*Proc. Nat. Acad. Sci. USA*
Vol. 72, No. 11, pp. 4293–4297, November 1975
Biochemistry

# Translation of RNA that contains polyadenylate from yeast mitochondria in an *Escherichia coli* ribosomal system

(cytochrome oxidase peptides/immunoprecipitation/acrylamide gel electrophoresis/hybridization/pulse labeling)

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*Communicated by Leon O. Jacobson, August 14, 1975*

**ABSTRACT** **RNA that contains poly(A) [poly(A)-RNA] has been isolated from yeast mitochondria by poly(U) Sepharose-4B column chromatography. Pulse-labeled poly(A)-RNA shows 8–10 discrete peaks by acrylamide gel electrophoresis. The specific activity of mitochondrial poly(A)-RNA is six to eight times greater than that of mitochondrial rRNA after pulse labeling of protoplasts with [$^3$H]uridine. Ethidium bromide inhibits incorporation by over 90%. The total mitochondrial RNA preparation was contaminated with 5–15% cytoplasmic rRNA as determined by gel electrophoresis, but RNA exhaustion hybridization experiments indicated little or no cytoplasmic contamination of the mitochondrial poly(A)-RNA. The poly(A)-RNA stimulates [$^3$H]leucine incorporation into protein in an *E. coli* cell-free system. A fraction of the labeled product is precipitated with antibody directed toward yeast cytochrome oxidase, but not with antibody directed toward bovine serum albumin. Sodium dodecyl sulfate gel electrophoresis of the immunoprecipitated material reveals labeled peptides having the mobility of the three larger cytochrome oxidase peptides, which are known to be translated by mitochondrial ribosomes.**

It is now well established that mitochondria are partially autonomous organelles and that mitochondrial DNA (mtDNA) codes for mitochondrial ribosomal RNA (rRNA) and transfer RNAs (tRNAs) (1, 2). Mitochondrial ribosomes synthesize several of the polypeptides that comprise the cytochrome oxidase and oligomycin-sensitive ATPase (ATP phosphohydrolase, EC 3.6.1.3) complexes (3, 4), and it is considered likely that the structural genes for these proteins are contained in mtDNA, inasmuch as transport of cytoplasmic messenger RNA (mRNA) into the mitochondria appears not to be significant (5). However, unequivocal evidence that mtDNA codes for these mitochondrially synthesized peptides is not yet available.

In mitochondria of higher eukaryotes, about 25–30% of the genome codes for mitochondrial rRNA and mitochondrial tRNA (6). The 7–8 poly(A)-RNA bands observed on gel electrophoresis (7), presumably represent mitochondrial mRNA and occupy most if not all of the remaining available sequences. The yeast mitochondrial genome is five times larger than the mammalian mitochondrial genome (1), and hence considerably more information is potentially available to code for mRNA. At the present time, however, it is not clear whether the additional sequences available in yeast mtDNA code for mRNAs, or whether they represent, at least in part, high(A+T)spacer regions (8).

Yeast offers several experimental advantages for the study of mitochondrial mRNA transcription. Mitochondrial development can be altered readily by glucose repression, oxygen deprivation, or oxygen deprivation with lipid depletion, and a large variety of petite (deletion) mutants as well as antibiotic resistance (point) mutants have been characterized (9). These mutants could be valuable in defining the mitochondrial origin of mRNAs and in the purification and mapping of mitochondrial genes that code for specific mRNAs.

Eukaryotic mRNAs can be isolated conveniently because of the presence of poly(A) segments at the 3′ terminus of most of these RNAs (10, 11). Discrete species of poly(A)-containing RNAs which hybridize to mtDNA have been detected in HeLa cell mitochondria (12, 13). These presumably are mRNAs coding for mitochondrially synthesized proteins, although no direct evidence for this is as yet available. Cooper and Avers (14) have indicated that yeast mitochondria contain poly(A)-RNA, and that the length of the poly(A) segment is similar to that of yeast cytoplasmic RNA (i.e., 40–60 nucleotides). Groot *et al.* (15), however, failed to detect mitochondrial poly(A)-RNA in yeast and attributed the earlier results to cytoplasmic contamination. We have recently reported that a fraction of yeast mitochondrial RNA definitely contains a short segment of poly(A) at the 3′ end, and that the size of this segment is approximately 20–30 nucleotides (16). The present study reports on the further characterization and messenger activity of yeast mitochondrial poly(A)-RNA. The poly(A)-RNA has been translated in an *Escherichia coli* cell-free system. The products include three peptides recognized by immunoassay as being those of components of cytochrome oxidase which were shown previously to be synthesized on mitochondrial ribosomes *in vivo* (17) and in mitochondria *in vitro* (18).

## METHODS

**Growth and Isolation of Mitochondria.** A haploid strain of *Saccharomyces cerevisiae*, D243-2B-R1 (R1), was harvested in the mid-logarithmic phase (19). Mitochondria were prepared from protoplasts according to the method of Grivell *et al.* (20), except that the cells were not preincubated before exposure to snail enzyme. The protoplasts were washed only once before disruption of the cells in a Waring blender.

**Labeling Conditions and Analysis of RNA.** Steady-state labeling of the RNA was achieved by growing the yeast with $^{32}$P (orthophosphate, carrier-free, Schwarz/Mann, Orangeburg, N.Y.) or [2-$^3$H]adenine (10–15 Ci/mM, New England Nuclear, Boston, Mass.) to a mid-exponential phase of growth. Pulse labeling was carried out with yeast protoplasts for 15 min in 1.5 M sorbitol that contained 2% galactose and 0.1% glucose. The mitochondria were lysed in NETS [100 mM NaCl, 2 mM EDTA, 10 mM Tris·HCl (pH 7.8), 0.2% sodium dodecyl sulfate] that contained 20 μg/ml of polyvinyl sulfate and 200 μg/ml of heparin. Total RNA was isolated from lysed mitochondria by the phenol/chloroform/isoamyl

* Operated by the University of Chicago for the U.S. Energy Research and Development Administration.

alcohol extraction procedure (21). Poly(A)-RNAs were isolated with poly(U) Sepharose-4B (Pharmacia, Piscataway, N.J.) by a combination of the methods of Lindberg *et al.* (22) and Firtel *et al.* (23). A 3 ml column of poly(U) Sepharose equilibrated with high salt buffer [400 mM NaCl, 2 mM EDTA, 10 mM Tris·HCl (pH 7.5), and 0.2% sodium dodecyl sulfate] was used. The mitochondrial RNA preparation was dissolved in high salt buffer, and 2 ml of the solution containing approximately 50 $A_{260nm}$ units/ml was applied to the column at 25°. After 10 min, the column was washed with 9 volumes of high salt buffer. The first 4 volumes were pooled and alcohol-precipitated (Fraction 1). The poly(A)-RNA was eluted with 3 volumes of formamide buffer [90% formamide, 2 mM EDTA, 10 mM potassium phosphate (pH 7.5)] (Fraction II). After precipitation with 2.5 volumes of ethanol, RNA was subjected to two additional cycles of poly(U) Sepharose chromatography. RNA fractions were electrophoresed on 2.7% gels with "E" buffer (24). Formaldehyde-denatured RNA samples were electrophoresed on 3% gels in the presence of formaldehyde, as described by Ojala and Attardi (13).

**RNA·DNA Hybridization.** RNA·DNA filter hybridization was carried out under conditions of considerable DNA excess (RNA exhaustion). mtDNA purified by CsCl isopyknic gradient centrifugation was denatured and bound to cellulose nitrate filters (22). Control filters were charged with *E. coli* DNA, or with nuclear DNA isolated from a petite yeast strain (IL8-8C/H71) which contained no mtDNA. Filters were incubated for 44 hr at 41° in scintillation vials containing 1 ml of 0.3 M NaCl, 0.03 M Na citrate, 8 M urea, and 0.03 μg/ml of RNA (25). The DNA:RNA ratio was 860 to 960. Filters were then washed, digested with $T_1$ and pancreatic RNase, dried, and counted in toluene scintillation fluid (19).

**Translation of Mitochondrial Poly(A)-RNA in Cell-Free Systems.** The poly(A)-containing RNA fraction from the poly(U) Sepharose column (Fraction II) was tested for its ability to direct protein synthesis in an *E. coli* cell-free system. The preparation of the S30 fraction from *E. coli* Q13 and the incubation conditions were as described by Nathans (26), except that leucovorin was included in the assay mixture. Synthesis was followed by measurement of [$^3$H]leucine incorporation into trichloroacetic acid-precipitable protein. Aliquots of the incubation mixture were reacted with antibodies to cytochrome oxidase [enzyme and antibodies prepared according to the method of Mason *et al.* (27)] for isolation of cytochrome oxidase polypeptides formed in the cell-free system. As a control, bovine serum albumin was added to an aliquot of the incubation mixture, and immunoprecipitation was carried out with antibody to bovine serum albumin. Immunoprecipitates were either counted in Triton X-100 scintillation fluid or dissociated by heating to 100° in 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol for 2 min, and analyzed on 12% acrylamide gels. Dissociated cytochrome oxidase, bovine serum albumin, D-aminoacid oxidase, ovalbumin, and cytochrome *c* were used as marker proteins.

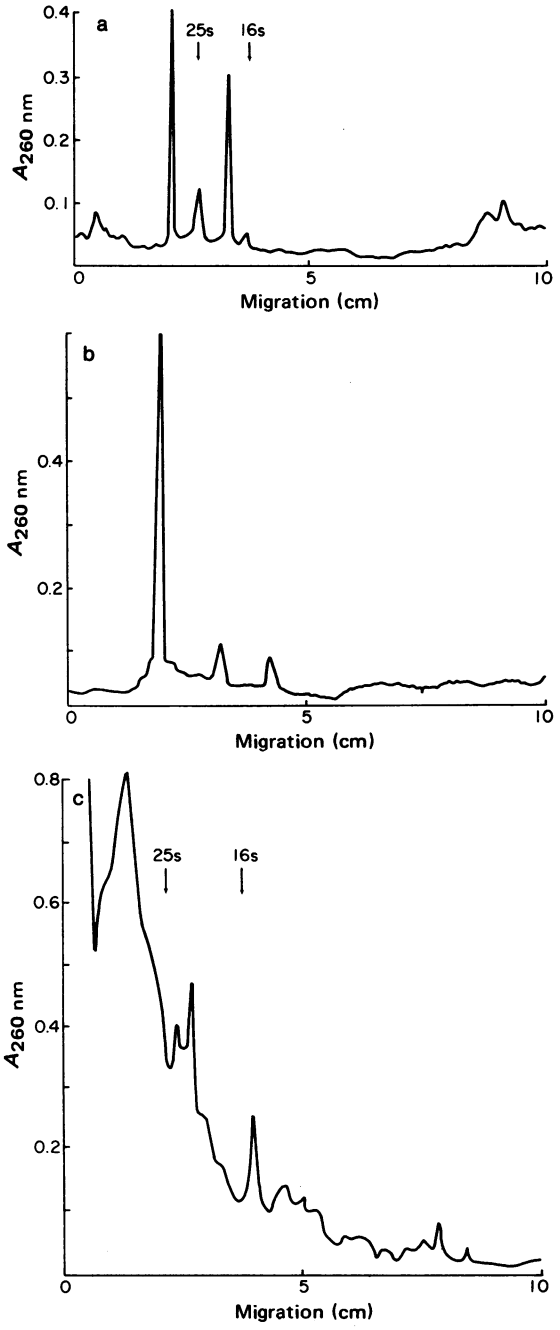


FIG. 1. Absorbance 260 nm profiles of mitochondrial RNA analyzed by acrylamide gel electrophoresis. (a) Total mitochondrial RNA; (b) mitochondrial poly(A)-RNA; and (c) formaldehyde-denatured mitochondrial poly(A)-RNA. In (a) and (b), RNA samples were electrophoresed on 2.7% gels in "E" buffer (24) at 5 mA/gel for 2.5 hr. The mitochondrial poly(A)-RNA in gel c was denatured in neutralized 3% formaldehyde [20 mM sodium phosphate (pH 7.8)] at 63° for 15 min and then run for 3 hr in the buffered formaldehyde at 5 mA/gel. Arrows denote the position of yeast cytoplasmic ribosomal RNA markers (25 S and 16 S). All gels were scanned with a Gilford gel scanner.

## RESULTS

Gel electrophoretic profiles ($A_{260nm}$) of total mitochondrial RNA and Fraction II RNA are shown in Fig. 1. The two conspicuous bands in Fig. 1a are those of mitochondrial rRNAs which characteristically have a slower mobility than the cytoplasmic rRNA species, even though they are smaller and sediment more slowly (28). There appeared to be 5–15% contamination with cytoplasmic rRNA in different mitochondrial RNA preparations. The $A_{260nm}$ profile of Fraction II (Fig. 1b) reveals three distinct peaks which can be assigned S values of 11–12, 14–15, and 23–24 on the basis of the electrophoretic mobilities of the marker yeast mitochondrial rRNAs. If the mobilities of yeast cytoplasmic or rat liver cytoplasmic rRNAs are taken as standards, the corre-

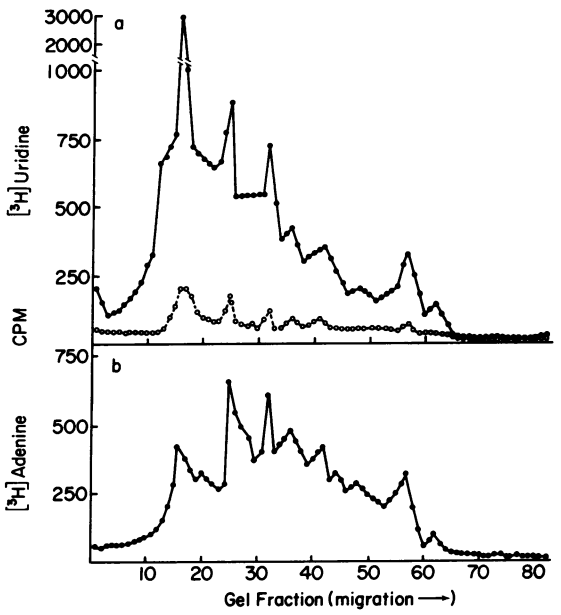


FIG. 2. Gel electrophoretic analysis of pulse-labeled mitochondrial poly(A)-RNA. (a) Protoplasts were pulse-labeled with [3H]uridine for 15 min at 28° (●– –●) or were pretreated with ethidium bromide (10 μg/ml) for 10 min before labeling (○——○). Unlabeled carrier uridine was added, and mitochondrial poly(A)-RNA was isolated as described under *Methods*. (b) Mitochondrial poly(A)-RNA was isolated from protoplasts pulse-labeled with [3H]adenine. The poly(A)-RNA was layered onto 2.7% acrylamide gels and run in "E" buffer for 2.5 hr at 25° and 5 mA/gel. Gels were sliced with a Savant Autogel Divider (Savant, Hicksville, N.Y.) and were counted in Instagel (Packard, Downers Grove, Ill.) in a Packard liquid scintillation counter.

sponding calculated S values would be 13–14, 15–16, and 32–35. This optical density pattern of Fraction II has been observed consistently, and the largest species is always present in at least three to four times the concentration of the two smaller species. To examine whether the RNA fractions seen in the gels are aggregates of smaller species, we denatured the preparation in the presence of formaldehyde. It can be seen in Fig. 1c that the three bands are again distinct, although they are broader and the largest band is possibly composed of more than one species of RNA. When compared to the migration of rRNA, the RNA of Fraction II appears to be intact and not composed of aggregates.

The radioactivity profiles of pulse-labeled RNA Fraction II analyzed by gel electrophoresis are shown in Fig. 2. [3H]Uridine pulse label is incorporated into the three major peaks seen in optical density profiles as well as into several additional discrete peaks. Ethidium bromide inhibits [3H]uridine incorporation into Fraction II by 90% (Fig. 2a). The [3H]adenine profile was closely similar to that of [3H]uridine, except that the incorporation into bands two and three was greater than that into the band with the largest optical density. About 8–10 discrete peaks of radioactive RNA material could be detected on the gels. The relative incorporation of pulse label into Fractions I and II was assessed from the ratio of the pulse label [3H]uridine or [3H]adenine to the steady-state label $^{32}P$ in the two fractions. The [3H]uridine:$^{32}P$ ratios for Fractions I and II were 0.12 and 0.72, respectively. The [3H]adenine:$^{32}P$ ratios were 0.08 and 1.00. Of the total isotope incorporation, Fraction II accounted for 25–30% of the uridine and 30–35% of the adenine label.

For assessment of the purity of the mitochondrial RNA, hybridization of the mitochondrial RNA (labeled under steady state conditions) to mtDNA and to nuclear and *E. coli* DNA was carried out (Table 1). Under the conditions of hybridization employed, more than 55% of Fraction II hybridized to mtDNA, with essentially no hybridization to either yeast nuclear or *E. coli* DNA. About 38% of Fraction I RNA hybridized with mtDNA and 12.5% with yeast nuclear DNA. Although it appears that the total RNA preparation is contaminated by cytoplasmic RNA label (see also Fig. 1a), Fraction II may contain little, if any, cytoplasmic contamination.

Table 1. Filter hybridization of mitochondrial RNA with mitochondrial, nuclear, and *E. coli* DNA under conditions of considerable DNA excess

| | Fraction I | | Fraction II | |
|---|---|---|---|---|
| DNA on filter | cpm bound | % | cpm bound | % |
| Mitochondrial | 202 | 38.2 | 250 | 55.4 |
| Nuclear | 66 | 12.5 | 3 | 0.5 |
| *E. coli* | 2 | 0.3 | 1 | 0.3 |

Filters contained 25 μg of mitochondrial, nuclear, or *E. coli* DNA. Mitochondrial RNA was labeled under steady state conditions with [3H]adenine and then fractionated on poly(U) Sepharose as described in *Methods*. Fraction I (0.029 μg, 530 cpm) or 0.026 μg of Fraction II [mitochondrial poly(A)-RNA] (451 cpm) in 1 ml of 0.3 M NaCl, 0.03 M sodium citrate, 8 M urea was hybridized with filters containing mitochondrial, nuclear, or *E. coli* DNA for 44 hr at 41° (25). The filters were processed as described in *Methods*.

As reported previously, Fraction II contained a short poly(A) segment at its 3′ terminus (16). An *E. coli* cell-free system was chosen to translate the mitochondrial poly(A)-RNA because of similarities between systems for mitochondrial and bacterial protein synthesis (3). Table 2 and Fig. 3 show that the addition of the mitochondrial poly(A)-RNA to the *E. coli* S30 system enhances [3H]leucine incorporation into protein about 4-fold over the endogenous level. Cytochrome oxidase antibody precipitated about 5% of the total trichloroacetic acid-precipitable radioactivity, whereas only 0.5% was recovered after precipitation with antibody to bovine serum albumin under identical conditions (Table 2). The endogenous proteins synthesized by the *E. coli* system

Table 2. Stimulation of [3H]leucine incorporation into an *E. coli* cell-free system by mitochondrial poly(A)-RNA

| | Radioactivity incorporated into protein precipitates (cpm) | | |
|---|---|---|---|
| Additions | Trichloroacetic acid | Cytochrome oxidase antibody | Bovine serum albumin antibody |
| None | 25,862 | 180 | 162 |
| Mitochondrial poly(A)-RNA | 109,534 | 3,950 | 158 |
| MS2 RNA | 303,275 | 195 | 190 |

Incubation was at 37° for 45 min in a total volume of 0.5 ml, consisting of the components of the cell-free system in amounts as described by Nathans (26). Additional leucovorin (1.37 μg) was also included. RNA (25 μg) was added as indicated. The preparation of cytochrome oxidase from yeast, characterization of the enzyme, antibody preparation and characterization, as well as the immune precipitation conditions, were essentially those of Mason *et al.* (27).

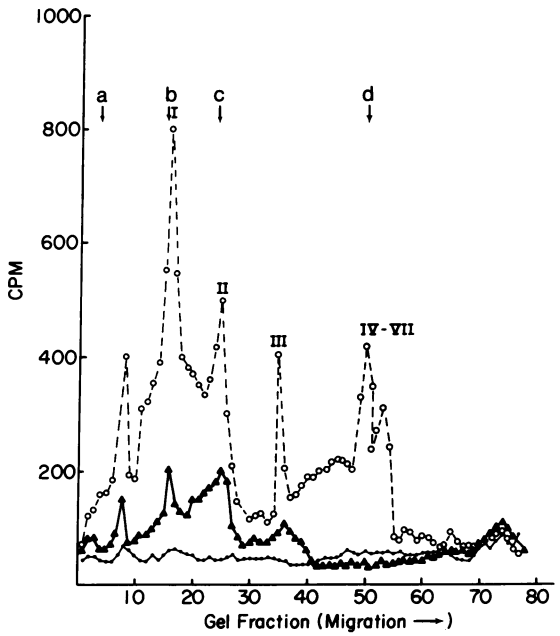


FIG. 3. Acrylamide gel electrophoretic analysis of cytochrome oxidase peptides synthesized by *E. coli* ribosomes directed by mitochondrial poly(A)-RNA. [$^3$H]peptides formed after the addition of 25 $\mu$g of mitochondrial poly(A)-RNA to an *E. coli* S30 system were precipitated with cytochrome oxidase antibody (▲——▲) or bovine serum albumin antibody (●——●) in the presence of 25 $\mu$g of the respective carrier antigen. The immune precipitates were heated to 100° in 2% sodium dodecyl sulfate for 2 min and analyzed on 12% acrylamide gels (50 mM Tris-glycine at pH 8.3, 0.03% sodium dodecyl sulfate) with a 2.5% stacking gel. Electrophoresis through the stacking gel was at 1 mA/gel ($\simeq$1 hr). Gels were then subjected to a current of 3 mA per tube for 2.5 hr. [$^{14}$C]Leucine-labeled cytochrome oxidase, prepared according to the methods of Mason *et al.* (27) from cells grown with [$^{14}$C]leucine, was run simultaneously (O– –O). Corrections for spillage were made. Numerals I, II, III, and IV–VII denote the positions of the cytochrome oxidase peptides as described by Mason *et al.* (27). Arrows indicate the position of marker peptides: (a) bovine serum; (b) ovalbumin; (c) D-amino-acid oxidase; and (d) cytochrome *c*. Similar results were obtained with three different preparations of mitochondrial poly(A)-RNA. Analysis of the products of the *E. coli* ribosomal system by gel electrophoresis with or without added [$^{14}$C]cytochrome oxidase marker gave similar peptide patterns.

or those synthesized in response to the addition of MS2 RNA do not contain measurable amounts of labeled material precipitable by cytochrome oxidase antibody.

Further support for the conclusion that the poly(A)-RNA from yeast mitochondria directs the synthesis of cytochrome oxidase peptides was obtained by electrophoresis of the dissociated immunoprecipitates on sodium dodecyl sulfate polyacrylamide gels (Fig. 3). Bands labeled I, II, and III in marker [$^{14}$C]cytochrome oxidase correspond in electrophoretic mobility to the three cytochrome oxidase peptides shown by Mason and Schatz (17) to be synthesized by mitochondrial ribosomes. An additional, more slowly moving band, which may represent incompletely dissociated peptides or may be a contaminant, is also present. Fig. 3 shows that the labeled peptides synthesized by *E. coli* ribosomes in response to mitochondrial poly(A)-RNA, and precipitated by cytochrome oxidase antibody, migrate with the mobility of the mitochondrially synthesized cytochrome oxidase peptides. The heavier band is also present. Control immunoprecipitates with antibody to bovine serum albumin showed no detectable radioactive bands.

## DISCUSSION

We have isolated, from yeast mitochondria, poly(A)-containing RNA that appears to represent mitochondrial mRNA. This poly(A)-RNA directs the synthesis of the three largest cytochrome oxidase peptides in an *E. coli* ribosomal system; it has been previously established that these same three peptides are translated by mitochondrial ribosomes *in vivo* (17) and in isolated yeast mitochondria (18). Several lines of evidence are consistent with the view that the poly(A)-RNA isolated are transcribed from mtDNA, and that the three cytochrome oxidase peptides are gene products of the mitochondrial genome. (*1*) Ethidium bromide, a specific inhibitor of mitochondrial transcription, inhibits the incorporation of [$^3$H]uridine into poly(A)-RNA by more than 90%. (*2*) Poly(A)-RNA hybridizes to mtDNA to a substantial extent, but not to nuclear DNA. This result could be due in part to the 200-fold difference in the complexity of the mitochondrial and nuclear genomes in yeast. The 5–20% contamination of the total mitochondrial RNA preparations detected by gel electrophoresis and by hybridization appears to be due to rRNA rather than to poly(A)-RNA. We believe that the cytoplasmic poly(A)-RNAs are degraded under the conditions used for mitochondrial RNA isolation. Degradation is probably caused by digestion with nucleases present in the glusulase that may adhere to the protoplasts which are only washed once in our experiments. (*3*) The short stretches (20 to 30 nucleotides) of poly(A) present at the 3′ terminus of the mitochondrial DNA readily distinguish this RNA from cytoplasmic poly(A)-RNA, in which the poly(A) contains 50–70 nucleotides (16). It is therefore likely that the poly(A)-RNA which we isolated is transcribed from mitochondrial DNA, and that the three larger cytochrome oxidase peptides are mitochondrial gene products. However, we cannot completely eliminate the possibility that imported nuclear transcripts are present in very small amounts in our mitochondrial poly(A)-RNA preparations, and are responsible for the specification of these peptides. Absolute proof that mtDNA codes for the three peptides requires the translation of RNA eluted from mtDNA·RNA hybrids, or, still more rigorously, requires studies of mitochondrial mutants that involve the structural genes for the cytochrome oxidase peptides.

Our pulse labeling experiments show that mitochondrial poly(A)-RNA (Fraction II) is being synthesized much more rapidly than ribosomal RNA, and support the view that it probably represents messenger RNA. The polyacrylamide gel analysis of pulse-labeled poly(A)-RNA reveals 8 to 10 discrete bands that have a pattern similar to those reported by Hirsch *et al.* (12) and Attardi (13) for mitochondrial poly(A)-RNAs from higher organisms. However, in contrast to Ojala and Attardi's (13) observation that HeLa cell poly(A)-RNAs behave as aggregates which convert to smaller species after formaldehyde denaturation, we found that this procedure did not significantly alter the mobilities of the yeast mitochondrial poly(A)-RNA species.

It has been suggested that mitochondrial translation products may be small (8,000–11,000 daltons) (29) and that the larger polypeptides synthesized by mitochondria may be aggregates. Schatz's group, however, has shown that yeast mitochondria are capable of synthesizing larger polypeptides both *in vitro* and *in vivo* (17, 18). Our findings that yeast mitochondria contain larger poly(A)-RNA ($\simeq$23 S), and that the poly(A)-RNA directs the synthesis of larger polypeptides in a cell-free system, support Schatz's view that the large polypeptides are the primary products of mitochondrial protein synthesis. It is likely that yeast mitochondria contain

larger polysomes than those isolated so far from mitochondria of HeLa cells (30) or from other organs.

The species with the largest molecular weight, observed on gel electrophoresis of the poly(A)-RNA (i.e., 23 S), has a mobility similar to that of the largest ribosomal RNA subunit. Similar poly(A)-RNAs of high molecular weight have been isolated from mitochondria of HeLa cells, hamsters, mosquitoes, and Drosophila (21). Although it is likely that these 23S species represent mRNA or precursors of mRNA, the presence of contaminating mitochondrial rRNA cannot be eliminated. In this context, it should be noted that the 23S fraction is assigned its value on the basis of the electrophoretic mobility of yeast mitochondrial rRNA, but that the S value would be approximately 35 if mobilities of yeast cytoplasmic rRNA or rat liver cytoplasmic rRNA were used as standards.

It is also possible that some yeast mitochondrial messages lack poly(A) segments and hence may be present in RNA Fraction I. Some species of cytoplasmic mRNA are thought to lack a poly(A) segment (31). Our designation of RNA Fraction II as a messenger-containing fraction is based both on its poly(A) content and on its ability to stimulate the synthesis of specific mitochondrial proteins in an *E. coli* cell-free system.

This work was supported in part by Grants HL0442, HL09172, and HL05673 from the National Institutes of Health, the U.S. Public Health Service, and the Louis Block Fund of the University of Chicago.

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