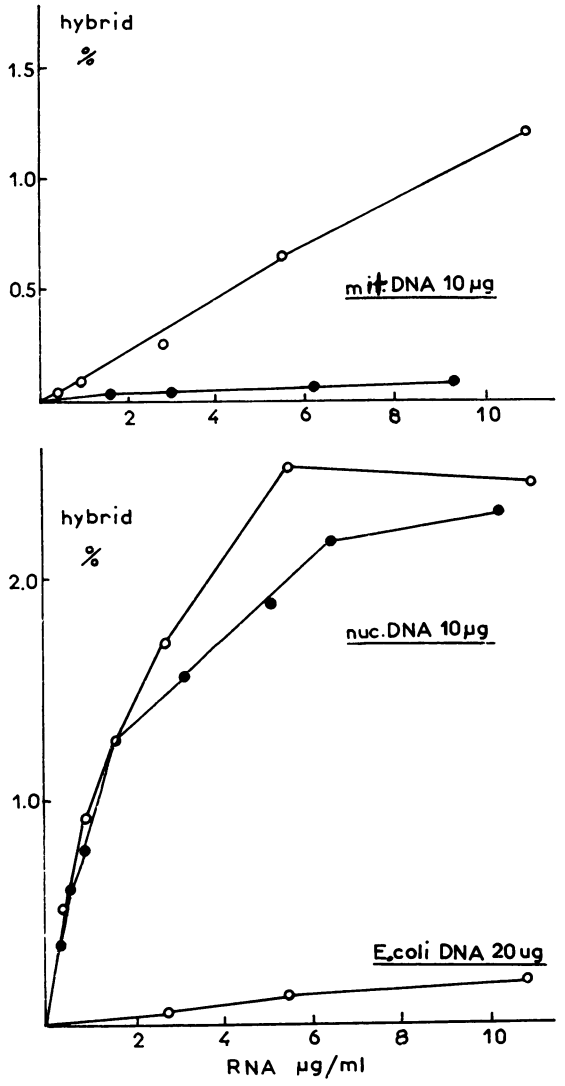

FIG. 2.—Hybridization of metabolically stable RNA with mit-DNA and nuc-DNA. Cells grown aerobically in the presence of $P^{32}O_4$ were chased with $P^{31}O_4$ for two generations, and "membrane-bound RNA" and "cytoplasmic ribosomal RNA" were extracted as described in *Materials and Methods*. The specific radioactivities were 7500 cpm/µg for membrane-bound RNA and 7700 cpm/µg for cytoplasmic ribosomal RNA. Ten µg of H^3-DNA were fixed on each nitrocellulose filter. Hybridization was carried out at 45°C for 20 hr. One per cent hybridization corresponded to about 760 cpm. The noise measured on blank filters, at the highest concentrations of RNA added, was about 40 cpm for membrane-bound RNA and about 20 cpm for cytoplasmic ribosomal RNA. The scintillation noise was 10 cpm. *Upper figure:* hybridization with mit-DNA; *lower figure:* hybridization with nuc-DNA.
O—O: Membrane-bound RNA; ●—●: cytoplasmic ribosomal RNA. "% Hybrid" is the per cent of total DNA that is saturated with RNA.

In Figure 4, we examined, by sucrose gradient centrifugation, the sedimentation of the metabolically stable RNA prepared from the membrane-rich fraction of aerobic cells. The sedimentation profile, determined by the distribution of radioactivity and by ultraviolet absorption, revealed only the presence of the two known species of ribosomal RNA; however, when each fraction of the gradient was hybridized with a fixed amount of mit-DNA, the maximal hybridization was not found in the ribosomal RNA region but in a region of less rapid sedimentation, the peak being approximately at the 13*S* region. This experiment indicated clearly that metabolically stable RNA which hybridized with mit-DNA contained a molecular species distinct from cytoplasmic ribosomal RNA, and its concentration was low compared to total RNA.

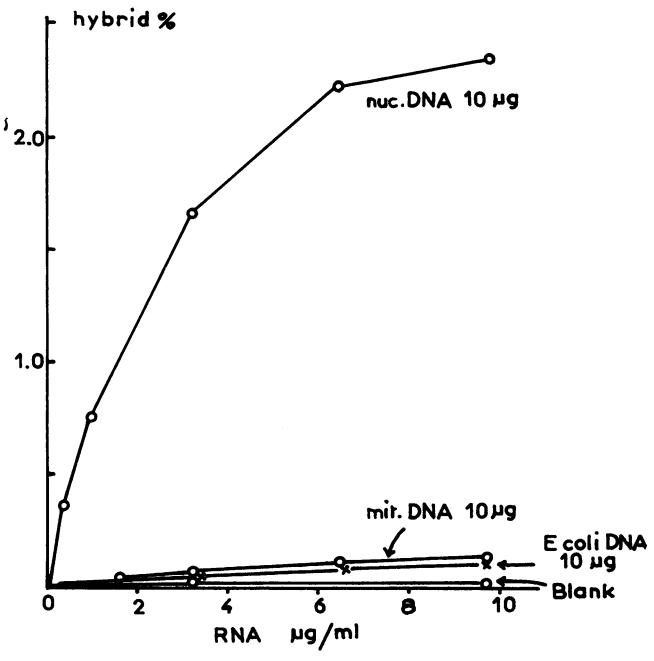

FIG. 3.—Hybridization of cytoplasmic ribosomal RNA with mit-DNA and nuc-DNA. The preparations of RNA and DNA were as in the experiment of Fig. 2, except that the P^{32}-RNA labeled cytoplasmic ribosomal RNA was extracted without Duponol. At each concentration of P^{32}-RNA added, a filter charged with nuc-DNA and a blank filter were incubated in the same vial. Other conditions were as in Fig. 2.

Discussion and Conclusion.—(1) Both aerobically growing cells and anaerobically growing cells contain an RNA species that hybridizes with mit-DNA. The total amount of this RNA is significantly greater in aerobic cells than in anaerobic cells. No such difference is observed with nuc-DNA. It is inferred from these data that mit-DNA codes for special classes of RNA and that preferential transcription of mit-DNA occurs during respiratory adaptation. In this regard, it should be mentioned that the R_1 strain we used contains, when cultured in strict anaerobiosis, an appreciable amount of satellite DNA having a very similar buoyant density to that in aerobic cells.

(2) RNA that hybridizes with mit-DNA contains a metabolically stable RNA. It is distinct from cytoplasmic ribosomal or transfer RNA, as judged by its sedimentation velocity. The RNA is found almost exclusively bound to membrane-rich subcellular fragments, and is extracted by detergents. The subcellular fragments, if prepared from aerobic cells, contain cytochrome-oxidase activity and show intense cytochrome spectra. These findings suggest the possible existence of mitochondrial RNA which is transcribed from mit-DNA, and which may be located originally on a ribosome-like particle.

(3) Mit-DNA appears not to contain segments coding for cytoplasmic ribosomal RNA if one assumes the molecular weight of yeast mit-DNA to be 10 to 20 million daltons[8] (cf. refs. 17, 18, and 19 for animal mitochondrial DNA). A single set of

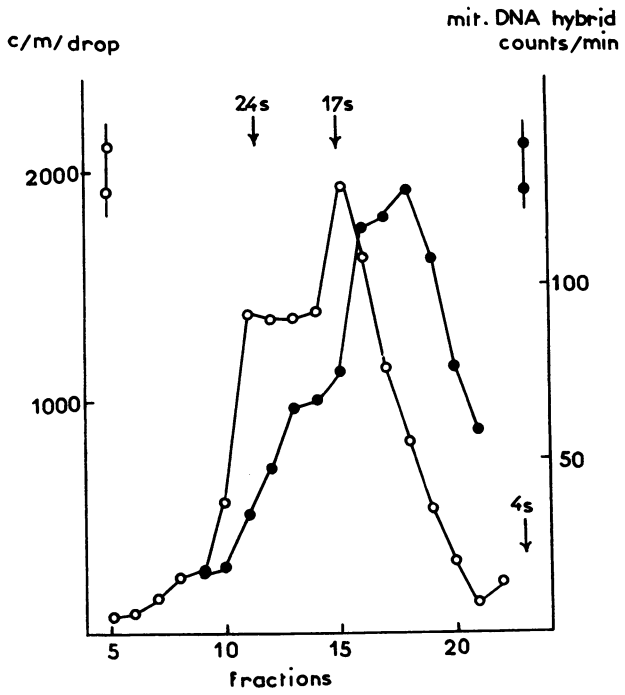

FIG. 4.—Hybridization of fractions from sucrose gradient centrifugation of metabolically stable RNA. Metabolically stable RNA was prepared from the membrane fraction of aerobically growing cells, as in Fig. 2. The purified P^{32}-RNA (194 μg, 20,000 cpm/μg) was centrifuged in a 4–20% sucrose gradient (0.1 *M* NaCl) at 23,000 rpm for 16 hr in a Spinco L SW25 head. After centrifugation, the RNA was collected, by puncture of the bottom of the tube, in 1-ml fractions. The RNA sedimentation profile was determined on one-drop aliquots of each fraction. The remainder was diluted to 2 ml with 2 × SSC and incubated at 45°C for 20 hr with 2 μg of immobilized mit-DNA; a blank filter was included in each incubation vial; less than 10 cpm was fixed on the blank filters.

O—O: Total radioactivity; ●—●: radioactivity found on mit-DNA after incubation.

ribosomal RNA cistrons would represent a saturation plateau of at least 20 per cent. We found at most 0.1 per cent, a low value which is similar to that found in the heterologous cross between *E. coli* DNA and yeast RNA. Nuc-DNA contains segments complementary to ribosomal RNA, representing about 2.5 ± 0.5 per cent of the total nuclear genome. The high proportion of ribosomal RNA cistrons in yeast nuclear genome may explain why this organism has a high content of ribosomal RNA per unit quantity of DNA.[10]

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