Mitochondrial and Cytoplasmic Ribosomes

DISTINGUISHING CHARACTERISTICS AND A REQUIREMENT FOR THE HOMOLOGOUS RIBOSOMAL SALT-EXTRACTABLE FRACTION FOR PROTEIN SYNTHESIS

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(Received 5 November 1973)

1. Mitochondrial and cytoplasmic ribosomes of *Euglena gracilis* differ in their total RNA and protein content. 2. Mitochondrial ribosomes dissociate to subunits at higher Mg^{2+} concentrations than do cytoplasmic ribosomes. 3. A separable 5S RNA is obtained from cytoplasmic and chloroplast ribosomes, but not from mitochondrial ribosomes. 4. For protein-synthesizing activity with a natural mRNA, mitochondrial ribosomes use tRNA from any cell compartment and are partly active with supernatant enzymes from cytoplasm. Cytoplasmic ribosomes are partly active with enzymes and tRNA from mitochondria or chloroplasts. 5. Both mitochondrial and cytoplasmic ribosomes show high specificity for the homologous salt-extractable ribosomal fraction for protein-synthesizing activity.

Much attention has focused on the origin of mitochondria and their evolutionary significance to eukaryotic cells in recent years (Ashwell & Work, 1970; Cohen, 1973). Consequently, the mode of synthesis by mitochondria of various macromolecules, especially proteins, has been studied in diverse cell types (Ashwell & Work, 1970; Küntzel, 1971; Borst & Grivell, 1971; Beattie, 1971; Borst, 1972; Perlman & Penman, 1970).

We have reported the isolation of polyribosomes active in protein synthesis *in vitro* from the mitochondria, chloroplasts and cytoplasm of the photosynthetic flagellate *Euglena gracilis* (Avadhani & Buetow, 1972a,b). This was the first isolation of mitochondrial polyribosomes free of significant cytoplasmic contamination from any cell type (Avadhani & Buetow, 1972a). The present paper reports further studies on the protein-biosynthetic machinery of mitochondria and cytoplasm. Presented here are physicochemical properties of ribosomes from different cellular compartments and the effects of homologous and heterologous tRNA, pH 5 enzymes (tRNA-free) and ribosome-associated factors (high-salt wash) on protein biosynthesis by cytoplasmic and mitochondrial ribosomes. The results show that tRNA and pH 5 enzymes are interchangeable, at least partially, for protein biosynthesis by these two ribosome types, but that the salt-extractable ribosomal factors are highly specific to the homologous ribosome.

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Materials and Methods

Organisms and growth conditions

Organisms used were a streptomycin-bleached chloroplast-lacking mutant of *Eug. gracilis* (strain SM-L1) and the photosynthetic form, *Eug. gracilis* (*bacillaris* variety). The cells were grown and harvested as described by Avadhani & Buetow (1972b). Strain SM-L1 was used for mitochondrial and cytoplasmic preparations. The photosynthetic *Euglena* was used for chloroplast preparations.

Buffers

Buffers A, B and C had the same concentrations of KCl (40 mM) and Tris-HCl (10 mM, pH 7.5 at 25°C). They differed only in $MgCl_2$ content as follows: buffer A, 3 mM- $MgCl_2$; buffer B, 0.5 mM- $MgCl_2$; buffer C, 0.2 mM- $MgCl_2$. Buffer D contained 20 mM-Tris-HCl (pH 7.5 at 25°C) and 0.3 M-KCl. BRS medium contained 40 mM-Tris-HCl (pH 7.8 at 25°C), 12.5 mM-magnesium acetate, 40 mM-KCl, 10 mM- NH_4Cl and 10 mM- β -mercaptoethanol.

Preparation of mitochondrial and cytoplasmic ribosomes

Mitochondrial and cytoplasmic polyribosomes were prepared from cells ruptured with glass beads as described by Avadhani & Buetow (1972b). Heparin and a protein fraction from rat liver were used as RNAase (ribonuclease) inhibitors (Avadhani & Buetow, 1972b). Mitochondrial ribosomes were prepared by suspending mitochondrial polyribosomes in buffer B for 3 h at 0–4°C and centrifuging the resultant

monoribosomes and ribosomal subunits at 230000g for 2 h at 2–4°C. Cytoplasmic ribosomes were similarly prepared from cytoplasmic polyribosomes except that buffer C was used. Mitochondrial and cytoplasmic ribosomal pellets were washed twice with buffers B and C respectively, suspended in BRS medium, and stored in liquid N₂.

Preparation of pH 5-enzyme fractions

Mitochondria and chloroplasts were isolated as described by Avadhani & Buetow (1972a,b). The 150000g supernatant fraction (S-150) was prepared from purified mitochondria and chloroplasts in BRS medium (Avadhani & Buetow, 1972b) with 0.5% sodium deoxycholate for lysis. A 150000g supernatant fraction from the whole cell lysate free of organelles was used to prepare the cytoplasmic S-150 fraction (Avadhani & Buetow, 1972b). The pH 5-enzyme fractions were prepared from the S-150 fractions as described by Fuhr *et al.* (1969) and suspended in buffer D at a concentration of 3–5 mg of protein/ml. The tRNA-free pH 5-enzyme fractions were prepared by treatment with DEAE-cellulose essentially as described by Gilbert & Anderson (1970). Cellex-D anion-exchange cellulose (high-exchange capacity; Bio-Rad Laboratories, Richmond, Calif., U.S.A.) was washed three times with buffer D by centrifugation at 2500g for 5 min each time. The pH 5-enzyme fraction (in buffer D) was mixed by a Vortex mixer with washed packed DEAE-cellulose (9.0 ml of pH 5 enzymes; 5.0 ml packed volume). After repeated mixing, the DEAE-cellulose was pelleted by centrifugation at 10000g for 10 min. The supernatant was aspirated out, mixed with 0.11 vol. of 10× BRS medium and dialysed overnight against BRS medium at 0–4°C. The non-diffusible material was finally stored in liquid N₂.

Preparation of unfractionated tRNA

The S-150 fractions from mitochondria, chloroplasts and cytoplasm were prepared as described above in BRS medium, mixed with 1 vol. of cold (–20°C) chloroform and 2 vol. of cold phenol, shaken for 30 min at room temperature, and centrifuged at 10000g for 15 min. The resultant aqueous phase was aspirated off and extracted twice more with chloroform and phenol as above. The final aqueous phase was mixed with 2 vol. of cold (–20°C) ethanol and kept at –20°C for 10 h. The RNA precipitate was pelleted at 5000g for 10 min and extracted with 10–15 ml of cold (2°C) 2.0M-NaCl. tRNA was precipitated from the NaCl extract with cold ethanol and pelleted by centrifugation as described above. The tRNA thus obtained was washed three times with cold ethanol, dried under a jet of N₂ gas, and dissolved in BRS medium at a concentration of 4.0 mg of RNA/ml (1 *E*₂₆₀ unit equals 40 µg of RNA/ml).

Salt extraction of ribosomes and preparation of the salt-extractable fraction

Ribosomes obtained as above were suspended in cold (2°C) 1.0M-KCl at a concentration of 5.0 mg of ribosomes/ml (11.2 *E*₂₆₀ units equals 1 mg of ribosomes/ml; Fuhr *et al.*, 1969) and centrifuged at 230000g for 3 h. The resultant ribosomal pellet was rinsed with 2–3 ml of buffer A and suspended in buffer A at a concentration of 2–3 mg of ribosomes/ml. These preparations are the 'salt-extracted ribosomes'.

(NH₄)₂SO₄ was added to the supernatant to 65% saturation and stored at 0–4°C for 12 h to precipitate the salt-extractable ribosome-associated factors. The precipitate was centrifuged down at 10000g for 10 min, dissolved in BRS medium at a concentration of 10–15 mg of protein/ml and dialysed overnight against BRS medium at 0–4°C. The non-diffusible material comprises the 'ribosomal salt-extractable fraction' and was stored in liquid N₂ until used.

Cell-free protein synthesis

Assays were run in a total volume of 0.2 ml and included 40 mM-Tris-HCl (pH 7.8), 12.5 mM-magnesium acetate, 40 mM-KCl, 10 mM-β-mercaptoethanol, 10 mM-NH₄Cl, 6.0 mM-phosphoenolpyruvate (Sigma Chemical Co., St. Louis, Mo., U.S.A.), 3.0 mM-ATP (Sigma Chemical Co.), 1.5 mM-GTP (Sigma Chemical Co.), 0.5 µCi of algal hydrolysate containing 15 ¹⁴C-labelled amino acids (New England Nuclear Corp., Boston, Mass., U.S.A.; 1.8 µCi/mg), 40 µM each of the following unlabelled L-amino acids (asparagine, glutamine, tryptophan, methionine and cysteine), 2.5 µg of crystalline pyruvate kinase (Sigma Chemical Co.), 0.1 mg of ribosomes, 20 µg of unfractionated tRNA (0.5 *E*₂₆₀ unit), 0.1 mg of tRNA-free pH 5-enzyme fraction, and, as messenger, 40 µg of 15S mRNA from *Euglena* (Avadhani *et al.*, 1971; Avadhani & Buetow, 1972c). Whenever salt-extracted ribosomes were used, 50 µg of the ribosomal salt-extractable fraction was also added. The reaction mixture was incubated at 37°C for 60 min. The reaction was stopped by adding 6–8 ml of cold (2°C) 5% (w/v) trichloroacetic acid. Preparation of samples for scintillation counting and all other details were as described previously (Avadhani & Buetow, 1970, 1972a,b,c).

Determination of RNA and protein

Total RNA and total protein contents of ribosomes were determined by the methods of Ceriotti (1955) and Lowry *et al.* (1951) respectively, with ribose and bovine serum albumin as standards.

Composition and Mg²⁺-sensitivity of ribosomes

The total RNA and protein content of mitochondrial ribosomes resembles that of *Escherichia coli*

Table 1. *Composition of ribosomes*
Results are means of three determinations.

Source	Content (% _w , w/w)	
	RNA	Protein
<i>Euglena</i> cytoplasm	42	58
<i>Euglena</i> mitochondria	57	43
<i>E. coli</i> A19	61	39

Fig. 1. *Ribosome sensitivity to Mg^{2+} concentration*

Cytoplasmic (●) and mitochondrial (○) monoribosomes (15 E_{260} units) were suspended in 0.5 ml of buffer containing 10 mM-Tris-HCl (pH 7.5 at 25°C), 40 mM-KCl and concentrations of Mg^{2+} ranging from 0.1 to 1.0 mM. After incubation at 0–4°C for 1 h, 0.2 ml samples were layered over 10.0 ml 5–25% linear sucrose density gradients containing the same buffer as the incubation medium and centrifuged in the SB 206 rotor of an International model B-60 ultracentrifuge at 230000 g_{max} for 3 h. Gradients were analysed as described by Avadhani & Buetow (1972b). In each case, the peak area for the monoribosomes was measured and compared with the total E_{260} -absorbing area of the gradient to determine percentage dissociation.

more closely than that of cytoplasmic ribosomes (Table 1). Mitochondrial ribosomes are more sensitive than cytoplasmic ribosomes to dissociation at low Mg^{2+} concentration (Fig. 1), a result supporting previous observations (Küntzel, 1969; Beattie, 1971; Avadhani & Buetow, 1972a).

Ribosomal RNA

In a previous study from this laboratory, the RNA of *Euglena* mitochondrial ribosomes was separated on a sucrose density gradient and 16S and 21S components as well as a small amount of a 4–5S component were resolved (Avadhani & Buetow, 1972b).

Fig. 2. *Analysis of mitochondrial, cytoplasmic and chloroplast rRNA on tandem gels*

Cells were grown in the presence of $H_3^{32}PO_4$ as described by Avadhani & Buetow (1973). ^{32}P -labelled ribosomes were isolated as described in the Materials and Methods section. Ribosomes were treated with sodium dodecyl sulphate at a final concentration of 2% (w/v) and layered (Avadhani & Buetow, 1973) on tandem gels containing 5.0 cm of 3.2% polyacrylamide and 2.0 cm of 7.5% polyacrylamide. Electrophoresis was done at 5 mA/gel. Gels were sliced into 2 mm-thick slices and counted for radioactivity as described previously (Avadhani & Buetow, 1973). (a) Mitochondrial rRNA; (b) cytoplasmic rRNA; (c) chloroplast rRNA. S values were determined with *E. coli* and rat liver rRNA as standards.

Table 2. *Requirements for protein synthesis by mitochondrial and cytoplasmic ribosomes*

Protein-synthesis assays were done in a final volume of 0.2 ml at 37°C for 60 min as described in the Materials and Methods section. Ribosomes extracted with 1 M-KCl as described in the Materials and Methods section are designated as 'salt-washed' ribosomes. The radioactivities obtained with control mitochondrial (14900 c.p.m.) and cytoplasmic (16200 c.p.m.) ribosomes in a complete system (no omissions) are regarded as 100% (columns 2 and 3 below). The 'salt-washed' mitochondrial and cytoplasmic ribosomes, however, incorporated 12600 and 15100 c.p.m. respectively, when the 'salt-extractable fraction' was added back. These radioactivities are also designated as 100% (columns 4 and 5 below). Except for one set of tubes as specifically mentioned (second line of the Table), the 'salt-washed' ribosomal assay system always contained the 'salt-extractable fraction'.

Omission	Activity (% of control)			
	Control ribosomes		'Salt-washed' ribosomes	
	Mitochondria	Cytoplasm	Mitochondria	Cytoplasm
None	100	100	100	100
Salt-extractable fraction	—	—	5	4
Supernatant enzymes	6	9	8	9
tRNA	1	0	1	1
Phosphoenolpyruvate and pyruvate kinase	16	13	14	15
ATP	18	21	20	15
15S mRNA	3	2	0	0

Table 3. *Ribosome specificity for supernatant enzymes, tRNA and ribosomal salt-extractable fractions*

Protein-synthesis assays were done as described in the Materials and Methods section with (a) homologous tRNA, but supernatant enzymes from different sources; (b) homologous supernatant enzymes, but tRNA from different sources; (c) homologous tRNA and supernatant enzymes, but ribosomal salt-extractable fraction from different sources. Ribosomes were salt-extracted in (c), but not in (a) or (b). Amino acid incorporations (c.p.m.) obtained with control and 'salt-washed' ribosomes were as given in Table 2.

	Activity (% of control)	
	Mitochondrial ribosomes	Cytoplasmic ribosomes
(a) Source of supernatant enzymes		
Cytoplasm	60	100
Mitochondria	100	53
Chloroplasts	105	51
(b) Source of tRNA		
Cytoplasm	97	100
Mitochondria	100	75
Chloroplasts	100	81
(c) Ribosomal source of salt-extractable fraction		
Cytoplasm	6	100
Mitochondria	100	18
Chloroplasts	65	27

Mitochondrial ribosomes from various cell types have been reported to lack a separable 5S rRNA component (Lizzard & Luck, 1971; Attardi & Attardi, 1971; Borst, 1972; Dubin & Friend, 1972). Therefore the possibility that preparations of mitochondrial ribosomes used in our previous studies contained small 'contaminating' RNA species such as tRNA was investigated. In the first experiment, mitochondrial ribosomes were washed twice either with BRS medium or with buffer A before RNA was extracted

by phenol (Schuit & Buetow, 1968) and then layered on sucrose density gradients. This treatment completely eliminated the peak in the region of 4–5S. In another experiment, cells were labelled with $^{32}\text{P}_i$ and used for the isolation of mitochondrial rRNA. When this labelled rRNA was subjected to electrophoresis on tandem gels of 3.2 and 7.5% polyacrylamide (Fig. 2a), only two species of RNA, 23S and 16S, were resolved. The 5S component detectable with ^{32}P -labelled cytoplasmic (Fig. 2b) and chloroplast (Fig.

2c) ribosomes was absent. Therefore the 4–5 S RNA component previously resolved from *Euglena* mitochondrial ribosomes (Avadhani & Buetow, 1972b) probably consisted of small contaminating RNA species.

Protein synthesis

Both mitochondrial and cytoplasmic ribosomes incorporated amino acids into the hot-trichloroacetic acid-insoluble fraction (Table 2). Similar incorporation activities were obtained with such ribosomes extracted with 1 M-KCl if the salt-extracted fraction was added back to the system (Table 2). Also, for protein synthesis, all ribosomes required mRNA, ATP, phosphoenolpyruvate and pyruvate kinase, tRNA and supernatant enzymes. These results are consistent with the characteristics of a typical system for protein synthesis *in vitro* (Nirenberg & Matthai, 1961; Noll *et al.*, 1963).

For maximal protein-synthesizing activity, mitochondrial ribosomes required the homologous supernatant enzymes or those from chloroplasts (Table 3a), but could use tRNA from any cell compartment (Table 3b). Mitochondrial ribosomes were partly active with supernatant enzymes from cytoplasm (Table 3a). Cytoplasmic ribosomes require the homologous supernatant enzymes and tRNA for full activity, but are partly active with enzymes and tRNA from mitochondria and chloroplasts (Table 3a,b).

Both mitochondrial and cytoplasmic ribosomes showed a high degree of specificity for the homologous ribosomal salt-extractable fraction for protein-synthesizing activity (Table 3c). The cytoplasmic and mitochondrial fractions are not interchangeable. Even the chloroplast ribosomal fraction was only 65% efficient in the mitochondrial system (Table 3c).

Discussion

The physicochemical properties of mitochondrial ribosomes from *Eug. gracilis* are quite different from their cytoplasmic counterparts. Mitochondrial ribosomes and rRNA species have lower sedimentation values (Fig. 2; also Avadhani & Buetow, 1972a,b) and are more sensitive to low concentrations of Mg^{2+} (Fig. 1) when compared with the cytoplasmic ribosomes. These results are in accordance with findings with mitochondria from various eukaryotes (Küntzel, 1969, 1971; Ashwell & Work, 1970; Beattie, 1971; Borst, 1972; Vesco & Penman, 1969; Vignais *et al.*, 1972). Also, as in previously reported cell types (Lizzardi & Luck, 1971; Attardi & Attardi, 1971; Borst, 1972; Dubin & Friend, 1972), mitochondrial ribosomes from *Euglena* are devoid of a separable 5 S RNA component (Fig. 2a). In contrast, the plastid-specific ribosomes contain a separable 5 S RNA component (Fig. 2c). This latter result parallels

the findings with chloroplast ribosomes from higher plants and *Chlamydomonas* (Payne & Dyer, 1971).

Euglena mitochondrial ribosomes show no specificity for homologous tRNA (Table 3b) and only limited specificity for homologous pH 5 enzymes (tRNA-free) (Table 3a) for protein-synthesizing activity. These ribosomes are less specific for their own tRNA and pH 5 enzymes than are the mitochondrial ribosomes of yeast and *Neurospora* (Ashwell & Work, 1970; Grandi & Küntzel, 1970; Scragg, 1971) and of the vertebrate *Xenopus laevis* (Swanson, 1973). The lower eukaryote, *Prototheca zopfii*, a colourless alga, contains both 80S and 70S ribosome-specific polymerizing enzymes in its cytoplasm (Ciferri & Parisi, 1970; Tiboni *et al.*, 1970). This also appears to be the case with *Euglena* (Tiboni *et al.*, 1970) and could explain the present results where the 70S mitochondrial ribosomes are functional with cytoplasmic tRNA and at least partly functional with cytoplasmic pH 5 enzymes. It was noted before that *Euglena* mitochondrial ribosomes are functional with pH 5 enzymes from *E. coli* but not from rat liver (Avadhani & Buetow, 1972a,b). It should be emphasized here that each ribosomal preparation used in this study was pure in that it was free of significant contamination by ribosomes from any other cellular compartment (Avadhani & Buetow, 1972a,b). The tRNA preparations used were obtained from mitochondrial, chloroplast and cytoplasmic isolates which give these pure ribosomal preparations. Each tRNA preparation then is unlikely to be contaminated significantly by tRNA from any other cellular compartment.

Cytoplasmic ribosomes are also partly functional with mitochondrial or chloroplast tRNA and pH 5 enzymes (Table 3a,b) and with supernatant fractions from *E. coli* and rat liver (Avadhani & Buetow, 1972b). These results have interesting evolutionary implications in that the cytoplasmic ribosomes of the eukaryote *Euglena* are at least partly capable of using tRNA and pH 5 enzymes from cells or organelles containing either 70S or 80S ribosomes.

The same mRNA is translated by both the mitochondrial and the cytoplasmic ribosomal systems (Table 2). Though some mRNA molecules are selectively translated on certain ribosomes (Berisi *et al.*, 1971; Nudel *et al.*, 1973), other mRNA molecules are translated on both prokaryotic-type and eukaryotic-type ribosomes (Laycock & Hunt, 1969; Klein *et al.*, 1972). The natural 15 S mRNA (Avadhani & Buetow, 1972c; Avadhani *et al.*, 1971) used in the present case appears to fit the latter category, since it is translated on both cytoplasmic ribosomes and mitochondrial ribosomes (Table 2).

The greatest specificity between cytoplasmic and mitochondrial ribosomes is in their requirement for the homologous ribosomal salt-extractable fraction (Table 3c). Further, in contrast with the results with

tRNA and pH 5 enzymes (Table 3a,b), even the salt-extractable fraction from the 70S chloroplast ribosomes is not fully efficient in the mitochondrial system (Table 3c). The 1M-KCl extract from the ribosomes has not been characterized yet. Therefore it cannot be decided definitively which components of the salt-extractable fraction confer this functional specificity on the ribosomes. It seems reasonable, however, to speculate that those proteins which initiate peptide biosynthesis and are known to be contained in this fraction (Lucas-Lenard & Lipmann, 1971) are involved. Since this fraction is not interchangeable between the ribosomal types under study, it is thus suggested that cytoplasmic and mitochondrial ribosomes contain at least one specific protein involved in the initiation process. As a whole, the present data indicate that ribosome-associated factors play a key role in the translational control of protein synthesis in various compartments of eukaryotic cells.

These studies were supported in part by grant HD 3163 from the National Institutes of Health.

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