Production of Mitochondrial Peptide-Chain Elongation Factors in Yeast Deficient in Mitochondrial Deoxyribonucleic Acid*

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ABSTRACT: Mutants of the yeast *Saccharomyces cerevisiae* lacking mitochondrial DNA contain the complete set of mitochondrial elongation factors; these enzymes are identical in their functional, protein chemical, and immunological properties with their counterparts in the parent strain.

In preceding studies (Richter and Lipmann, 1970a; Perani *et al.*, 1971) it was shown that a set of elongation factors T and G could be isolated from yeast mitochondria which complemented ribosomes from mitochondria and *Escherichia coli* but not those from the cytoplasm. These factors could then be identified relatively easily in chromatograms of whole cell homogenates, and the mitochondrial T and G factors could thus be separated from their cytoplasmic counterparts. The preparation of mitochondrial factors without isolating the mitochondria has now been improved and rather highly purified T and G factors have been obtained.

In order to determine if these mitochondrial factors were mapped on mitochondrial DNA, advantage was taken of the availability of yeast mutants free of this DNA. Mutants of the yeast *Saccharomyces cerevisiae*, which are deficient in mitochondrial DNA, may be produced by treatment with ethidium bromide (Slonimski *et al.*, 1968; Perlman and Mahler, 1971). Marmur and his colleagues showed such mutants to have no detectable mitochondrial DNA (Goldring *et al.*, 1970); they were therefore well suited for our purpose. Two of these mutants were used in the following experiments. The results to be reported show that the content of elongation factors in these mutants is identical with that of the wild-type yeast.

Experimental Section

The following strains from *S. cerevisiae* were used: the wild-type strain 18A, and the "petite" mutants II-1-40 and III-1-7; all three strains were obtained from Dr. Marmur, Albert-Einstein College of Medicine. The two "petite" mutants lack mitochondrial DNA (J. Marmur, personal communication).

Growth Conditions. Strain 18A was grown in a yeast extract–peptone–salt medium containing, per l.: 5 g of yeast extract, 10 g of peptone, 6 g of $(NH_4)_2HPO_4$, 2 g of $MgSO_4$, 9 g of KCl, and 33 ml of a 60% lactate syrup. The medium was adjusted to pH 4.5 with HCl. A 10-l. carboy was inoculated with 300 ml of an overnight culture containing 7.0 A_{450} units/ml. The cells were grown at 30° in a New Brunswick fermentor under aeration (2 l. of air/min) with stirring (800 rpm). Growth was stopped at a turbidity of 8.0 as measured from the 450-nm absorbance; the culture was quickly cooled and centrifuged in a continuous-flow rotor. The "petite" mutants were grown under similar conditions, except that the lactate was replaced by 1.5% glucose. The yield for all three strains was about 7–10 g of cells (wet weight) per l. of medium.

Preparation of Mitochondrial Elongation Factors. The same procedure for isolating the T and G factors may be applied to the wild-type strain 18A and the mutants II-1-40 and III-1-7. Mitochondrial T and G factor activity, respectively, were assayed with 50 μg of *E. coli* ribosomes supplemented with complementary bacterial factor (Richter and Lipmann, 1970a). The progress of purification is described in Table I for the wild-type strain, and in Table II results are compared with those obtained with the mutants. The total amount of T and G enzyme units was calculated for each purification step, and then expressed per gram of protein originally present in the 105,000*g* supernatant fraction. The data indicate that the mutant contains slightly less enzyme units per gram of supernatant protein than the wild-type strain.

Yeast cells (200 g wet weight) were mixed with 400 ml of buffer 1 (20 mM Tris-HCl (pH 7.4)–1 mM DTT[1]) and were homogenized in a Manton-Gaulin homogenizer as described (Richter and Lipmann, 1970a). The homogenate was centrifuged at 5000*g* for 10 min, and the cell pellet was reextracted with 300 ml of buffer 1 and centrifuged as above. The supernatant fractions were combined and centrifuged twice, first at 18,000*g* for 20 min, then at 78,000*g* for 2 hr. This fraction is referred to as S-100 (step 1 of Table I).

The S-100 fraction (1040 ml) was adjusted to pH 6.8, precipitated with 70 g of ammonium sulfate/100 ml of solution, and centrifuged at 18,000*g* for 1 hr. The protein pellet was reextracted three times with 150 ml of 25%, then three times with 22%, and finally three times with 18% ammonium sulfate solutions (w/w); all solutions contained 1 mM DTT; the pH was adjusted to 6.8. The supernatant fractions of each step were combined and reprecipitated with 20 g of ammonium sulfate/100 ml of solution. The 25% fraction contained no mitochondrial elongation factors and was discarded. The 22% fraction contained most of the mitochondrial T factor activity and was almost free of mitochondrial G factor. The latter was found in the 18% fraction, together with some T factor activity (step 2 of Table I). The two ammonium sulfate fractions were purified separately, and were dialyzed against buffer 1.

Purification of the Mitochondrial T Factor. The ammonium sulfate fraction (22%) with most of the T activity was treated

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[1] The abbreviation used is: DTT, dithiothreitol.

TABLE I: Purification Steps of the Mitochondrial T and G Factors from the Wild-Type Strain 18A.[a]

	Total Protein (mg)		Specific Activity (nmoles/10 min per mg)		Total Enzyme Units (nmoles/10 min)	
	T	G	T	G	T	G
1. S-100	9310		0.100		931	
2. Ammonium sulfate	2720	1860	0.187	0.250	509	465
3. Protamine sulfate	2480	1815	0.190	0.252	471	457
4. Hydroxylapatite	424	211	0.420	0.430	178	90.7
5. DEAE-cellulose	31.1	8.4	5.5	7.0	171	58.8
6. Sephadex G-150	12.1	3.2	9.2	12.7	111	40.6

[a] Activities for the G and T factors were determined by polyphenylalanine synthesis in the presence of an excess of the complementary *E. coli* factor and *E. coli* ribosomes (Richter and Lipmann, 1970a). The activities are derived from experiments with a linear dependence on either G or T factor concentration.

with protamine sulfate and further purified on hydroxylapatite, DEAE-cellulose, and Sephadex G-150 columns. To 10 ml of protein solution, 0.8 ml of a 1% neutralized protamine sulfate solution was added and the resulting precipitate removed by centrifugation (step 3 of Table I). After dialysis against 5 mM phosphate buffer (pH 7.2), containing 1 mM DTT, the protein solutions were clarified by centrifugation at 105,000*g* for 3 hr. The T factor was then applied to a hydroxylapatite column (4.5 × 9 cm) that was equilibrated with 5 mM phosphate buffer (pH 7.2); 1 mM DTT was present in this and all the following buffers. After washing the column with 500 ml of both the 10 and 30 mM phosphate buffers, the T activity was eluted with 70 mM phosphate buffer (step 4 of Table I). Fractions containing the T factor were combined, and 43 g of ammonium sulfate/100 ml of solution was added. After dialysis against buffer 1, the T factor was subjected to a DEAE-cellulose column (1.3 × 23 cm) equilibrated with 0.1 M KCl of buffer 1; the column was washed with 200 ml of the same buffer. T factor activity was eluted with a linear gradient from 0.1–0.5 M KCl (200 × 200 ml). The fractions with T factor activity were pooled, concentrated, and dialyzed as described for the hydroxylapatite step (step 5 of Table I).

TABLE II: Purification of Mitochondrial T and G Factors from Wild-Type Strain 18A and the Petite Mutants II-1-40 and III-1-7.[a]

	Enzyme Units (nmole/10 min)/g of Total S-100 Protein					
	18A		II-1-40		III-1-7	
Purification Steps	T	G	T	G	T	G
1. S-100	100	100	76	94	65	82
2. Ammonium sulfate	55	50	45	47	37	43
3. Protamine sulfate	51	49	39	45	32	31
4. Hydroxylapatite	19	10	12	8		
5. DEAE-cellulose	18	6	10	5		
6. Sephadex G-150	12	4	6	3		

[a] Purification of the T and G factors were carried out as outlined in the Experimental Section. For assay of the T and G factors, see Table I.

TABLE III: Comparison of the Function of the Mitochondrial Elongation Factors from the Wild-Type Strain 18A and the Petite Mutant II-1-40.[a]

	Sp Act. of T or G Factors (pmoles/10 min per mg)			
	Wild-Type Strain		Petite Mutant	
Function Assayed	T	G	T	G
Experiment 1				
Formation of Phe-tRNA–GTP–T factor complex[b]	30.9		29.1	
Binding of Phe-tRNA to ribosomes[c]	48.1		47.2	
Experiment 2				
Ribosome-dependent GTPase activity[d]		154.4		167.7
Formation of ribosome–GDP–G factor complex[e]		35.3		36.1

[a] T and G factors from the Sephadex G-150 step were used. The activities were obtained from values representing a linear dependence on the factor concentrations. [b] The Phe-tRNA GTP–T factor complex was formed with 50 μg of T factor, 300 pmoles of [^{3}H]GTP (specific activity 6.05 Ci/mmole), and 100 pmoles of [^{14}C]Phe-tRNA (350 pmoles of [^{14}C]-phenylalanine/mg of tRNA) and isolated on Sephadex G-50 columns (Richter and Lipmann, 1970b). [c] The binding reaction was performed with 50 μg of T, 50 μg of *E. coli* ribosomes, 100 μg of poly(U), 20 pmoles of [^{14}C]Phe-tRNA, 200 pmoles of GTP; the Phe-tRNA bound to the ribosomes was isolated by the Millipore filter technique (Richter, 1970). [d] Ribosome-dependent GTPase activity was measured with 20 μg of *E. coli* ribosomes, 50 μg of G, and 570 pmoles of [γ-^{32}P]GTP (specific activity 150 cpm/pmole) (Nishizuka and Lipmann, 1966). [e] Formation of the ribosome–GDP–G factor complex was carried out with 50 μg of *E. coli* ribosomes, 20 μg of G factor, and 15 pmoles of [^{3}H]GTP (Bodley *et al.*, 1970).

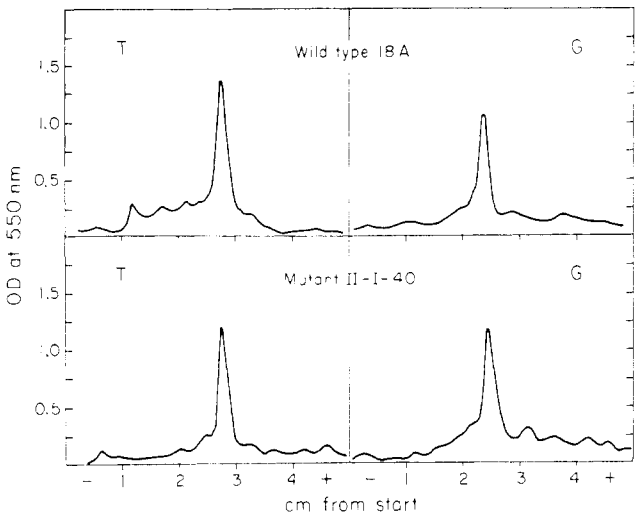

FIGURE 1: Electropherograms of the mitochondrial elongation factors T and G from the wild-type strain 18A and the petite mutant II-1-40. To the gels were added 100 μg of T factors or 80 μg of G factors (see Experimental Section). After running the gels for 1 hr at 3.5 mA/tube, they were stained with coomassie brilliant blue R-250, and destained overnight in methanol–acetic acid (20:7.5, v/v). The gels were densitometered in a Gilford spectrophotometer.

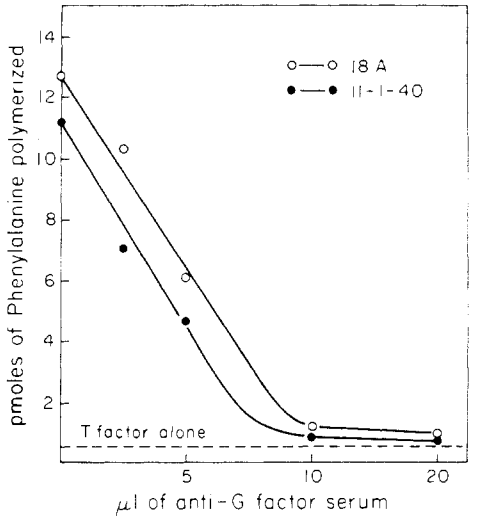

FIGURE 3: Inhibition of polyphenylalanine synthesis by anti-G-factor serum. Antiserum was prepared against the G factor from the wild-type strain 18A, and was added to the reaction mixtures as indicated. The experiments without antiserum represent the linear dependence on G-factor concentration from either strain; *E. coli* T factor (0.5 μg/assay) and *E. coli* ribosomes (50 μg/assay) were present (Richter and Lipmann, 1970a).

The T factor was further purified by passing it through a Sephadex G-150 column (1.3 × 75 cm), and eluting it with buffer 1. The fractions containing T factor were concentrated in a Diaflo Model 40 ultrafiltration cell using a UM-10 membrane (step 6 of Table I).

Purification of the Mitochondrial G Factor. The 18% ammonium sulfate fraction was purified in a similar manner to that described for the T factor (Table I). The G factor was eluted from the hydroxylapatite column (4.5 × 6.0 cm) with 30 mM phosphate buffer. DEAE-chromatography (column size 1.2 × 24 cm) was carried out with a linear gradient from 0.1 to 0.4 M KCl in buffer 1 (150 × 150 ml). Gel filtration on Sephadex G-150 was carried out as described for the T factor. All mitochondrial elongation factors used in this study were free of contamination by cytoplasmic elongation factors.

In some experiments, the T and G factors were additionally purified by sucrose gradient centrifugation and analyzed by gel disc electrophoresis. A 5-ml sucrose gradient from 5 to 18% in buffer 1 was used. On top of this gradient was layered 4–6 mg of either T or G protein, which was then centrifuged in a SW50L rotor at 200,000*g* for 18 hr at 0°. Four-drop fractions were collected and assayed for activity. The active fractions were combined, concentrated, and subjected to polyacrylamide gels (0.6 × 10 cm). Electrophoresis was carried out with 7.5% polyacrylamide running gels (Gordon, 1970). After these steps, the T and G factors were approximately 80% pure.

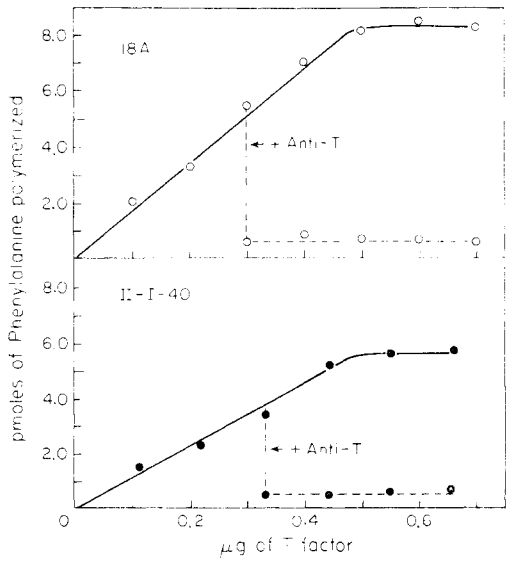

FIGURE 2: Effect of anti-T factor serum on polyphenylalanine synthesis. Where indicated, 20 μl of antiserum prepared against the wild-type T factor was used (Richter and Lipmann, 1970a). The assay system for polyphenylalanine contained T factor either from the wild type or from the petite mutant as indicated, and was completed with 0.1 μg of *E. coli* G factor and 50 μg of *E. coli* ribosomes (Richter and Lipmann, 1970a).

Preparation of Antisera against the T and G Factors from Strain 18A. From the Sephadex G-150 column step, 3 mg of T factor or 4 mg of G factor from the wild-type strain 18A were emulsified with complete Freund's adjuvant and injected subcutaneously into New Zealand white rabbits (Richter and Lipmann, 1970a). Antisera produced against the mitochondrial T and G factors did not give a cross-reaction with either the cytoplasmic T and G factors or the *E. coli* T and G factors.

Assays. The poly(U)-dependent binding of phenylalanine to *E. coli* ribosomes (Richter, 1970), the formation of the ribosome–GDP–G factor complex (Bodley *et al.*, 1970), the ribosome-dependent GTPase reaction (Nishizuka and Lipmann, 1966), as well as the formation of the ternary complex (Richter and Lipmann, 1970b) were as described. Protein concentration was determined as reported (Richter and Lipmann, 1970a).

Results

Comparison of the Mitochondrial Elongation Factors from the Wild-Type Strain and the "Petite" Mutants. The described

experiments with DNA-deficient mutants show that the mitochondrial T and G factors are coded by nuclear rather than by mitochondrial DNA (Table II). In the following experiment, the T and G factors from the different strains were compared with respect to their functional, chemical, and immunological properties. Experiment 1 of Table III compares the function of the two T factors in binding phenylalanyl-tRNA to ribosomes, and in the formation of the GTP–T factor–phenylalanyl-tRNA complex. Both T preparations show the same specific activity for these reactions. Similar results were obtained for the two G factors using as assay system either the formation of the ribosome–GDP–G-factor complex or the ribosome-dependent hydrolysis of GTP (expt 2 of Table III). Both G factors react specifically with bacterial or mitochondrial ribosomes, whereas the T factors also interact with cytoplasmic ribosomes (Richter and Lipmann, 1970a).

Further purification of the elongation factors from the normal strain and the "petite" mutant was carried out by sucrose gradient centrifugation and analyzed by polyacrylamide gel electrophoresis. Figure 1 shows the electrophoretic patterns of the mitochondrial enzymes from the normal strain and the mutant. Both T factors and both G factors were identical in their mobilities.

In addition, antisera prepared against the T and G factors from the wild-type strain 18A could interact with the T and G factors from the "petite" mutant. As indicated in Figures 2 and 3, the response of the antisera to the T or G factors from the mutant was quite similar to that of the wild-type factors.

Discussion

Recent experiments with chloramphenicol and cycloheximide have suggested that some or all of the ribosomal proteins from *Neurospora* mitochondria may be of extramitochondrial rather than of intramitochondrial origin (Küntzel, 1969; D. Luck, personal communication). Similar results have been obtained for a mitochondrial RNA polymerase (H. Küntzel, personal communication), and for the mitochondrial elongation factors using the antibiotics mentioned above (D. Richter, unpublished results; Parisi and Cella, 1971). The experiments with a petite mutant from yeast with no detectable mitochondrial DNA strongly indicate that the mitochondrial elongation factors T and G are not coded by mitochondrial DNA, and that they are identical with those of the parent strain in all properties tested so far. However, it remains to be seen whether these enzymes are synthesized in the cytoplasm on cytoplasmic ribosomes and transported into the mitochondria. Furthermore, the possibility that mRNA may be transported into the organelles to be translated on mitochondrial ribosomes is indicated by a report by Swanson (1971), who showed that mRNA could enter mitochondria but DNA or tRNA could not.

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