Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 6192–6196, October 1983
Biochemistry

Molecular cloning and sequence determination of the nuclear gene coding for mitochondrial elongation factor Tu of *Saccharomyces cerevisiae*

(protein synthesis/*tuf* gene probe/Southern hybridization/sequence conservation)

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Communicated by Severo Ochoa, July 8, 1983

ABSTRACT A 3.1-kilobase *Bgl* II fragment of *Saccharomyces cerevisiae* carrying the nuclear gene encoding the mitochondrial polypeptide chain elongation factor (EF) Tu has been cloned on pBR327 to yield a chimeric plasmid pYYB. The identification of the gene designated as *tufM* was based on the cross-hybridization with the *Escherichia coli tufB* gene, under low stringency conditions. The complete nucleotide sequence of the yeast *tufM* gene was established together with its 5'- and 3'-flanking regions. The sequence contained 1,311 nucleotides coding for a protein of 437 amino acids with a calculated M_r of 47,980. The nucleotide sequence and the deduced amino acid sequence of *tufM* were 60% and 66% homologous, respectively, to the corresponding sequences of *E. coli tufA*, when aligned to obtain the maximal homology. Plasmid YRpYB was then constructed by cloning the 2.5-kilobase *Eco*RI fragment of pYYB carrying *tufM* into a yeast cloning vector YRp-7. A mRNA hybridizable with *tufM* was isolated from the total mRNA of *S. cerevisiae* D13-1A transformed with YRpYB and translated in the reticulocyte lysate. The mRNA could direct the synthesis of a protein with M_r 48,000, which was immunoprecipitated with an anti-*E. coli* EF-Tu antibody but not with an antibody against yeast cytoplasmic EF-1α. The results indicate that the *tufM* gene is a nuclear gene coding for the yeast mitochondrial EF-Tu.

The polypeptide chain elongation factor Tu (EF-Tu) promotes a GTP-dependent binding of an aminoacyl-tRNA to the A site of ribosomes (1). EF-Tu from *Escherichia coli* consists of a single polypeptide chain with M_r 43,000, and the primary structure comprised of 393 amino acid residues has been determined (2). The protein is encoded by two nearly identical genes on the *E. coli* chromosome (3), *tufA* at 73 min and *tufB* at 89 min (4). Both *tufA* (5) and *tufB* (6) have been cloned and their nucleotide sequences determined. The sequences of *tufA* (7) and *tufB* (8) are nearly homologous and differ only in 13 positions but the gene products, EF-TuA and EF-TuB, are identical except for the COOH-terminal amino acid (2).

In eukaryotes, the counterpart of prokaryotic EF-Tu, designated as EF-1α, has been purified from various sources, including pig liver (9), rabbit reticulocytes (10), *Artemia salina* (11), wheat germ (12), and yeast (13), and was shown to consist of a single polypeptide chain, M_r 47,000–53,000. The partial amino acid sequence of EF-1α from rabbit reticulocytes (14) and *A. salina* (15) was determined, and sequence conservation between *A. salina* EF-1α and *E. coli* EF-Tu has been reported (15).

In addition to EF-1α, which functions in the cytoplasmic fraction in conjunction with 80S ribosomes, eukaryotic cells possess mitochondrial EF-Tu (designated as mEF-Tu) that functions in the mitochondrial translational apparatus (16, 17). Translational factors as well as ribosomal proteins in the mitochondria are encoded by nuclear genes, synthesized in cytoplasmic fractions, and transported into the mitochondria (18). The translational machineries of mitochondria have been thought to be closer to the prokaryotic ones than to the machinery present in eukaryotic cytoplasm (17, 19). However, the organization of the nuclear genes for the mitochondrial translational apparatus, their expression, and the incorporation of the products into the mitochondria are not well understood.

In this report, we describe the molecular cloning of the yeast mitochondrial EF-Tu gene (*tufM*) utilizing hybridization with the *E. coli tufB* gene under low stringency conditions. The nucleotide sequence as well as the deduced amino acid sequence of the *tufM* gene indicate that the structures of *E. coli* EF-Tu and yeast mitochondrial EF-Tu are remarkably conserved during evolution. The mRNA for *tufM* can be translated in the cell-free system from rabbit reticulocytes and the product has been identified as mEF-Tu by immunoprecipitation with an antibody against *E. coli* EF-Tu.

MATERIALS AND METHODS

Enzymes. Endonuclease *Eco*RI and T4 polynucleotide ligase were gifts from Takashi Yokota. Other restriction enzymes and T4 polynucleotide kinase were purchased from Takara Shuzo (Kyoto, Japan) or Bethesda Research Laboratories and were used essentially as recommended by the supplier (except for the use of 200 μg of gelatin per ml instead of bovine serum albumin). *E. coli* DNA polymerase I (large fragment) was purchased from Boehringer Mannheim.

Southern Hybridization. DNA was prepared from various sources including *Saccharomyces cerevisiae* 106A (mating type α, arginine requiring) as described by Cryer *et al.* (20) and completely digested with several restriction enzymes. Fragments were separated by electrophoresis on 1% agarose gel (21) and transferred to a nitrocellulose filter (Sartorius, 0.45-μm pore) as described by Southern (22). The 1.5-kilobase (kb) *Hpa* I fragment of pTUB1 (6) was subcloned at the *Pvu* II site of pBR322 to yield a hybrid plasmid pYT-1 which was used as a probe. This fragment covers the entire coding sequence of *E. coli* EF-TuB except for the NH_2-terminal 12 amino acids (see ref. 8). pYT-1 was labeled by nick-translation (23) with [α-^{32}P]dCTP (3,000 Ci/mmol; 1 Ci = 3.7×10^{10} Bq; Amersham Japan) to yield $\approx 1 \times 10^8$ cpm/μg and was hybridized with yeast DNA fragments essentially as described by Wahl *et al.* (24), except that the hybridization temperature was lowered to 28°C, and the filter was washed at 44°C with 15 mM NaCl/1.5 mM sodium citrate, pH 7.0/0.1% $NaDodSO_4$.

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Abbreviations: EF, polypeptide chain elongation factor; bp, base pairs; kb, kilobase(s).

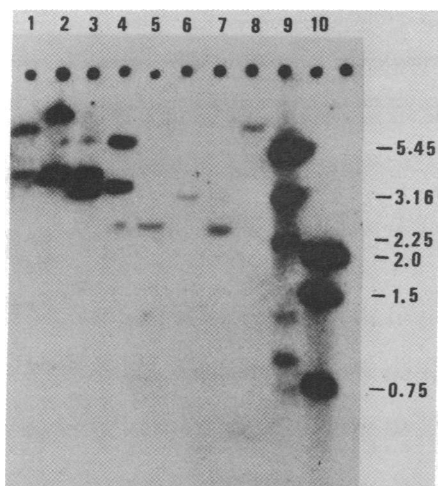

FIG. 1. Southern hybridization analysis of *E. coli* and yeast DNA. *E. coli* DNA (100 ng) (lanes 1–4) or yeast DNA (10 μg) (lanes 5–8) was digested with several restriction enzymes and electrophoresed on a 1% agarose gel. DNA was transferred to a nitrocellulose membrane filter and hybridized with nick-translated pYT-1 (1×10^8 cpm/μg). Restriction enzymes used were *Eco*RI (lanes 1 and 5), *Bgl* II (lanes 2 and 6), *Eco*RI and *Bgl* II (lanes 3 and 7), and *Pst* I (lanes 4 and 8). A mixture of the *Sma* I single-digested and the *Sma* I and *Eco*RI double-digested pYT-1 (lane 9) and the *Eco*RI, *Pvu* II, and *Pst* I triple-digested pBR322 (lane 10) were run as size markers. Sizes of marker DNA fragments are given in kb.

Restriction Mapping and DNA Sequence Determination. Restriction sites were determined by a single or double digestion of the cloned DNA. In some instances the arrangement of the restriction fragments was determined by Smith–Birnstiel mapping (25) of the labeled fragments. DNA sequence analysis was carried out by the chemical degradation method (26) with restriction fragments labeled either at the 5′ end with [γ-^{32}P]-ATP (prepared as described in ref. 27) and T4 polynucleotide kinase or at the 3′ end with [α-^{32}P]dCTP and *E. coli* DNA polymerase I (large fragment).

Hybridization-Translation. *S. cerevisiae* D13-1A (a *his*3-532 *trp*1 *gal*2) was transformed with YRpYB. This was constructed by cloning of the 2.5-kb *Eco*RI fragment containing the yeast nuclear gene for mitochondrial EF-Tu [cloned in pYYB (see *Results*)] into the yeast vector YRp7 (28). A transformant was grown on a minimal essential medium as described (29), except that 2% glucose was replaced by 2% lactate. Total RNA was prepared according to Struhl and Davis (30) and poly(A)-containing mRNA was selected by oligo(dT)-cellulose column chromatography (31).

The 2.5-kb *Eco*RI fragment of pYYB or *Eco*RI-digested pBR327 DNA was bound to a nitrocellulose filter (Schleicher & Schüll, 7 × 7 mm, 0.45-μm pore) and the filters were hybridized with 20 μg of mRNA prepared as above (32). The filter-hybridized mRNA was eluted and translated in the reticulocyte lysate system (33). Immunoprecipitation of the translational products with anti-sera and protein A adsorbent was carried out as described (34). The anti *E. coli* EF-Tu antibody (35) was a gift from A. Miyajima. The antibody against yeast cytoplasmic EF-1α was a generous gift from M. Miyazaki (Nagoya University).

RESULTS

Presence of a Sequence Homologous to the Gene for *E. coli* EF-Tu in Eukaryotes. First, we searched for a sequence homologous to *E. coli tufB* in DNA from various eukaryotic sources, including yeast, mouse liver, and human leukocytes utilizing low stringency conditions (36). Because the original plasmid pTUB1 containing *tufB* possessed other *E. coli* sequences, such as a part of *rrnB*, genes for four tRNAs (*thrT*, *glyT*, *tyrU*, and *thrU*), a gene for an unidentified protein "U," and a part of *rplK* (6), we constructed plasmid pYT-1 by subcloning the 1.5-kb *Hpa* I fragment of pTUB1 into pBR322 and used this as a probe. The fragment covered almost the entire coding sequence of *tufB* as well as about 300 nucleotides of the 3′-flanking region.

As shown in Fig. 1, *E. coli* DNA (lanes 1–4) gave two bands hybridizing with the *tufB* probe due to the presence of two genes for EF-Tu (*tufA* and *tufB*) in *E. coli* (3). On the other hand, a single DNA band was detected with yeast DNA (lanes 5–8), indicating that at least one sequence homologous to the *E. coli* EF-Tu gene is present in yeast. Mouse liver and human leukocyte DNA did not give any detectable band hybridizing with pYT-1 under the same conditions (data not shown), suggesting that the sequence of the gene in mouse or human cells is more divergent.

Isolation of the Yeast Gene Homologous to the *E. coli* EF-Tu Gene. Two hundred micrograms of DNA from *S. cerevisiae* 106A was digested with *Bgl* II, and fragments (3.1 kb in length) hybridizing with pYT-1 were isolated by agarose gel electrophoresis. The fragments were ligated to pBR327 at the *Bam*HI

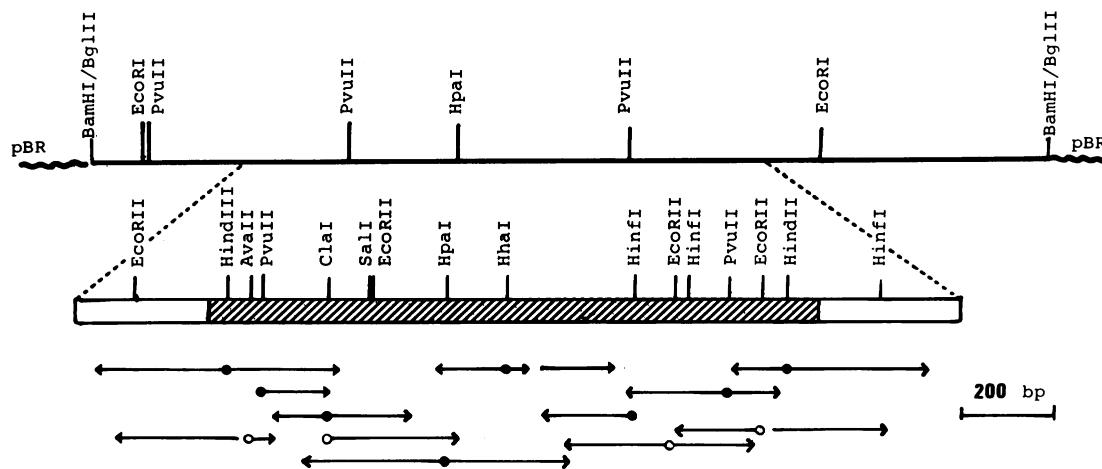

FIG. 2. Restriction map of the yeast DNA fragment cloned on pYYB and the strategy for nucleotide sequence determination. Restriction map of the yeast *Bgl* II fragment cloned at the *Bam*HI site of pBR327 is shown in the upper part of the figure with major restriction sites. The lower part shows the expanded structure of the yeast *tufM* gene. The sequence corresponding to the coding region is indicated by the shadowed box; the direction of transcription is left to right. The circles represent labeled termini at 5′ (●) or 3′ (○) ends, and the solid arrows show the sequence read off of the labeled fragments. bp, base pairs.

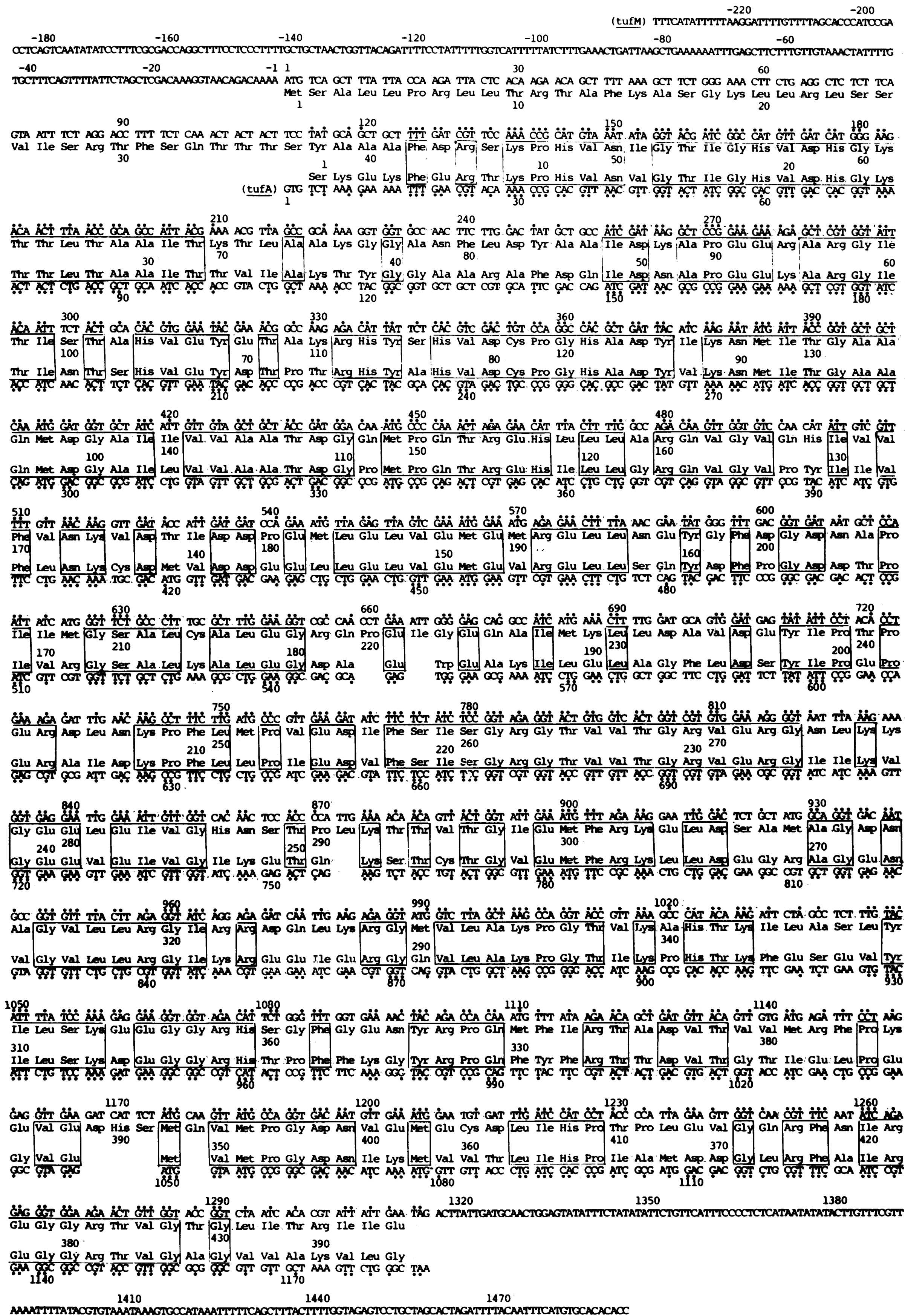

FIG. 3. Comparison of the nucleotide sequences of yeast *tufM* and *E. coli tufA* gene and the deduced amino acid sequences. The sequence of the *E. coli tufA* gene is from Yokota *et al.* (7). The sequences of *tufM* and *tufA* are aligned to give maximal homology by introducing several gaps in *tufA*. Identical amino acids are framed and identical nucleotides are marked by a dot.

site, and the hybrid plasmids were used to transform *E. coli* SK1592 (F *gal thi* T1 *endA sbc15 hsdR*[4] *hsdM*) by the method of Hoeijmakers *et al.* (37), to yield 768 ampicillin-resistant and tetracycline-sensitive clones.

Clones containing the yeast *tufM* gene were identified by Southern hybridization of plasmid DNA with a *tufB* probe under low stringency conditions. Plasmid DNA was prepared (38) from pools of 24 bacterial clones, and 0.5–1.0 μg of DNA from each pool was electrophoresed on a 1% agarose gel, to separate host DNA, and transferred to a nitrocellulose membrane filter. As a probe, we used the 1.5-kb *Hpa* I fragment containing *E. coli tufB* prepared from plasmid pTUB311 (unpublished data). Because pTUB311 is an R-derivative plasmid containing *tufB*, any vector DNA that might contaminate the *Hpa* I fragment would not hybridize with the sequence of pBR327 (a colE1 derivative) (39) in the hybrid plasmids.

One of 32 groups of 24 clones gave a positive hybridization with the ^{32}P-labeled *Hpa* I fragment, whereas DNA from other groups or pBR327 gave no hybridization, even at the low stringency conditions used for the present experiments. Plasmid DNA from each individual clone of this group was then prepared and analyzed as above, and a clone that gave a positive result was designated as pYYB.

Nucleotide Sequence Analysis. Fig. 2 shows the physical restriction map of pYYB constructed as described in *Materials and Methods* and the strategy for the DNA sequence analysis. Southern hybridization analysis of pYYB with *E. coli tufB* showed that most of the yeast sequence homologous to *tufB* resides within the 1.0-kb *Pvu* II fragment (data not shown). Therefore we determined the sequence of this region as well as the regions flanking it, as indicated in Fig. 2. The nucleotide sequence (1,712 bp) thus obtained is shown in Fig. 3 together with the sequence of the *E. coli tufA* gene (7). The translation initiation site for yeast *tufM* was assigned to the methionine codon AUG at nucleotide positions 1–3, because this was the first AUG triplet downstream of the nonsense codon UAA (positions −12 to −10) found in the open reading frame of the nucleotide sequence aligned for maximal homology with *E. coli tufA* (e.g., positions 124–149). The termination codon for translation was assigned to UAG at positions 1,318–1,320 in the same reading frame. Because we could not find any intron in the coding sequence of *tufM* (unpublished data), the gene codes for a protein of 437 amino acids including the NH_2-terminal methionine with a calculated M_r of 47,800, in a single open reading frame of 1,311 nucleotides.

A comparison of the sequence of yeast *tufM* with that of *E. coli tufA* reveals that the homology is 60% and 66% for the nucleotide and amino acid sequences, respectively. To obtain maximum homology, the initiator methionine for *tufA* was aligned to Ser-37 of *tufM*, and a limited number of gaps were introduced into the sequence of *tufA*. As seen in Fig. 3, several distinct regions are highly conserved between *tufM* and *tufA*. A most remarkable homology was found in amino acid residues 74–118 of *tufA* and 111–155 of *tufM*, where 41 of 44 amino acid residues were identical (91% homology). Because this region is supposed to be an active site for interaction with aminoacyl-tRNAs (reviewed in ref. 1; see also ref. 40), the sequence conservation of this region might be due to a functional requirement. Other homologies were found in amino acid sequences 5–32, 208–234, and 371–386 of *tufA* with the corresponding sequences of *tufM*, where 25 of 28, 24 of 27, and 13 of 16 amino acid residues are identical, respectively. Whether or not these conserved regions constitute functional domains of the protein remains to be determined.

The Yeast *tufM* Gene Codes for the Mitochondrial Factor. To determine whether the yeast *tufM* gene homologous to bacterial *tufA* and *tufB* codes for the cytoplasmic or the mitochondrial factor, the mRNA specified by *tufM* was isolated by hybridization to the cloned *tufM* gene and translated in the reticulocyte cell-free system (33). As shown in Fig. 4, the *tufM* mRNA markedly stimulated the incorporation of [^{35}S]methionine into a protein of M_r 48,000 (lane 3), whereas little increase of incorporation was observed with the mRNA hybridized to pBR322 (lane 2) as compared to the incubation without added mRNA (lane 1). The product synthesized under the direction of the *tufM* mRNA was immunoprecipitable with antibody against *E. coli* EF-Tu (lane 5) but not with antibody against yeast cytoplasmic EF-1α (lane 4). Furthermore, as will be described elsewhere, the M_r 48,000 protein was transported into yeast mitochondria on incubation in a cell-free system (unpublished data). From these results, it was concluded that the yeast *tufM* gene codes for the mitochondrial EF-Tu.

DISCUSSION

In the present study, a hybrid plasmid containing the yeast mitochondrial EF-Tu gene was isolated by subcloning and identified by Southern hybridization by using the *E. coli tufB* sequence as a probe. This procedure was found to be relatively simple and very effective. It may be applicable for isolation of other eukaryotic genes having a homology with *E. coli* genes—for example, the eukaryotic genes encoding for the mitochondrial translation factors and ribosomes.

Southern hybridization analysis of the yeast restriction fragments with labeled DNA containing the *E. coli tufB* gene revealed only a single band that hybridized with the probe, suggesting that the mitochondrial EF-Tu is coded by a single nuclear

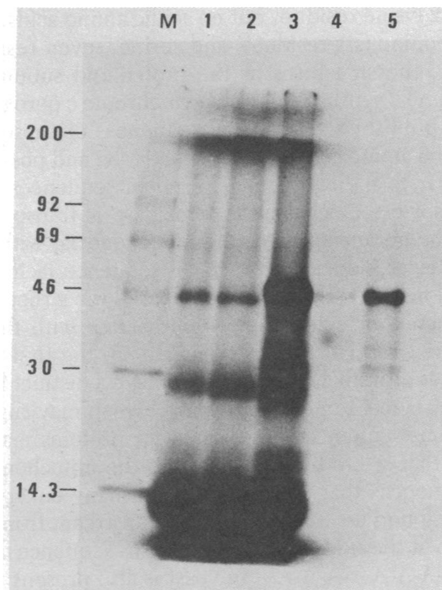

FIG. 4. *In vitro* translation of the hybridization-selected mRNA and immunoprecipitation of the product. The 2.5-kb *Eco*RI fragment of pYYB containing *tufM* gene or *Eco*RI-digested pBR322 was bound to nitrocellulose membrane filters and hybridized with mRNA obtained from yeast transformed with YRpYB. The hybridized mRNA was eluted and translated in 20 μl of reticulocyte lysate in the presence of 12 μCi of [^{32}S]methionine (1,490 Ci/mmol, Amersham Japan). One third of the product was electrophoresed on 15% polyacrylamide gels in the presence of $NaDodSO_4$ (41) and visualized by fluorography (42). Translation products without added mRNA (lane 1) or with mRNA hybridized to pBR322 (lane 2) or *tufM* (lane 3). One third each of the product used for the experiment of lane 3 was immunoprecipitated by antibodies against either yeast cytoplasmic EF-1α (lane 4) or *E. coli* EF-Tu (lane 5), and the precipitates were analyzed as above. Lane M, ^{14}C-labeled molecular weight standards (Amersham Japan) shown as $M_r \times 10^{-3}$ (from top to bottom): myosin, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, and lysozyme.

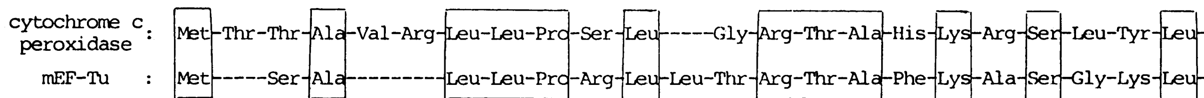

FIG. 5. Comparison of the NH_2-terminal sequence of yeast mitochondrial EF-Tu and the signal sequence of yeast cytochrome *c* peroxidase. The amino acid sequence of yeast mitochondrial EF-Tu is from Fig. 3 and the signal sequence of yeast cytochrome *c* peroxidase is from Kaput *et al.* (45). The boxed amino acids indicate identical amino acids; dashes show gaps introduced to obtain the maximal homology.

gene and that the gene for the cytoplasmic factor may be more divergent. This agrees with the previous finding that yeast mitochondrial EF-Tu is functionally interchangeable with *E. coli* EF-Tu but not with yeast cytoplasmic EF-1α (16). More recently, Piechulla and Küntzel (17) have shown that an antibody against yeast mitochondrial EF-Tu crossreacts with *E. coli* EF-Tu. As we have shown, the deduced amino acid sequence of yeast *tufM* is remarkably homologous to that of *E. coli* EF-Tu, especially in some regions. Amons *et al.* (15) have recently determined the partial amino acid sequence of EF-1α from *A. salina* and pointed out the homology with *E. coli* EF-Tu. However, the homology of *E. coli* EF-Tu with *A. salina* EF-1α is far less pronounced than that with yeast mitochondrial EF-Tu (compare figure 4 of ref. 15 with Fig. 3 of the present paper).

In general, nuclear-coded mitochondrial proteins are synthesized as precursors in the cytoplasm and transported into mitochondria (43). Although it is not known whether yeast mitochondrial EF-Tu is synthesized as a precursor that is processed to the mature protein during transport into the mitochondria, it is noteworthy that the *tufM* gene codes for a protein 37 amino acids longer than *E. coli* EF-Tu at the NH_2-terminal end. The 37-amino acid peptide is strongly basic, having 4 arginine and 2 lysine residues but no acidic amino acids. It is also rich in threonine (six residues) and serine (seven residues).

Recently, the structures of the proteolipid subunit of mitochondrial ATP synthase (44) and cytochrome *c* peroxidase (45) have been published. The signal sequences of these proteins are 68 and 66 amino acids long, respectively, and possess properties similar to the above NH_2-terminal sequences. Because the precursor of cytochrome *c* peroxidase is transported into the inner membrane space of the mitochondria, with the first 18 amino acid residues of the signal sequence facing the mitochondrial matrix (45), we have compared the structure of the signal sequence of cytochrome *c* peroxidase with that of the mitochondrial EF-Tu. As shown in Fig. 5, the first 20 amino acid residues of mitochondrial EF-Tu have a distinct homology with that of cytochrome *c* peroxidase, which may suggest that a homologous sequence is important for proteins made in the cytoplasm that are to be imported into the mitochondria.

The sequences that are conserved in many eukaryotic promoters are found at −225 nucleotides upstream from the first ATG codon of the *tufM* gene. A consensus sequence for poly(A) addition T-A-A-A-T-A-A-$^{A}_{G}$ in yeast is also present at 96–103 nucleotides downstream from the TAG termination codon. More detailed characteristics of the sequence as well as the analysis of the transcriptional and translational products will be discussed elsewhere.

We thank Dr. M. Miyazaki (Nagoya University) for his generous gift of the antibody against yeast cytoplasmic EF-1α. Thanks are also due to Dr. R. W. Davis for providing us with vector plasmid YRp7 and strain D13-1A.

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