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EVIDENCE OF INVOLVEMENT OF MITOCHONDRIAL POLYSOMES AND MESSENGER RNA IN SYNTHESIS OF ORGANELLE PROTEINS

Carole S. Cooper* and Charlotte J. Avers

*Department of Biological Sciences, Douglass Campus -
Rutgers University, New Brunswick, New Jersey 08903,
U.S.A.*

In the last few years much attention has been focussed on exploring the components and capacity of the mitochondrial genome (1). The mitochondrion may also differentiate, a phenomenon most clearly observed in organisms such as yeast, which undergo respiratory adaptation. The role of the mitochondrial genome in these developmental changes is complicated to analyze because the mitochondrion relies heavily on the nucleocytoplasmic system for much of its structural and enzymatic components, and perhaps for its regulation as well. We therefore developed an experimental design to study the contribution of the mitochondrial system to respiratory adaptation without inhibiting the nucleocytoplasmic compartment and, conceivably, altering the natural sequence of events.

Using intact mitochondria isolated from derepressing spheroplasts of yeast (2), we have been able to demonstrate that protein synthesis occurs on mitopolysomes and that changes in polysome activity may accompany respiratory adaptation. The mitopolysomes are programmed, at least in part by mRNA synthesized from mtDNA. The mitochondrial mRNA molecules contain tracts of poly-A, which marks an essential difference between mitochondrial and prokaryotic protein synthesis (3).

MASTER

*Present address: Biology Department, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, U.S.A.

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MATERIALS AND METHODS

Cell growth conditions and isolation of mitochondria. Experimental details were as reported previously (4). Cells of *Saccharomyces cerevisiae*, strain iso-N, were grown in medium containing 10 % glucose and harvested for conversion to spheroplasts in 10 min using glucanase (Endo Labs). The spheroplasts then were allowed to derepress for 90-120 min in medium containing 2 % ethanol; then chilled, lysed, and nuclei sedimented at $3500 \times g$ for 10 min. Mitochondria were washed three times in TMK buffer containing 0.5 M sorbitol, followed each time by sedimentation at $9000 \times g$ for 15 min. Isolations were carried out at 0-5° C under sterile conditions.

Labeling of mitochondria and isolation of mitopolysomes. Mitochondria were suspended, at a concentration of 1 mg protein/ml, in 0.05 M Bicine, pH 7.4; 0.1 M KCl; 0.01 M $MgCl_2$; 0.01 M succinate; 0.005 M phosphoenol pyruvate; 0.04 M spermidine; 0.005 M proline; 0.002 M ATP; 0.01 M KH_2PO_4 ; 0.5 M sorbitol; and 2 enzyme units/ml pyruvate kinase. The suspension was incubated, with shaking, at 30° C. The reaction was terminated with 10 % trichloroacetic acid when total mitochondrial incorporation was examined, or by chilling to 0-5° C when polysomes were to be isolated. In the latter case the mitochondria were recovered by centrifugation at $9000 \times g$, then suspended and lysed in TMK containing 2 % Triton X-100. Membrane fragments were removed by sedimentation at $27,000 \times g$ for 20 min, and the supernatant was layered onto gradients of 15-40 % (w/v) ribonuclease-free sucrose (Mann Research Corp.) in TMK. For experiments analyzing the rRNA constituents, the polysome region of the gradient was pooled and polysomes sedimented in the Spinco Type 65 rotor for 1.5 h at 60,000 rpm and RNA was extracted from the pellets by dissolving in 0.01 M Tris, pH 7.5; 0.01 M NaCl; 0.001 M EDTA; 1 % SDS. For studying [3H] labeled polysomal RNA the polysome fractions of the gradient were pooled without further sedimentation and RNA was extracted by the hot phenol-SDS method (5).

Fig. 1. Case sedimentation of purified ribosomes from mitochondria and cytoplasm. 2.7 A_{260} units of unlabeled mitochondrial ribosomes (obtained from centrifugation of mitochondrial lysate at $210,000 \times g$ for 1 h) were layered onto a sucrose density gradient along with 0.02 A_{260} units of radioactive cytoplasmic ribosomes, labeled with [3H]uracil to contain 2000 cpm. The gradient (4.6 ml, linear 15-30 % sucrose in 0.02 M Tris, pH 7.4; 0.005 M $MgSO_4$) was centrifuged in the Spinco SW50 rotor for 90 min, 46,000 rpm, 0-5°. Radioactivity:; A_{260} : —.

Preparation of cytoplasmic ribosomes. Cytoribosomes were obtained from strain iso-N grown in medium containing 10 % glucose, as previously reported (4).

Binding polysomal RNA to poly-U sepharose. A 5 x 0.6 cm column was prepared of sepharose covalently linked to poly-U, according to the method of Firtel *et al.* (6). Polysomal RNA was layered on the column in "binding buffer" (0.01 M Tris, pH 7.4; 0.12 M NaCl; 0.5 % SDS) and elution of the column was continued in this buffer at 18° C until fractions had no measurable radioactivity or absorbance at 260 nm. Then the column was eluted with 12 ml of "eluting buffer" (0.01 M Tris, pH 7.4; 0.5 % SDS) at 18° C. The temperature finally was raised to 45° C and the column eluted with 50 % elution buffer plus 50 % formamide. RNA was precipitated from fractions with 2.5 volumes of ethanol.

RESULTS

Mitoribosomes can be isolated free of cytoribosome contamination. The success of the experimental design greatly on recovering mitochondria free of contamination by nuclei or cytoribosomes. Various independent lines of evidence showed the purity of our isolated mitochondrial preparations, as follows: (a) nuclei were not detected in electron micrographs; (b) RNA synthesis was sensitive to rifampicin, 250 µg/ml; (c) mitoribosome monomers sedimented more slowly than cytoribosomes, at an estimated sedimentation coefficient of 75S when compared with 80S yeast cytoribosomes and 70S *E. coli* ribosomes (Fig. 1); (d) RNA extracted from either mitoribosomes or mitopolysomes sedimented more slowly in sucrose gradients (Fig. 2) and migrated more slowly in polyacrylamide gels (Fig. 3) than the analogous RNA from cytoribosomes, as also reported by others (7,8); and (e) erythromycin (2 mg/ml) inhibited 60 % of the amino acid incorporation, while the cytoribosome inhibitor cycloheximide (100 µg/ml) was completely ineffective in blocking amino acid incorporation (Table I).

The clarity by which their characteristics could be

Fig. 2. Sedimentation of ribosomal RNAs derived from cytoplasmic ribosomes or mitochondrial polysomes. RNA prepared from pellets of mitopolysomes or cytoribosomes was layered onto identical sucrose gradients (5-20 % sucrose in TMS), centrifuged in the Spinco SM25.1 rotor at 24,500 rpm for 16 h at 0-5°. The absorbency profiles for rRNA extracted from mitochondrial monoribosomes was identical to the polysomal RNA profile presented.

Fig. 3. Electrophoretic analysis of cytoplasmic ribosomal RNA and RNA extracted from mitopolysomes. RNA from the two sources was layered onto identical 5 cm gels of 2.4 % acrylamide, 0.12 % bis-acrylamide. Electrophoresis was accomplished at 4.5 mA/tube for 90 min at 20°.

TABLE I.

ANTIBIOTIC SENSITIVITY OF AMINO ACID INCORPORATION IN ISOLATED MITOCHONDRIA. Mitochondria were incubated for 10 min with 5 μ Ci/ml [14 C] amino acids.

Additions to medium	% inhibition relative to control
None (control)	—
+ 2 mg/ml erythromycin	59.8 %
+ 100 μ g/ml cycloheximide	0 %

resolved was another line of evidence that the mitochondria were essentially uncontaminated. The precise level of contamination was assessed by a reconstruction experiment, performed by adding an excess of extraneous, [3 H]labeled cytoribosomes to lysed spheroplasts and purifying the mitochondria from the mix using standard procedures. The mitoribosomes showed less than 1.5 % level of contamination.

The incorporation of amino acids increased linearly with time throughout the incubation intervals in all the experiments, indicating the continuing activity of the mitochondrial system. Contamination by bacteria never exceeded 100 cells per mg mitochondrial protein (based on plating aliquots of the incubation suspension on nutrient agar), far below the concentrations which would affect amino acid incorporation.

Characteristics of mitochondrial polysomes. When isolated mitochondria were incubated for a short time with [3 H]amino acids, acid-insoluble radioactivity became associated with mitochondrial polysomes (Fig. 4). To demonstrate that these products were nascent polypeptides rather than ribosomal proteins, puromycin was added to the incubation mixture for 8 min leading to the release of about 40 % of the polysome-associated radioactivity (Fig. 5). As has been noticed in other systems (9,10), this effect depended on NH_4Cl in place of KCl in the incubation medium. While most experiments yielded radioactivity profiles comparable to those in Fig. 4, we sometimes observed an enormous increase in amino acid incorporation by polysomes composed of 7 to 10 ribosomes. On other occasions amino acid incorporation occurred primarily in dimer and trimer polysomes, even though larger polysomes also appeared

Fig. 4. Incorporation of [3 H]amino acids by isolated mitochondria into peptides associated with mitochondrial ribosomes and polysomes. Mitochondria were incubated in incorporation medium with 20 μ Ci/ml [3 H]amino acids for 7 min. Polysomes were prepared as described in Methods; gradient fractions were collected and precipitated by cold 10 % trichloroacetic acid and counted in a toluene-Triton-X-100 scintillation cocktail. Radioactivity: —; A₂₆₀: —. Centrifugation was performed in the SW27 rotor for 3.5 h. at 27,000 rpm.

Fig. 5. Effect of puromycin on incorporation of [3 H]amino acids by polysomes in isolated mitochondria. Mitochondria were incubated in incorporation medium, modified by the substitution of 0.1 M NH_4Cl for KCl, with 10 μ Ci/ml [3 H]amino acids. After 5 min puromycin (0.5 mM) was added to one half of the mitochondria. Incubation of both samples was continued for an additional 8 min. Subsequent procedures are the same as detailed in the legend to Fig. 4. Radioactivity in puromycin treated sample: ····; radioactivity in untreated sample: —; A₂₆₀ of untreated sample: —.

Fig. 6. Electron microscopy of mitochondrial polysomes. The polysome region of the sucrose gradient was collected and drops of the suspension were placed onto a clean, plastic Petri dish. Nickel grids (from Fullam, 100 mesh, coated with parlodion and stabilized with a thin layer of carbon) were placed onto the drops and allowed to remain for 10 min at 0-5°. The grids were then rinsed successively in 5 changes of 0.1 M sucrose in 10% formalin and 3 changes of 0.4% Phosfolo (Kodak), then stained in 0.05 M uranyl acetate, 0.05 M HCl, freshly diluted 1:1000 with 90% ethanol. Arrows indicate presumptive mRNA strand connecting the ribosomes.

in the absorbance profile of the gradient. These variations are currently under study.

Electron microscope examination of mitopolysomes taken from sucrose gradient fractions revealed ribosomes aggregated into coiled clusters. Occasionally the ribosomes were separated slightly and a strand of material, presumably mRNA, was exposed (Fig. 6). We previously had shown that mitopolysomes treated with ribonuclease sedimented as monomers in a sucrose gradient (4). Additional evidence that an RNA molecule was responsible for the structural integrity of the mitopolysomes was provided by collecting the polysome region of a gradient and treating it with ribonuclease then examining the fraction with the electron microscope. Subsequent scoring of the polysome sizes demonstrated that this treatment had induced breakdown of most of the polysomes, bringing about a concomitant increase in the number of monomers and small polysomes (Fig. 7).

RNA synthesis in isolated mitochondria. RNA synthesis, measured by incorporation of [3 H]uracil into isolated mitochondria, was sensitive to rifampicin (Table I), an antibiotic reported to affect prokaryotic-type RNA polymerase (11). We then sought to test the activity of the mitochondrial RNA polymerase as a function of respiratory adaptation. Mitochondria obtained from derepressing spheroplasts were 21% more active in synthesizing RNA than mitochondria from spheroplasts kept in catabolite repression (Table II).

The utilization of some of the mitochondrially synthesized RNA as mRNA was indicated when rapidly-labeled RNA (synthesized in isolated mitochondria during a 5 min incubation with [3 H]uracil) was found with mitopolysomes (Fig. 8). There was no significant radioactivity in rRNA or tRNA extracted from the mitopolysomes after such a short period of labeling (data not shown). However, 95% of the radioactivity in phenol-extracted polysomal RNA bound to a column of poly-U-sepharose under high salt conditions (Table III). The radioactivity was removed by elution in low salt plus 50% formamide at 50° C. This behavior has been shown

Fig. 7. Sensitivity of polysome structure to ribonuclease. The region of a sucrose gradient containing mitopolysomes was collected and divided into two portions. One portion was left untreated and the other incubated with 20 $\mu\text{g/ml}$ pancreatic ribonuclease at $0-5^\circ$ for 20 min. Samples were then fixed by addition of 10% formalin, 0.1 M sucrose. Subsequent procedures as described in Fig. 6. The data presented in the graph were calculated by examining photographs of 1000 mitoribosomes and scoring the number of ribosomes in polysomes of different sizes.

Fig. 8. Association of rapidly-labeled RNA with mitochondrial ribosomes and poly-somes. Isolated mitochondria were incubated in incorporation medium with 20 $\mu\text{Ci/ml}$ [^3H]uracil for 5 min. Subsequent procedures as in Fig. 4. Radioactivity:—; A260:---

MITOCHONDRIAL POLYSOMES, MESSENGER RNA, AND ORGANELLE PROTEINS

TABLE II.

RNA SYNTHESIS IN ISOLATED MITOCHONDRIA.

A. Inhibition of [^3H]uracil incorporation into whole mitochondria by rifampicin. Mitochondria were incubated with 10 $\mu\text{Ci/ml}$ [^3H]uracil for 10 min.

Additions to medium	% inhibition relative to control
None (control)	—
+ 250 $\mu\text{g/ml}$ rifampicin	28%

B. Comparison of [^3H]uracil incorporation by isolated mitochondria obtained from derepressed or repressed spheroplasts. Mitochondria were incubated with 10 $\mu\text{Ci/ml}$ [^3H]uracil for 5 min.

Respiratory state of spheroplasts from which mitochondria were isolated	c.p.m. per μg mit. protein	% difference
Derepressed	8,558	—
Repressed	7,089	21%

TABLE III.

BINDING OF POLYSOMAL RNA TO POLY-U-SEPHAROSE.

[^3H]labeled RNA was extracted from mitopolysomes and put over a column of poly-U-sepharose. Table information was calculated by pooling total cpm and A260 from samples eluted under the following conditions. Details in Methods.

Eluting Conditions	% A260 eluted	cpm eluted	% cpm eluted
"Binding buffer" and "Eluting buffer" at 18°	100%	70	4%
"Eluting buffer" and 50% "eluting buffer" + 50% formamide, at 50°	---	1509	96%

by Singer *et al.* (12) and by control experiments with [^{14}C]poly-A (Kaufman, unpub. data) to be characteristic of the presence in the RNA of tracts of poly-A which form hydrogen bonds with the poly-U on the column. In recent experiments the RNA from mitopolysomes has been hybridized to [^{14}C]poly-dT, and has been demonstrated to protect the [^{14}C]label from digestion with single-strand-nuclease. These results show that the rapidly-labeled, polysomal RNA contains poly-A sequences. Moreover, since these RNA species were labeled in purified, isolated mitochondria they must originate as a transcript from mtDNA.

DISCUSSION

The experiments reported here demonstrate that protein synthesis in yeast mitochondria occurs on mitopolysomes which are directed by mRNA transcribed from mtDNA.

Mitopolysomes have been reported in yeast (4,13), *Euglena* (14), and HeLa cells (15). We have used several lines of evidence to characterize the mitopolysomes: (a) sedimentation as large units in sucrose density gradients, (b) composition of rRNA, (c) direct visualization by electron microscopy as ribosomes connected by a strand of RNA, (d) sensitivity of ribosome association to ribonuclease, (e) function in protein synthesis. The functional criterion was demonstrated by amino acid incorporation and subsequent release of nascent polypeptides from the polysomes when treated with the tRNA analogue puromycin.

Protein synthesis in the isolated mitochondria was found to be completely resistant to cycloheximide, a potent inhibitor of cytoplasmic ribosomes, while sensitive to erythromycin, an inhibitor of prokaryotic-type ribosomes. We have also shown that mitoribosomes can be purified away from radio-labeled cytoribosomes in a reconstruction experiment, and that the rRNA constituents isolated from the mitoribosomes form discrete bands in gradients or gels, with no visible "shoulder" contributed by cytoribosomes. These sets of data affirm that the components of the mitochondrial system can be isolated free from contamination by the cytoplasmic system.

Evidence that mtDNA transcribes mRNAs has previously been indirectly implied by experiments showing non-Mendelian mutations affecting mitochondrial structure or function or antibiotic sensitivity (16,17), and by inhibition of mitochondrial protein synthesis by drugs or by treatments which block RNA synthesis (18-20). Recently, Perlman *et al.* (21) labeled HeLa cells in the presence of camptothecin, an inhibitor of nuclear RNA synthesis, and found poly-A-containing RNA in the mitochondria. Our present results clearly demonstrate

that native mitochondrial mRNA is used to program protein synthesis on mitopolysomes. These RNA molecules are synthesized in isolated mitochondria, completely free of nucleocytoplasmic contamination. They fulfill several criteria expected for messenger molecules: (a) they are labeled more rapidly than rRNA or tRNA, (b) they are found in association with polysomes, and (c) they have binding and hybridization characteristics which indicate the presence of poly-A sequences. Poly-A sequences have been identified as an integral portion of all eukaryotic mRNA molecules, with the exception of histone mRNAs (22).

The experimental system we have described takes advantage of the phenomenon of facultative respiration in yeast. The increase in RNA synthesis in mitochondria obtained from yeast spheroplasts undergoing respiratory adaptation is one indication that the isolated organelle retains the capacity for differentiation induced while in the cellular environment. We also have observed a varying level of amino acid incorporation in certain sizes of mitopolysomes. Although these data are preliminary, we speculate that these changes in polysome activity represent an important event in respiratory adaptation. Critical analysis of polysomes may thus prove to be a sensitive approach to studying mitochondrial differentiation. The lack of cytoplasmic contamination in this system would also facilitate the future isolation of pure mitochondrial components, such as mitopolysomes or mitochondrial mRNA, for *in vitro* studies to identify the proteins encoded in mtDNA. In addition, this experimental system may be exploited to test the effects on mitochondria of cytoplasmic extracts taken from cells in different metabolic or respiratory states.

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Abbreviations used: mtDNA, mitochondrial DNA; mRNA, messenger RNA; rRNA, ribosomal RNA; tRNA, transfer RNA; poly-A, polyadenylic acid; poly-U, polyuridylic acid; SDS, sodium dodecyl sulfate; EDTA, ethylenediamine tetraacetic acid; TMK buffer, 0.01 M Tris, pH 7.5, 0.01 M KCl, 0.01 M MgCl₂.