Mitochondrial Polypeptide Elongation Factor EF-Tu of Saccharomyces cerevisiae
Functional and Structural Homologies to Escherichia coli EF-Tu

Birgit PIECHULLA and Hans KÜNTZEL
Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, Göttingen

(Received November 5, 1982/January 12, 1983) – EJB 6174

A complex mitochondrion-specific genetic apparatus is required to express a few mitochondrial genes coding for hydrophobic proteins of the inner mitochondrial membrane [1]. The RNA components of this genetic system (rRNAs and tRNAs) are coded by mitochondrial genes [2], whereas all enzymes involved in the replication, transcription and processing of mitochondrial nucleic acids (with the exception of intron-coded splicing maturases), all ribosomal proteins (one exception), ribosomal initiation and elongation factors and hydrophobic proteins of the inner mitochondrial membrane are required to express a few mitochondrial genes coding for these abundant proteins [10–12].

The polypeptide elongation factor EF-Tu was isolated from a mitochondrial 100000 × g supernatant of the yeast Saccharomyces cerevisiae and purified over 880-fold by DEAE-Sephadex chromatography and gel filtration. The factor efficiently replaces bacterial EF-Tu in a phenylalanine polymerizing cell-free system of Escherichia coli, it binds GDP and it protects phenylalanyl-tRNA against hydrolysis of the ester bond in the presence of 10 mM GTP. The polymerizing activity of the mitochondrial factor is inhibited to 90 % by 50 μM N-ethylmaleimide and to 50 % by 2.5 μM kirromycin.

The purified factor contains two major polypeptides of apparent molecular weights 48000 and 34000. Antibodies raised against the 48000-Mr protein react with EF-Tu_E. coli as revealed by immune blotting and by the inhibition of phenylalanylation. No reaction was observed between anti-(34000-Mr) and 48000-Mr proteins or EF-Tu_E. coli. The 48000-Mr protein has the same isoelectric point (pI = 6.2) and a content of cysteine and basic amino acids similar to the bacterial EF-Tu.

It is concluded that the 48000-Mr protein is the analogue to EF-Tu_E. coli, and that yeast mitochondrial EF-Tu is functionally and structurally more related to bacterial EF-Tu than cytosolic EF-1 of the same cell.

MATERIALS AND METHODS

Chemicals

[14C]Phenylalanine (specific activity 10 Ci/mol) and [3H]GDP (specific activity 10 Ci/mmol) were purchased from Amersham/Buchler (Braunschweig, FRG). Phosphoenolpyruvate, GTP, ATP, poly(U), pyruvate kinase and tRNA_Phe were obtained from Boehringer (Mannheim, FRG). DEAE-Sephadex CL-6B, protein-A-Sepharose CL-4B, and standard proteins for isoelectric focussing were from Pharmacia (Uppsala, Sweden). AcA 44 was from LKB (Broma, Sweden). Molecular weight standard proteins for sodium dodecyl-sulfate/polyacrylamide gel electrophoresis were obtained from...
Bio-Rad Laboratories (München, FRG). Fluoresceinisothiocyanate-labeled goat anti-rabbit antibodies were obtained from Nordic Immunological (London, Great Britain). Complete and incomplete Freund’s Adjuvant was from Behring (Marburg, FRG). Nitrocellulose filter discs were from Schleicher & Schuell (Dassel, FRG). Nitrocellulose filter sheets were from Sartorius (Gottingen, FRG). Acrylamide, bisacrylamide, and dithio-riitol were from Biomol (Ilvesheim, FRG). All other reagents were of analytical grade and purchased from Merck (Darmstadt, FRG). Escherichia coli C6 cells (M 1157, 1/2 log) were also from Merck. S. cerevisiae was obtained from ABC All-Back (Hamm, FRG).

Isolation of Mitochondria and Mitochondrial Supernatant Protein

3 kg frozen yeast cells (S. cerevisiae, commercial baker’s yeast) were suspended in 6000 ml buffer A (2 mM ethylendiaminetetraacetic acid (EDTA), 50 mM Tris/HCl pH 7.5, 0.05 M EDTA, 0.5 M dithiothreitol, 20 mM phenylmethylsulfonyl fluoride, 0.22 M sucrose, 5% glycerol) and disrupted in a Dyno mill (glass beads: 0.5 mm, flow rate 5 I/h) [20]. All further steps were at 4°C. After removing cell debris by two centrifugations (GS 3 rotor, 8000 rev./min, 10 min) mitochondria were pelleted by centrifugation at 13000 rev./min, 45 min, and washed twice with an equal volume of buffer A. The yield was about 80 g mitochondria (wet weight)/3 kg yeast.

Mitochondria were disrupted by grinding with two volumes of alumina (Alcoa), and a 100000 x g supernatant was prepared by centrifugation at 20000 x g for 20 min, followed by centrifugation at 100000 x g for 2 h.

Purification of Polypeptide Elongation Factors

EF-Tu was isolated from E. coli and from yeast mitochondrial 100000 x g supernatant protein by the two-step method (DEAE-Sephadex CL-6B chromatography and AcA 44 gel filtration) described by Leberman et al. [21], with the following modifications: E. coli cells were lysed by lysozyme in buffer B (50 mM Tris/HCl pH 7.5, 0.05 M EDTA, 0.5 M dithiothreitol, 20 mM phenylmethylsulfonyl chloride, 5 mM EDTA, 5% glycerol) and treated with DNase I (5 μg/ml) in the presence of 30 mM MgSO4. A 100000 x g supernatant of E. coli or yeast mitochondria was applied to a DEAE-Sephadex CL-6B column (4 x 20 cm) equilibrated with buffer C (1 mM dithiothreitol, 10 mM MgSO4, 10% glycerol, all other components as in buffer B without EDTA). The active fractions (assayed by phenylalanine polymerization) were precipitated by 70% (NH4)2SO4, dialyzed against buffer C containing 50 mM KCl, resuspended in a small volume of this buffer and subjected to AcA 44 gel filtration (3 x 150-cm column). Active fractions of mitochondrial origin were concentrated by DEAE-Sephadex CL-6B chromatography (1 x 5 cm), and the bacterial and mitochondrial EF-Tu fractions were precipitated by 70% (NH4)2SO4, dialyzed against buffer C containing 50 mM KCl and 50% glycerol and stored at -60°C.

Isolation of EF-G and Ribosomes from E. coli

EF-G was isolated as described [21] and assayed by complementation of EF-Tu in the phenylalanine polymerizing system. 70-S ribosomes were isolated, washed three times with 1 M NH4Cl, 10 mM Tris/HCl pH 7.5, 50 mM KCl, 10 mM MgCl2, resuspended in the same buffer without NH4Cl and stored in 50-μl aliquots at -60°C.

**EF-Tu Assays**

Poly(U)-Dependent Polyphenylalanine Synthesis. The reaction mixture (100 μl) contained 60 mM Tris/HCl pH 7.6, 70 mM KCl, 10 mM MgCl2, 1 mM dithiothreitol, 1 mM GTP, 1 mM ATP, 1 mM phosphoenolpyruvate, 0.2 unit pyruvate kinase, 50 μg poly(U), 10 mM [14C]phenylalanine, 4 A260 units E. coli tRNAphe-C-C-A, 0.73 μg E. coli phenylalanyl-tRNA synthetase and 36 μg partially purified EF-G. The concentrations of EF-Tu are given in the legends to the figures. The reaction was started by the addition of ribosomes and incubated at 37°C. 20-μl aliquots were withdrawn at indicated time intervals and spotted onto Whatman 3MM filter discs. The formation of polyphenylalanine was determined by washing the filters for 5 min in 10% trichloroacetic acid at 90°C, followed by two washings in 10% trichloroacetic acid (5 min at room temperature), one wash in ethanol and one wash in ether. The residual radioactivity on the filters was measured in a Tricarb liquid-scintillation counter.

[^3H]GDP Binding. The reaction mixture (20 μl) containing 50 mM Tris/HCl pH 7.5, 10 mM MgSO4, 250 mM NH4Cl, 10 mM dithiothreitol, 10 μl [3H]GDP (50 μM, 100 counts-min-1 pmol-1) and 70 μl of EF-Tu was incubated for 10 min at 37°C. The reaction was stopped by the addition of 1 ml ice-cold buffer and the mixture was filtered through the nitrocellulose filter discs (0.45 μm). Filters were washed with 5 ml buffer and counted in a Tricarb liquid-scintillation counter.

Formation of a Terney Complex EF-Tu · GTP · Phe-tRNAphe and Spontaneous Hydrolysis of the Ester Bond. 100 μg EF-Tu (corresponding to a concentration of 13 μM) was incubated with 1 mM GTP, 60 mM Tris/HCl pH 7.6, 70 mM KCl, 10 mM MgCl2, 1 mM dithiothreitol, 1 mM phosphoenolpyruvate and 0.2 unit pyruvate kinase for 10 min at 37°C in a total volume of 150 μl, in order to form an EF-Tu · GTP complex [22]. [14C]Phe-tRNAphe (E. coli) was added to a final concentration of 3 μM, and the rate of decylation at 37°C was measured by determination of the remaining amount of Phe-tRNAphe in the reaction mixture. 15-μl aliquots were removed at indicated periods and applied to Whatman 3MM filters. Filters were treated with 10% trichloroacetic acid, and the acid-insoluble radioactivity was determined.

**Gel Electrophoresis of Protein**

Sodium dodecylsulfate gel electrophoresis was performed in 0.1 M Tris/HCl pH 8.3, 0.1 M glycine, 0.1% sodium dodecylsulfate, with 10% acrylamide (cross-linking factor 2.7); running conditions: 40 min at 70 V. Isoelectric focussing was performed according to the LKB manual 1804-101, using a pH gradient between 3.5 and 9.5.

**Amino Acid Composition**

Denatured 48000-Mr protein was isolated by preparative sodium dodecylsulfate gel electrophoresis, precipitated by six volumes of acetone to remove electrophoresis buffer and suspended in 20% performic acid. Samples were hydrolyzed with 6 M HCl for 24 h and analyzed with a Durrum amino acid analyzer.

**Preparation of Antiserum and Antibodies (IgG)**

About 500 μg denatured or native antigen together with Complete Freund’s Adjuvant was injected intracutaneously into both sides of the backbone of a rabbit (Chinchilla rabbit:
Table 1. Purification of the mitochondrial EF-Tu from yeast

Total protein represents the yield from 500 g wet weight mitochondria from yeast. The total activity is given by the [14C]polyphenylalanine polymerized under standard conditions after 30 min.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein</th>
<th>10^-6 x Total activity</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>counts min^-1</td>
<td>counts min^-1 mg^-1</td>
<td>%</td>
<td>-fold</td>
</tr>
<tr>
<td>100000 x g supernatant</td>
<td>40 300</td>
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<td>0.115</td>
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<tr>
<td>Eluate of DEAE-Sephadex CL-6B chromography</td>
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<td>5.4</td>
<td>9.52</td>
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<td>82.8</td>
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<tr>
<td>Eluate of AcA 44 gel filtration</td>
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<td>5.2</td>
<td>72.84</td>
<td>96.7</td>
<td>633.4</td>
</tr>
<tr>
<td>Dialysate</td>
<td>47.6</td>
<td>4.8</td>
<td>102.2</td>
<td>90.2</td>
<td>888.8</td>
</tr>
</tbody>
</table>

Ch bb, female, 2.5 − 3.0 kg from Thomae, Biberach, FRG. About 14 days after the first injection blood was taken from the artery of the ear. After determination of the titer of antibodies a second injection with 200 μg incomplete Freund’s Adjuvant was performed. After 4 days about 30 ml blood was taken from the artery of the ear. For coagulation the blood was left 1 − 2 h at room temperature and overnight at 4°C. After centrifugation the antiserum could be decanted.

Immunoglobulines (IgG) were prepared on a protein-A-Sepharose CL-4B column. The procedure was followed as described in the Pharmacia manual (Affinity Chromatography – Principles and Methods).

Immunological Detection of Blotted Proteins on Nitrocellulose Filters

The electrophoretic transfer of proteins from sodium dodecylsulfate/polyacrylamide gels to nitrocellulose sheets was carried out as described by Towbin et al. [23]. The blotted and stained nitrocellulose filters were incubated in 3% bovine serum albumin in saline (0.9% NaCl, 10 mM Tris/HCl pH 7.4) for 1 h at 37°C, and afterwards rinsed twice in buffer D (0.2% sodium dodecylsulfate, 0.5% Triton-X-100, 0.5% bovine serum albumin, 0.01% NaN₃ in saline). Antibodies were diluted in buffer D (1 μg purified IgG/ml). The blots were shaken in the antibody solution for 5 − 10 h at room temperature, washed with buffer D for 30 min (five changes) and reincubated again for 5 − 10 h at room temperature with fluorescein isothiocyanate-labeled goat anti-rabbit antibodies (diluted 1/100 in buffer D). The blot was washed in buffer D for 30 min with five changes, air-dried and screened under ultraviolet light.

RESULTS AND DISCUSSION

Mitochondria were isolated from mechanically disrupted yeast cells, washed with EDTA-containing buffer to remove cytosolic ribosomes and homogenized by grinding with alumina (Alcoa). Alternative methods of extracting mitochondria (sonication or French press treatment) were found to be much less efficient, since the final yield of EF-Tu was only about 1/4 of that of alumina-treated mitochondria.

A mitochondrial high-speed supernatant (100000 × g) was further purified by a two-step procedure developed for isolating Escherichia coli EF-Tu [21]. The mitochondrial protein was applied to DEAE-Sephadex CL-6B column and eluted by a linear KCl gradient (0 − 0.4 M). The fractions active in a poly(U)-programmed phenylalanine-polymerizing cell-free system of E. coli elute well behind the bulk of mitochondrial matrix proteins at a similar KCl concentration as EF-Tu_E. coli. The peak fractions (about 80-fold enriched) were pooled and further purified by AcA 44 gel filtration. Most of the phenylalanine polymerizing activity elutes at about the same volumes as the bacterial EF-Tu, and the final preparation was enriched over 880-fold, as summarized in Table 1.

Fig. 1 demonstrates that the mitochondrial factor efficiently replaces the bacterial EF-Tu in an E. coli ribosomal system. The specific activities of bacterial and mitochondrial factors varied between 22 pmol and 80 pmol phenylalanine polymerized in 30 min at 37°C/μg factor on 1 A260 unit of 70-S ribosomes, depending on the quality of the ribosomal preparations. However, the specific activity of the heterologous
mitochondrial factor was always slightly higher than that of the homologous bacterial factor, if tested under identical conditions.

Fig. 2 shows the stimulation of the polymerizing activity of mitochondrial factor and *E. coli* EF-Tu by adding *E. coli* EF-G. Both factors are stimulated to about the same extent, and since the *E. coli* EF-Tu is free of EF-G, the polymerizing activity in the absence of added EF-G has to be explained by ribosome-bound EF-G. Furthermore, the 70-S ribosomes used in these experiments contain enough bound EF-Tu to regenerate the EF-Tu·GTP complex during protein synthesis. However, it is not clear whether the mitochondrial factor is regenerated by the *E. coli* EF-Ts or by a mitochondrial EF-Ts co-purifying with EF-Tu.

The polymerizing activity of *E. coli* EF-Tu is extremely sensitive to N-ethylmaleimide (90% inhibition by 50 μM N-ethylmaleimide [24]. Fig. 3 demonstrates that the mitochondrial factor is as sensitive as the bacterial one, suggesting the presence of at least one essential cysteine residue in the GTP or aminoacyl-tRNA binding site.

Another characteristic property of prokaryotic EF-Tu is the sensitivity against the antibiotic kirromycin, which appears to prevent the release of EF-Tu from the ribosome [25]. According to the data of Fig. 4 the mitochondrial factor is about ten times less sensitive (50% inhibition by 6 μM) than the bacterial factor (50% inhibition by 0.6 μM kirromycin).

Finally two other functional tests were used to compare mitochondrial and bacterial EF-Tu [5]: the binding of GDP and the protection of aminoacyl-tRNA against hydrolysis in the presence of GTP. Both factors bind comparable amounts of [3H]GDP (13.2 μM and 8.8 μM GDP/μg protein of bacterial and mitochondrial factor respectively), and both factors form a ternary complex with GTP and aminoacyl-tRNA, as revealed by the GTP-dependent protection of [14C]Phe-tRNA against the spontaneous hydrolysis of the ester bond at pH 7.6 (Fig. 5). The mitochondrial factor requires about 20 times more GTP than the bacterial one for a comparable protection of the aminoacyl ester linkage.

The bacterial and mitochondrial EF-Tu preparations, obtained by the same two-step procedure [21], were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. As seen in Fig. 6, the *E. coli* factor contains a single protein species of apparent molecular weight 45000, whereas the mitochondrial preparation contains two major components of $M_r = 48000$ and $M_r = 34000$, as well as a minor band of $M_r = 38000$.

In order to test structural and functional relationships between EF-Tu$_{E.coli}$ and the two mitochondrial proteins, we have prepared antibodies (IgG) against the native bacterial factor and the denatured 48000-$M_r$ and 34000-$M_r$.

Fig. 7 demonstrates that the polymerizing activity of EF-Tu$_{E.coli}$ and EF-Tu$_{mit}$ is inhibited up to 70% by the heterologous

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**Fig. 2.** Dependence of EF-Tu activity on addition of EF-G$_{E.coli}$. The polymerization activity of EF-Tu$_{E.coli}$ (black columns) or EF-Tu$_{E.coli}$ (white columns) was measured in the absence or presence of EF-G$_{E.coli}$ (36 μg, partially purified). The assay conditions were the same as in Fig. 1. EF-G$_{E.coli}$ had no activity in the absence of EF-Tu.

**Fig. 3.** Inhibition of EF-Tu from *E. coli* and yeast mitochondria by N-ethylmaleimide. 10 μg EF-Tu$_{E.coli}$ (A) or 10 μg of yeast EF-Tu$_{mit}$ (B) were pre-incubated for 5 min at room temperature in the absence (×—×) or presence of 50 μM N-ethylmaleimide (■—■) and assayed as in Fig. 1, except that dithiothreitol was absent from all buffers.

**Fig. 4.** Kirromycin sensitivity of EF-Tu from *E. coli* and yeast mitochondria. The polymerization activity of 5.6 μg EF-Tu$_{E.coli}$ (A) and 7 μg EF-Tu$_{mit}$ (B) was measured in the absence (●—●) and presence of 0.63 μM (×—×), 6.3 μM (○—○), 63 μM (■—■) and 176 μM kirromycin (▲—▲) as described in the legend to Fig. 1.
antibodies [anti-(48000-Mr) and anti-(EF-TuE,E.coli)] respectively, whereas anti-(34000-Mr) does not affect the activity of both factors. The observation that the bacterial EF-Tu activity is more sensitive to the heterologous than to the homologous antibody may be explained by the possibility that more antigenic sites were exposed in the denatured 48000-Mr polypeptide than in the native EF-TuE,E.coli protein during immunization of rabbits. Similar results were obtained by immune blotting, as seen in Fig. 8: EF-TuE,E.coli and 48000-Mr protein strongly interact with anti-(48000-Mr), but not with anti-(34000-Mr). The second mitochondrial polypeptide interacting with anti(48000-Mr) (lane b of Fig. 9) is believed to be a proteolytic fragment of 48000-Mr protein co-migrating with 34000-Mr protein, because a second band is almost invisible in other blotting experiments, using different mitochondrial preparations.

The data of Fig. 7 and 8 clearly demonstrate that EF-TuE,E.coli and 48000-Mr protein share antigenic sites, whereas the 34000-Mr component is immunologically unrelated to both EF-Tu factors. The latter protein possibly represents the analogue to EF-TsE,E.coli, which has the same size [26] and also forms a ternary complex with EF-Tu at low GTP concentrations [27]. Since the isolation procedure used for both the bacterial and mitochondrial factor results in a dissociation of the bacterial EF-Tus complex, the mitochondrial complex would be more stable than the bacterial one. Further functional tests are required to elucidate the role of the 34000-Mr protein.

Another structural similarity between bacterial and mitochondrial EF-Tu is revealed by isoelectric focussing: both factors band at a pH of 6.2, whereas the functionally analogous cytosolic EF-1 of yeast is reported to have an isoelectric point of 8.5–8.75 [14].

In Table 2 the amino acid composition of the mitochondrial EF-Tu (48000-Mr protein) is compared to that of EF-TuE,E.coli [7] and yeast cytosolic EF-1 [14], and it is not unexpected that the mitochondrial factor differs both from its cytosolic counterpart and from the bacterial factor. It should be noted, however, that cysteine residues are found in the N-ethylmaleimide-sensitive factors of bacteria and mitochondria, but are absent from the N-ethylmaleimide insensitive cytosolic factor [14]. Furthermore the mitochondrial and bacterial factors have a lower content of basic amino acids (56–57 residues) than the cytosolic factor (77 residues). This may be correlated with the differential pl values observed, although the number of acidic residues is not known for the two heterotopic yeast factors.

In summary, we conclude from the following observations that the yeast mitochondrial EF-Tu is functionally and structurally more related to the bacterial EF-Tu than to the yeast cytosolic EF-1.

1. The bacterial and mitochondrial factors are interchangeable: on bacterial and mitochondrial ribosomes [17–19], and both factors can replace cytosolic EF-1 on cytosolic ribosomes, whereas EF-1 does not function on bacterial and mitochondrial ribosomes [18].
2. The bacterial and mitochondrial factors are sensitive to kirromycin, although the mitochondrial factor requires an about ten times higher kirromycin concentration for 50% inhibition (6 μM) than the bacterial factor (0.6 μM). The effect of kirromycin on yeast cystosolic EF-1 has not been reported to our knowledge. However, the cytosolic EF-1 of Euglena gracilis is unaffected by the drug of concentrations up to 10 μM [15].

3. The bacterial and mitochondrial factors show immunological cross-reactivity in the functional test (polymerization activity) and in the immune blotting assay (Fig. 7 and 8), whereas antibodies against yeast EF-1 do not react with mitochondrial EF-Tu of the same organism [18].

4. The bacterial and mitochondrial factors contain cysteine residues, and the strong inhibition by N-ethylmaleimide indicates the presence of at least one cysteine in the nucleotide-binding and/or RNA-binding site [5]. In contrast, the cytosolic EF-1 is insensitive to N-ethylmaleimide and does not contain cysteine in the active site [14].

5. The bacterial and mitochondrial factors are acidic proteins (pI = 6.2), the cytosolic factor is a basic protein (pI = 8.5–8.75) [14].

We further conclude that the two heterotopic EF-Tu factors of yeast are different proteins encoded by different genes. Since mitochondrial elongation factors cannot be coded by mitochondrial genes [28, 29] both EF-Tu genes must reside in the nucleus. They could have originated by duplication of an ancestral nuclear gene, but the prokaryotic properties of the mitochondrial factor would rather point to an independent endosymbiotic origin and a subsequent transfer of a mitochondrial EF-Tu gene to the nuclear genome.

Phenylalanyl-tRNA synthetase was a kind gift of E. Graeber. We thank Dr H. Kratzin for performing amino acid analyses and Dr H. Sternbach for helpful discussions.

REFERENCES