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Effects of domain exchanges between *Escherichia coli* and mammalian mitochondrial EF-Tu on interactions with guanine nucleotides, aminoacyl-tRNA and ribosomes

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Abstract

Escherichia coli elongation factor (EF-Tu) and the corresponding mammalian mitochondrial factor, EF-Tu_{mt}, show distinct differences in their affinities for guanine nucleotides and in their interactions with elongation factor Ts (EF-Ts) and mitochondrial tRNAs. To investigate the roles of the three domains of EF-Tu in these differences, six chimeric proteins were prepared in which the three domains were systematically switched. *E. coli* EF-Tu binds GDP much more tightly than EF-Tu_{mt}. This difference does not reside in domain I alone but is regulated by interactions with domains II and III. All the chimeric proteins formed ternary complexes with GTP and aminoacyl-tRNA although some had an increased or decreased activity in this assay. The activity of *E. coli* EF-Tu but not of EF-Tu_{mt} is stimulated by *E. coli* EF-Ts. The presence of any one of the domains of EF-Tu_{mt} in the prokaryotic factor reduced its interaction with *E. coli* EF-Ts 2–3-fold. In contrast, the presence of any of the three domains of *E. coli* EF-Tu in EF-Tu_{mt} allowed the mitochondrial factor to interact with bacterial EF-Ts. This observation indicates that even domain II which is not in contact with EF-Ts plays an important role in the nucleotide exchange reaction. EF-Ts_{mt} interacts with all of the chimeras produced. However, with the exception of domain III exchanges, it inhibits the activities of the chimeras indicating that it could not be productively released to allow formation of the ternary complex. The unique ability of EF-Tu_{mt} to promote binding of mitochondrial Phe-$tRNA^{Phe}$ to the A-site of the ribosome resides in domains I and II. These studies indicate that the interactions of EF-Tu with its ligands is a complex process involving cross-talk between all three domains.

Keywords: Mitochondrion; Translation; Elongation factor Tu; Protein synthesis; Elongation; Chimeric protein

1. Introduction

Elongation factor Tu (EF-Tu) plays a central role in protein biosynthesis by delivering aminoacyl-tRNA (aa-tRNA) to the A-site of the ribosome [1]. *Escherichia coli* EF-Tu forms a ternary complex with GTP and aa-tRNA. The ternary complex binds the A-site of an actively translating ribosome in a mRNA-dependent manner. Once the cognate ternary complex is bound to the ribosome, the GTP is hydro-

Abbreviations: EF-Tu, elongation factor Tu; his-tagged, histidine tagged molecule; MEE, EMM, EEM, MME, EME and MEM, chimeric proteins with the letter E or M designating that domain I, II or III are from *E. coli* EF-Tu or EF-Tu_{mt}, respectively; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PK, pyruvate kinase; PEP, phosphoenolpyruvate

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lyzed to GDP by activation of the GTPase activity of EF-Tu [2]. The EF-Tu·GDP complex dissociates from the ribosome and is recycled to EF-Tu·GTP in a nucleotide exchange reaction catalyzed by elongation factor Ts (EF-Ts) [3].

The crystal structures of *E. coli* and *Thermus thermophilus* EF-Tu reveal that both proteins fold into similar three-dimensional structures composed of three domains connected by highly conserved flexible spacer peptides (Fig. 1) [4,5]. Domain I encompasses the N-terminal 200 amino acids and contains the guanine nucleotide binding site and the catalytic site for the GTPase activity. The functions of domain II (residues 208–295, *E. coli* numbering) and domain III (residues 300–393) are less precisely defined, but domains I and III interact with EF-Ts while all three domains are involved in aa-tRNA binding [4,6,7]. A great deal of movement occurs at the interfaces of these domains when different ligands bind to EF-Tu and all three domains contribute to the function of EF-Tu [8–11]. A number of experimental results indicate that cross-talk between the domains allows each to carry out its role in achieving the overall biological function of EF-Tu [12–14].

The basic features of the elongation cycle in mammalian mitochondria are similar to those of *E. coli* [15,16]. There are, however, subtle differences in the manner in which EF-Tu_{mt} interacts with the components of the translational machinery. First, EF-Tu_{mt} unlike *E. coli* EF-Tu does not readily bind guanine nucleotides [17]. Second, the activity of *E. coli* EF-Tu can be stimulated by both *E. coli* EF-Ts and EF-Ts_{mt}. In contrast, the activity of EF-Tu_{mt} is not stimulated by *E. coli* EF-Ts [18]. Finally, EF-Tu_{mt} readily forms ternary complexes with mitochondrial or bacterial aa-tRNA and delivers them to the A-site of the ribosome. In contrast, although *E. coli* EF-Tu can form ternary complexes with mitochondrial aa-tRNAs, it cannot promote binding of these complexes to the A-site of either *E. coli* or mammalian mitochondrial ribosomes [19].

A complex consisting of EF-Tu·Ts_{mt} has been isolated and characterized from bovine liver mitochondria [17,20]. EF-Tu_{mt} and EF-Ts_{mt} from this complex have been cloned, sequenced and expressed in *E. coli* [21,22]. Sequence comparisons show that the mature form of EF-Tu_{mt} is 56% identical to *E. coli* EF-Tu and to the *T. thermophilus* factor. This conservation of primary sequence suggests that the three dimensional structure of EF-Tu_{mt} is similar to that of the bacterial factors.

To better understand the differences between *E. coli* EF-Tu and EF-Tu_{mt}, and the role that interdomain interactions play in these differences, six chimeric proteins were constructed. These chimeric proteins were used to examine the roles of different domains in governing the strength of guanine nucleotide binding, in ternary complex formation, in interactions with EF-Ts and in allowing the productive use of mitochondrial tRNA in translation. To produce these chimeras, the domains of *E. coli* EF-Tu have been systemically replaced with those from EF-Tu_{mt}, and the domains of EF-Tu_{mt} have been replaced with those from *E. coli* EF-Tu.

2. Materials and methods

2.1. Materials

Restriction enzymes were from Promega. Primers and oligonucleotides were synthesized at the Lineberger Comprehensive Cancer Research Center at the University of North Carolina at Chapel Hill. All other chemicals were obtained from either Sigma or Mallinckrodt. Ribosomes from *E. coli* were prepared as described in [23] with the modifications outlined in [24]. Elongation factor EF-G was purified from *E. coli* as described [25] and further purified by chromatography on Sephadex G-75. Mitochondrial tRNA was isolated from purified bovine mitochondrial pellets by extraction with phenol and chloroform and further purified by DEAE-Sepharose column chromatography. Human mitochondrial $tRNA^{Phe}$ synthetase has been cloned, expressed and purified in our laboratory [26].

2.2. PCR primers

Forward primer Vec-2 (5′-taggggaattgtgagcggataac-3′) is complementary to a sequence of pET24C(+) outside of the region coding for domain I of both *E. coli* EF-Tu and EF-Tu_{mt}. Reverse primer Vec-3 (5′-attgctcagcggtggcagcagcc-3′) is complementary to a sequence of pET24C(+) outside of the region coding for domain III of both *E. coli* EF-Tu

and EF-Tu_{mt}. Forward primers ETu480 (5′-tacgacttcccgggcgac-3′) and FSP-STAA (tgtggagtatagcacagcc) are complementary to sequences within domain I of *E. coli* EF-Tu and EF-Tu_{mt}, respectively, allowing extension toward domain III. The 3′ half of the reverse chimeric primer Tud1-2EM (5′-ccacgggcagcagaaaaggaatgtaagaatccagg-3′) is complementary to a sequence of the *E. coli* EF-Tu gene in the conserved region between domains I and II allowing extension toward domain I, while the 5′ half of this primer (underlined) is complementary to the adjacent sequence of the EF-Tu_{mt} gene. The reverse chimeric primer Tud1-2ME (5′-tcgcacgctctggttccggcttctccaggtcccgg) is complementary to the same region as Tud1-2EM, except the 3′ half is complementary to the EF-Tu_{mt} gene (underlined) while the 5′ half is complementary to the *E. coli* EF-Tu gene. The 3′ half of the reverse chimeric primer Tud2-3EM (5′-tgcctccaccttctggtgcggcttgatggtgcc-3′) is complementary to a sequence of the *E. coli* EF-Tu gene in the conserved region between domains II and III and allows extension toward domain I, while the 5′ half (underlined) is complementary to the adjacent region of the EF-Tu_{mt} gene. The reverse chimeric primer Tud2-3ME (5′-cagattcgaacttggtgtgaggctggatggaacc-3′) is complementary to the same region as Tud2-3EM, except that the 3′ half is complementary to the EF-Tu_{mt} gene (underlined) while the 5′ half is complementary to the *E. coli* EF-Tu gene.

2.3. Preparation of chimeras of E. coli EF-Tu and EF-Tu_{mt}

Clones encoding His-tagged forms of *E. coli* EF-Tu and EF-Tu_{mt} in pET24C(+) have been previously prepared [18,21]. Preparation of the chimeric genes encoding portions of the *E. coli* and mitochondrial EF-Tu genes was a multi-step process based on the procedures described previously [18] that allow the formation of chimeric genes without the need to introduce restriction sites. First, PCR was used to produce templates to be used in the third step. A truncated form of the *E. coli* EF-Tu gene (E23) containing all of domains II and III and a short segment of domain I was prepared using primers Etu480 and Vec-3 and the *E. coli* EF-Tu gene as a template. A corresponding derivative of the EF-Tu_{mt} gene (M23) was prepared in an analogous manner using FSP-STAA and Vec-3 and the EF-Tu_{mt} gene as a template. Second, to produce large primers for the third step, four different PCR reactions were carried out. Primers Vec-2 and Tud1-2EM were used with the *E. coli* EF-Tu gene as a template and yielded DNA coding for domain I of *E. coli* EF-Tu as a product (E1). Primers Vec-2 and Tud2-3EM were used with the *E. coli* EF-Tu gene as a template and yielded DNA coding for domains I and II of *E. coli* EF-Tu (E12). Each product retained the 3′ half of the original primer complementary to the EF-Tu_{mt} gene as described above. Primers Tud1-2ME and Tud2-3ME were used in the same way except with the EF-Tu_{mt} gene as a template and yielded the same products from EF-Tu_{mt}, (M1 and M12). These PCR products also retained the 3′ half of the original primers complementary to the *E. coli* EF-Tu gene as described above. Third, in different PCR reactions the E1 and E12 DNAs along with Vec-3 were used as primers with M23 as a template to produce the genes for the chimeric proteins EMM and EEM (Fig. 2). Genes for the chimeric proteins MEE and MME were produced with the same method using primers M1 and M12 along with Vec-3 and E23 as a template (Fig. 2).

The chimera EME was prepared by PCR using E1 and Vec-3 as primers and the gene for the chimera MME as a template. Chimera MEM for unknown reasons could not be prepared by this method, so a new strategy was developed. First, PCR using primers Etu480 and Vec-3 was carried out with the gene for chimera EEM as a template and yielded E2M3. Next, PCR using E2M3 as a template and M1 and Vec-3 as primers was carried out. Vec-2 was added as an additional primer to boost amplification of the final PCR product and resulted in the gene for chimera MEM. All PCR reactions conditions were as previously described [18].

All final PCR products were cut with *Nde*I and *Xho*I, inserted into pET24C(+) plasmids and transformed into *E. coli* DH5α. Plasmids were purified using Qiagen midi-prep kits and the sequences were confirmed at the Automated DNA Sequencing Facility at The University of North Carolina at Chapel Hill.

2.4. Expression and purification of the chimeric proteins

The plasmids containing the EF-Tu chimeras were transformed into *E. coli* BL21 (DE3) for expression [27]. Cultures were grown in LB media containing 25 μg/ml of kanamycin at 25°C until the concentration of the bacteria reached 0.6 A_{600}. Expression of the chimeric proteins was then induced by the addition of 50 μM IPTG at 25°C for 1 h. The chimeric proteins were purified through Ni-NTA columns as described [21]. Protein concentrations were determined by the Micro-Bradford method (Bio-Rad).

2.5. Construction of biotinylated derivatives of E. coli EF-Ts and EF-Ts_{mt}

Double stranded oligonucleotides coding for a hexahistidine sequence and a biotinylation signal [28] were inserted into pET24C(+) containing the gene for either *E. coli* EF-Ts or EF-Ts_{mt} [27]. These sequences were inserted so that the added residues were at the COOH-terminus of the protein. The sequences were verified and the plasmids were transformed into *E. coli* BL21 (DE3) for expression [27]. The His-tagged, biotinylated proteins were purified as described [28].

2.6. Assays

The abilities of the chimeric proteins to bind GDP were determined using a nitrocellulose filter binding assay [25]. In this assay radiolabeled GDP is incubated with EF-Tu and the labeled EF-Tu·GDP complex is detected using a nitrocellulose filter binding assay which retains the complex but not free GDP. This assay can only be used to assay GDP binding to a protein when the equilibrium dissociation constant (K_d) of the complex is small (about 10^{-7} M or lower). The assay can be used to detect GDP-binding to *E. coli* EF-Tu (K_d = 8 nM [29]). Although *E. coli* EF-Tu is isolated as an EF-Tu·GDP complex, this measurement is possible since the dissociation of this complex is rapid enough to allow measurement of the exchange of the cold GDP bound to the factor with the added radiolabeled GDP. However the binding of GDP to EF-Tu_{mt} is too weak (K_d = 1 μM) to be detected by this method (Y.-C. Cai and L. Spremulli, manuscript in preparation and [30]).

The abilities of the chimeric proteins to bind EF-Ts were analyzed by a modified Western procedure. Chimeric proteins (2 μg) were resolved on SDS-PAGE for 1 h at 100 V and then transferred to nitrocellulose membranes. The membranes were probed with biotinylated *E. coli* EF-Ts (1.4 mg/ml) or EF-Ts_{mt} (0.4 mg/ml). Binding was detected using alkaline phosphatase-conjugated streptavidin [28].

Ternary complex formation was assayed by examining the abilities of wild-type and chimeric EF-Tu to protect [^{14}C]Phe-tRNA^{Phe} from digestion by RNase A. For these experiments, any GDP present in the preparation buffer was converted to GTP just prior to the measurement of ternary complex formation [31,32]. Wild-type and chimeric EF-Tu (0.25, 0.5, 1.0, and 1.5 μM) were incubated in reaction mixtures (100 μl) containing 0.14 μM [^{14}C]Phe-tRNA^{Phe}, 25 mM Tris-HCl, pH 7.5, 0.5 mM dithiothreitol, 50 mM NH_4Cl, 10 mM $MgCl_2$, 2.5 mM phosphoenolpyruvate (PEP) and 0.17 units of pyruvate kinase (PK) for 15 min at 4°C. RNase A was added to a final concentration of 0.02 mg/ml and incubation was continued for 30 s. The reactions were stopped by the addition of 3 ml of ice cold 5% trichloroacetic acid (TCA) and filtered through nitrocellulose membrane filters to test for remaining [^{14}C]Phe-tRNA as described [32].

The abilities of EF-Tu and the chimeric proteins to participate in poly(U)-dependent polymerization of [^{14}C]phenylalanine was determined using precipitation of the polypeptide with hot TCA as described [17].

In an attempt to determine the domains of EF-Tu_{mt} responsible for its ability to use mitochondrial Phe-tRNA^{Phe}, a coupled aminoacylation-polymerization assay was used. In a 50 μl reaction, 5 pmol of active mitochondrial tRNA^{Phe} and the indicated amounts of EF-Tu or chimeric proteins were incubated for 1 h at 37°C in a buffer containing 50 mM Tris-HCl, pH 7.5, 40 mM KCl, 9 mM $MgCl_2$, 2.5 mM PEP, 0.17 units PK, 0.1 mM spermine, 2.5 mM ATP, 0.5 mM GTP, 7.5 μg poly(U), 50 units *E. coli* EF-G, 5 μM [^{14}C]phenylalanine, 0.1 μM *E. coli* ribosomes, 0.2 μM of the appropriate EF-Ts and 1.3 μM of human mitochondrial tRNA^{Phe} synthetase. The

amount of phenylalanine polymerized was quantitated using hot TCA precipitation as described [17].

3. Results and discussion

3.1. Construction of the chimeric proteins

In the first set of constructs, domains I, II and III of *E. coli* have been individually replaced with the corresponding regions of EF-Tu_{mt} leading to the formation of the chimeric proteins MEE, EME, and EEM (the letter designates the origin of the domain, with M referring to the mitochondrial and E to the *E. coli* factor, and the placement of the letter representing domains I, II or III) (Fig. 2). In the second set of constructs, domains I, II and III of EF-Tu_{mt} have likewise been replaced with the corresponding domains from *E. coli* EF-Tu yielding the chimeric proteins EMM, MEM and MME. The three domains of EF-Tu are connected by random coils (Fig. 1) which serve as hinges allowing the domains to move relative to each other [33]. The linking regions connecting the domains are highly conserved and almost identical between EF-Tu_{mt} and *E. coli* EF-Tu. To maintain the integrity of these hinge regions and to mitigate against the possibility of structural perturbations, these regions were selected as junction sites for constructing the chimeric proteins.

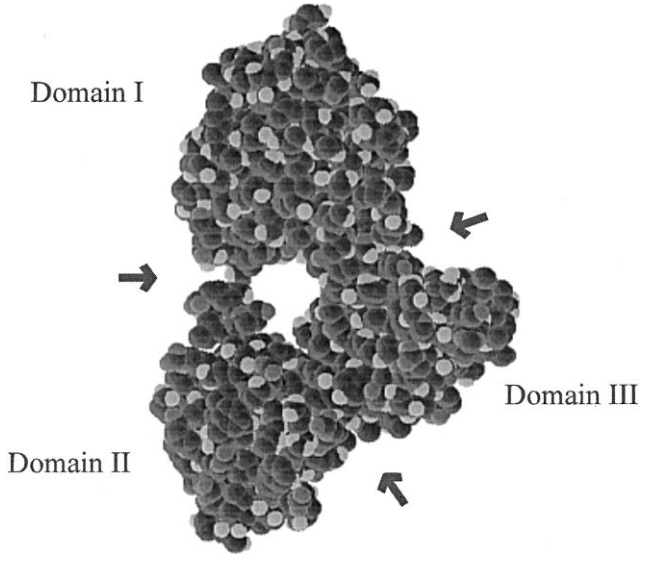

Fig. 1. Three-dimensional representation of the structure of *E. coli* EF-Tu. A space-filling model of *E. coli* EF-Tu in which the domains are indicated with roman numerals. The conformation shown corresponds to the conformation of *E. coli* EF-Tu complexed with *E. coli* EF-Ts. This conformation is similar to the conformation of EF-Tu bound to GDP. Arrows indicate domain interface regions. Coordinates were kindly provided by Dr. T. Kawashima and are displayed using RasMol.

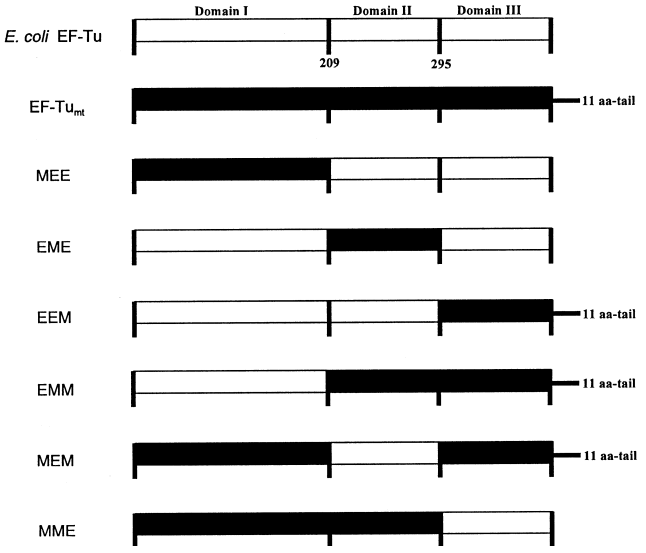

Fig. 2. Schematic representations of *E. coli* EF-Tu, EF-Tu_{mt} and the chimeric constructs. Areas depicting regions of *E. coli* EF-Tu are shown as open areas, and areas depicting regions of EF-Tu_{mt} are shown as filled areas. An eleven amino acid extension is found at the C terminus of EF-Tu_{mt} and is designated as the '11 aa tail'. The junction between domains I and II is located at residue 209 (*E. coli* numbering), and the junction between domains II and III is located at residue 295.

3.2. Ability to bind guanine nucleotides

E. coli EF-Tu has a high affinity for GDP (K_d = 8 nM, [29]) and is normally assayed by its ability to bind guanine nucleotides (Fig. 3). In contrast, EF-Tu_{mt} has a much lower affinity for GDP (K_d = 1 μM, Y.-C. Cai and L. Spremulli, manuscript in preparation) and GDP binding to this factor cannot be directly detected using nitrocellulose filter binding assays (Fig. 3). The weak binding of guanine nucleotides to EF-Tu_{mt} makes it impractical to saturate this factor with nucleotide in the filter binding assay which, in turn, makes it difficult to assess the percentage of active molecules in the preparation. Similar difficulties are encountered with mutants of *E. coli* EF-Tu that affect nucleotide binding [30]. However, the low level of binding most likely reflects

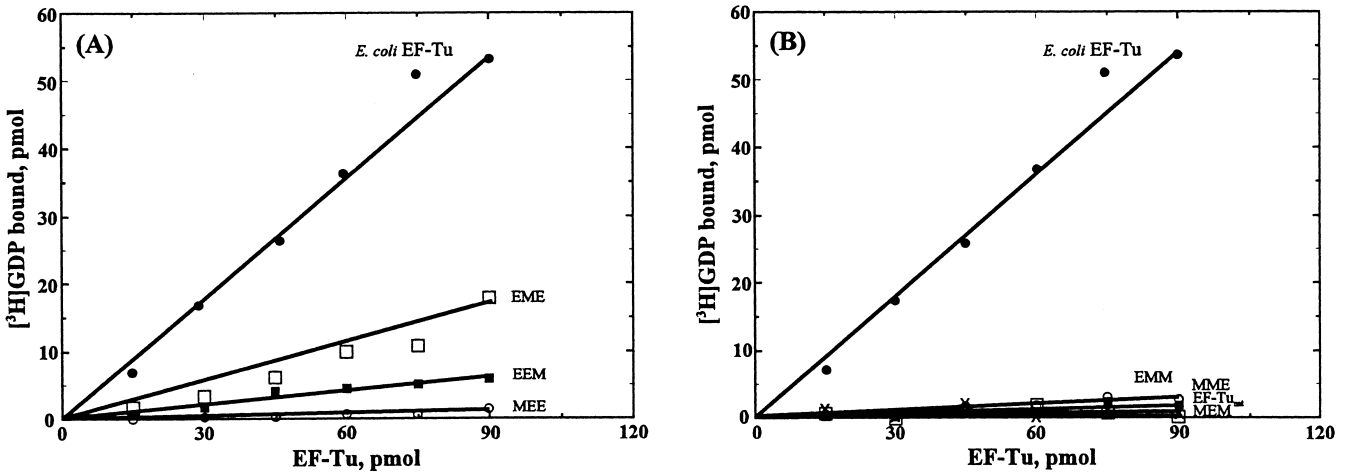

Fig. 3. Abilities of the chimeric proteins to bind guanine nucleotides. Nucleotide binding assays were carried out as described in Section 2. *E. coli* EF-Tu is shown in both graphs. (A) Chimeric derivatives of *E. coli* EF-Tu. (B) Wild-type and chimeric derivatives of EF-Tu_{mt}.

a weak interaction of EF-Tu_{mt} with GDP rather than a low percentage of active molecules since experiments using equilibrium dialysis indicate that EF-Tu_{mt} is almost fully active in binding GDP (Y.-C. Cai and L. Spremulli, manuscript in preparation). Furthermore, EF-Tu_{mt} is as active in ternary complex formation as *E. coli* EF-Tu (see below).

The guanine nucleotide binding pocket is located entirely in domain I of EF-Tu [34]. All of the residues in *E. coli* EF-Tu generally making direct contact with GDP have been conserved in EF-Tu_{mt}. Hence, the difference in nucleotide binding constants observed cannot be accounted for by changes in residues forming the nucleotide binding pocket. However, the exact conformation of the nucleotide binding pocket could influence the strength of the interaction occurring. This conformation could be an intrinsic property of domain I or could arise, at least in part, from the influences of domain II and/or domain III on the precise geometry of domain I. This question was first examined by replacing each domain of *E. coli* EF-Tu with the corresponding domain of EF-Tu_{mt} resulting in distinct chimeric proteins (MEE, EME and EEM, Fig. 2). The ability of each chimera to bind GDP was examined and compared to that of *E. coli* EF-Tu (EEE) and EF-Tu_{mt} (MMM) using the nitrocellulose filter binding assay. As indicated above, this assay allows detection of [^{3}H]GDP binding when the binding constants are strong such as observed with *E. coli* EF-Tu. However, weak nucleotide binding such as observed with EF-Tu_{mt} is not readily detectable by this method.

As indicated in Fig. 3A, nucleotide binding to *E. coli* EF-Tu was readily detectable. Replacing domain I of *E. coli* EF-Tu with domain I of EF-Tu_{mt} resulted in a chimera (MEE) which had no detectable ability to bind GDP suggesting that it has a low affinity for guanine nucleotides. This observation suggests that the weak binding of GDP to EF-Tu_{mt} could be an intrinsic property of domain I of the mitochondrial factor. Domain II of *E. coli* EF-Tu was then replaced by the corresponding domain of EF-Tu_{mt}. This chimera (EME) showed a detectable level of GDP binding, approximately 25% of that observed with *E. coli* EF-Tu (Fig. 3A). This observation suggests that the interaction between domains I and II of EF-Tu has a modest influence on the conformation of the nucleotide binding site in domain I of *E. coli* EF-Tu, and has some influence on the strength with which the prokaryotic factor binds GDP. This modest effect is in keeping with the very limited contact between domains I and II observed in the GDP bound conformation of the factor (Fig. 1). Replacing domain III of *E. coli* EF-Tu with the corresponding domain from EF-Tu_{mt} (EEM) resulted in a reduction of GDP binding to approximately 10% of that seen with the wild-type bacterial factor (Fig. 3A). This observation indicates that contacts between these two domains

influences the affinity of domain I for GDP significantly. This observation is in keeping with the more extensive contacts between domains I and III in the GDP-bound conformation of EF-Tu (Fig. 1).

As explained above, essentially no binding of GDP by EF-Tu_{mt} can be detected in the filter binding assays used here (Fig. 3B), reflecting the weak interaction of the mitochondrial factor with guanine nucleotides. The chimera in which domain I of EF-Tu_{mt} is replaced by domain I of *E. coli* EF-Tu (EMM, Fig. 2) might be expected to have a reasonable affinity for GDP since domain I of the bacterial factor has a high affinity for GDP. However, as indicated in Fig. 3B, essentially no guanine nucleotide binding was observed with this chimera. The chimeras MEM and MME also failed to bind GDP (Fig. 3B). This latter observation was expected since domain I of EF-Tu_{mt} appears to have a conformation that leads to an inherently low affinity for GDP. Clearly, neither the limited contact with domain II of *E. coli* EF-Tu nor the more extensive contacts possible with domain III of the prokaryotic factor are able to overcome the lack of binding observed with domain I of EF-Tu_{mt}.

The data provided above clearly indicate that domain III and, to a certain extent domain II, exert a regulatory effect on the binding of GDP to domain I of *E. coli* EF-Tu. These effects may arise through a stabilization of the conformation of domain I required for GDP binding. Other experiments have shown that domain I of *E. coli* EF-Tu expressed alone has a reduced affinity for GDP. Indeed the isolated domain I of *E. coli* EF-Tu has an affinity for GDP similar to that of intact EF-Tu_{mt} [35].

3.3. The ability of chimeric forms of EF-Tu to form ternary complexes with E. coli aa-tRNA

The ability of EF-Tu to form a ternary complex with GTP and aa-tRNA is essential for its ability to function in protein synthesis. The conformation of EF-Tu in the ternary complex is different than when the factor is bound to EF-Ts or GDP. Domains II and III are tightly packed against domain I, and the large opening (Fig. 1) between the domains present when EF-Tu is complexed with EF-Ts or GDP is closed [5,36,37]. The binding of aa-tRNA by EF-Tu involves all three domains with the aa-CCA end of the aa-tRNA binding primarily at the interface of domains I and II and the TΨC stem interacting primarily with domain III [7]. A cleft corresponding to the size of the tRNA acceptor helix is formed by the junction of the three domains, which in *T. thermophilus* EF-Tu is lined with positively charged amino acids [5]. The 5′ end of the tRNA is also bound at the junction of the three domains and interacts primarily with residues from domains I and III [7]. Clearly the interface between the domains must be correctly aligned for EF-Tu to form the ternary complex. Hence, it was of interest to examine the abilities of the chimeric proteins to bind aa-tRNA in the presence of GTP.

Nuclease protection assays were used to examine the abilities of the normal and chimeric proteins to form ternary complexes with *E. coli* Phe-$tRNA^{Phe}$ [32]. This assay measures the formation of a ternary complex by the ability of EF-Tu to protect the 3′ end of the tRNA with the covalently attached [^{14}C]Phe from nuclease digestion [38]. *E. coli* EF-Tu and EF-Tu_{mt} were both effective in protecting Phe-$tRNA^{Phe}$ from digestion (Fig. 4) indicating that they are both active in ternary complex formation. This observation is in agreement with previous data indicating that the mitochondrial factor forms a ternary complex that is sufficiently stable to be detected in the nuclease-protection assay [19,39]. All of the chimeras in which one of the three domains of *E. coli* EF-Tu was replaced with the corresponding domain from EF-Tu_{mt} (MEE, EME and EEM) were active in forming ternary complexes (Fig. 4). In a qualitative sense, replacing domain I of *E. coli* appeared to have a slightly negative effect on ternary complex formation while replacing either domains II or III had a positive effect on the ability to form the ternary complex. The chimeras in which the three domains of EF-Tu_{mt} were systematically replaced by the corresponding domains of *E. coli* EF-Tu were also tested for the ability to form ternary complexes (Fig. 4). All of these constructs were within 2-fold of the native construct in this assay.

Efforts were made to use the nuclease protection assay to determine the equilibrium dissociation constants for ternary complex formation by the chimeras. For this assay to be applicable for the determination of the dissociation constant, the release of the aminoacyl-tRNA from EF-Tu must be slow com-

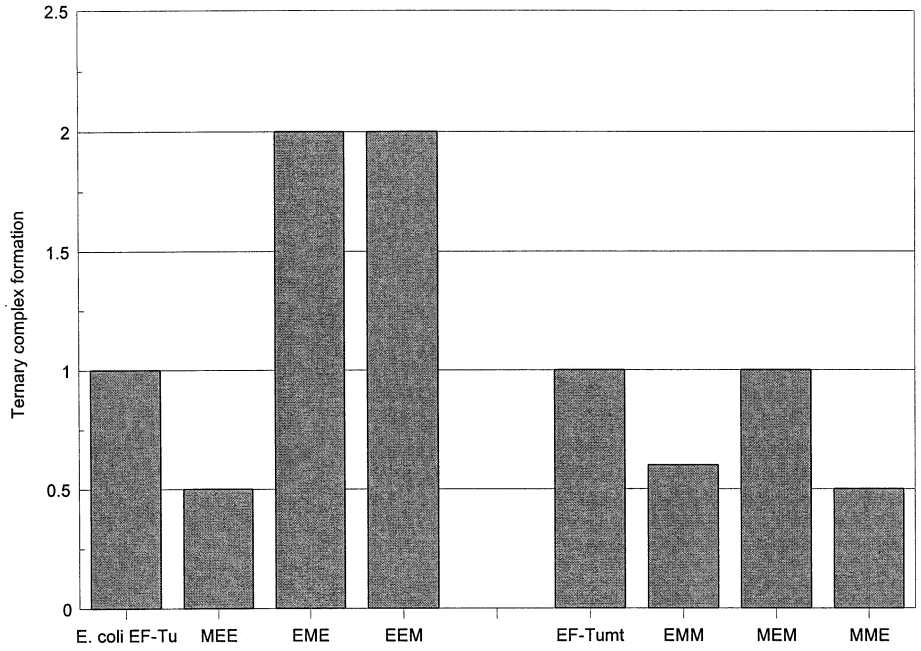

Fig. 4. Ternary complex formation measured by protection of *E. coli* Phe-tRNA^{Phe} from nuclease digestion. Nuclease protection assays were carried out as described in Section 2. Several levels of the EF-Tu derivatives were tested for the ability to form ternary complexes. The amounts of ternary complexes formed by *E. coli* EF-Tu and EF-Tu$_{mt}$ were set at a relative value of 1. These values represented approximately 7 pmol of Phe-tRNA protected from nuclease digestion. The amount of ternary complex formed by the corresponding levels of the chimeric proteins were normalized to this value. Wild-type and derivatives of *E. coli* EF-Tu are shown on the left and wild-type and derivatives of EF-Tu$_{mt}$ are shown on the right. Blanks representing background amounts of [^{14}C]Phe retained on the nitrocellulose filters in the absence of EF-Tu (about 0.2 pmol) have been subtracted from each value. The relative values were reproducible within ±10%.

pared to the time scale of the nuclease action. Unfortunately, this criterion did not appear to be met for several of the constructs preventing us from assessing the strength of ternary complex formation in a quantitative manner.

3.4. Ability to interact with EF-Ts

The catalytic function of EF-Tu is facilitated by EF-Ts which promotes the guanine nucleotide exchange reaction. The abilities of the various chimeric proteins to interact with EF-Ts were tested by examining their activities in poly(U)-directed polymerization using *E. coli* Phe-tRNA in the presence or absence of *E. coli* EF-Ts or EF-Ts$_{mt}$. This study was of particular interest because of the observation that the activity of EF-Tu$_{mt}$ can be stimulated by EF-Ts$_{mt}$ but not by *E. coli* EF-Ts. In contrast, as reported previously [18] and as indicated in Table 1, the activity of *E. coli* EF-Tu is stimulated by either *E. coli* EF-Ts or EF-Ts$_{mt}$. It should also be noted that EF-Ts$_{mt}$ binds *E. coli* EF-Tu with a 100-fold greater binding constant than does *E. coli* EF-Ts [18].

EF-Ts interacts with both domains I and III of *E. coli* EF-Tu [4]. The role of domain I of *E. coli* EF-Tu in the interaction with EF-Ts was first examined using the chimeric protein in which domain I of the bacterial factor was replaced by domain I of EF-Tu$_{mt}$ (MEE) (Table 1). The activity of this chimera was stimulated 3-fold by *E. coli* EF-Ts compared to a 10-fold stimulation observed with the wild-type *E. coli* EF-Tu ([18] and Table 1). This observation suggests that *E. coli* EF-Ts interacts more effectively with domain I of *E. coli* EF-Tu than domain I of EF-Tu$_{mt}$. The data also suggest that the failure of *E. coli* EF-Ts to stimulate the activity of EF-Tu$_{mt}$ does not reside exclusively in its interaction with domain I.

Surprisingly, the activity of the chimera MEE was completely inhibited by EF-Ts$_{mt}$ (Table 1). This observation suggests that EF-Ts$_{mt}$ binds tightly to MEE and is not effectively released in the presence

Table 1
Stimulation of the activities of *E. coli* EF-Tu and its chimeric derivatives in polymerization by *E. coli* EF-Ts and EF-Ts_{mt}[a]

	E. coli EF-Tu	MEE	EME	EEM
Minus EF-Ts[b]	1	1	1	1
E. coli EF-Ts	10	3	4	4
EF-Ts_{mt}	6	<0.1	0.5	1

[a]The abilities of *E. coli* EF-Tu and its derivatives to interact productively with prokaryotic and mitochondrial EF-Ts were investigated by testing the abilities of these nucleotide exchange factors to promote the activity of EF-Tu in the poly(U)-directed polymerization of phenylalanine using *E. coli* Phe-tRNA and a saturating amount of *E. coli* ribosomes (21 pmol).
[b]Several different levels of each construct were tested alone or in the presence or absence of the EF-Ts as indicated. The value for the amount of [^{14}C]Phe incorporated at 0.2 μM EF-Tu or chimera (in the linear region of the dose-response curve) in the absence of any EF-Ts was set at a relative value of 1. The actual amount of incorporation varied from 3–10 μM [^{14}C]Phe polymerized depending on the specific enzyme tested. The stimulation in reactions containing either *E. coli* EF-Ts or EF-Ts_{mt} were then normalized to this value.

of GTP and aa-tRNA. Thus, EF-Ts_{mt} would inhibit the formation of the ternary complex by MEE and its subsequent activity in protein synthesis. To test this theory a biotinylated derivative of EF-Ts_{mt} was prepared. The ability of this derivative to bind *E. coli* EF-Tu, EF-Tu_{mt} and all of the chimeric proteins was tested using a modified Western procedure. This analysis indicated that EF-Ts_{mt} could indeed bind wild-type and chimeric EF-Tu proteins equally well (data not shown).

Although domain II does not make direct contact with EF-Ts, the important role of interdomain contacts in the activity of EF-Tu made it of interest to examine the effect of replacing domain II of *E. coli* EF-Tu with the corresponding domain from EF-Tu_{mt} (EME). The activity of this chimera was stimulated 4-fold by *E. coli* EF-Ts (Table 1). Surprisingly, the activity of EME was still substantially inhibited by EF-Ts_{mt} (Table 1). Since EF-Ts_{mt} has no direct contacts with domain II of EF-Tu, these results suggest that contacts of domain I and/or III with domain II actually play a role in the ability of EF-Ts to promote guanine nucleotide exchange and emphasizes the importance of interdomain contacts in all of the conformational transitions observed during the function of EF-Tu in protein synthesis.

The role of domain III of *E. coli* EF-Tu in interactions with EF-Ts was examined next by replacing that domain with the corresponding domain from EF-Tu_{mt} (EEM) (Table 1). The activity of this chimera was stimulated 4-fold by *E. coli* EF-Ts. EF-Ts_{mt} neither stimulated or inhibited the activity of EEM.

Taken together, observations with these *E. coli* EF-Tu derivatives suggest that all three domains influence the ability of *E. coli* EF-Ts to stimulate the activity of *E. coli* EF-Tu. These observations also suggest that the binding of EF-Ts_{mt} with EF-Tu stems predominantly from interactions with domain I and that correct alignment with domain II is necessary to regulate the release of EF-Ts_{mt} in the presence of GTP and aa-tRNA.

As indicated above and in Table 2, the activity of EF-Tu_{mt} is not stimulated by *E. coli* EF-Ts, but is stimulated 10-fold by EF-Ts_{mt}. The activity of the chimera EMM, in which domain I of EF-Tu_{mt} had been replaced with that of the prokaryotic factor, was stimulated 3-fold by *E. coli* EF-Ts (Table 2). The ability of *E. coli* EF-Ts to stimulate the activity of EMM but not EF-Tu_{mt} indicates that one of the barriers for productive interaction of the mitochondrial EF-Tu with the bacterial EF-Ts lies in domain I. The stimulation of the activity of EMM by *E. coli*

Table 2
Stimulation of the activities of EF-Tu_{mt} and its chimeric derivatives in polymerization by *E. coli* EF-Ts and EF-Ts_{mt}[a]

	EF-Tu_{mt}	EMM	MEM	MME
Minus EF-Ts[b]	1	1	1	1
E. coli EF-Ts	1	3	3	3
EF-Ts_{mt}	10	<0.1	1	5

[a]The abilities of EF-Tu_{mt} and its derivatives to interact productively with prokaryotic and mitochondrial EF-Ts were investigated by testing the abilities of these nucleotide exchange factors to promote the activity of EF-Tu in the poly(U)-directed polymerization of phenylalanine using *E. coli* Phe-tRNA and a saturating amount of *E. coli* ribosomes (21 pmol).
[b]Several different levels of each construct were tested alone or in the presence or absence of the EF-Ts as indicated. The value for the amount of [^{14}C]Phe incorporated at 0.2 μM EF-Tu or chimera (in the linear region of the dose-response curve) in the absence of any EF-Ts was set at a relative value of 1. The actual amount of incorporation varied from 3–10 μM [^{14}C]Phe polymerized depending on the specific enzyme tested. The stimulation in reactions containing either *E. coli* EF-Ts or EF-Ts_{mt} were normalized to this value.

EF-Ts is lower than the 10-fold stimulation seen with *E. coli* EF-Tu supporting the idea that domain I is only partially responsible for the differences observed in the abilities of *E. coli* and mitochondrial EF-Tu to interact with *E. coli* EF-Ts. EF-Ts_{mt} again completely inhibited the activity of EMM suggesting that this chimera has a tight association with EF-Ts_{mt} and is not effectively released in the presence of GTP and aa-tRNA.

Surprisingly, the activity of the chimeric protein (MEM) is also stimulated 3-fold by *E. coli* EF-Ts. This observation emphasizes the idea that domain II plays an important role in the nucleotide exchange reaction despite that fact that it does not contact EF-Ts directly. This conclusion was strengthened by the observation that the activity of MEM was not stimulated by EF-Ts_{mt} although the native mitochondrial factor is stimulated 10-fold by the presence of EF-Ts_{mt} (Table 2).

Finally, the activity of the chimera MME was stimulated 3-fold by the presence of *E. coli* EF-Ts (Table 2). Interestingly, the activity of MME was stimulated 5-fold by EF-Ts_{mt}. The results obtained with these three chimeras suggest that both domains I and II must be derived from EF-Tu_{mt} for productive interaction with EF-Ts_{mt} while domain III appears to have little influence.

The crystal structure of the *E. coli* EF-Tu·Ts complex shows that EF-Ts interacts with domains I and III of EF-Tu [4]. In this conformation the domains form interfaces, composed of conserved residues between domains I and III, and domains II and III. Domains I and II do not make extensive contacts (Fig. 1) [34,40]. If domain I and II are both from EF-Tu_{mt} (MME) and are spatially aligned in the correct orientation, the tight association between domains II and III may pull domain III into position allowing the formation of a proper interface with domain I, thereby promoting a productive interaction with EF-Ts_{mt}. In chimeras EMM and MEM, in which domains I and II are not both from EF-Tu_{mt}, the spatial arrangement of domains I and II may preclude the formation of the interface between domains I and III. This is thought to be the case in other studies in which mutations in domain II of *E. coli* EF-Tu are thought to cause a shift in domain II relative to domain I resulting in a change of the binding constants between EF-Tu and GTP and aminoacyl-tRNA [41].

3.5. Ability to utilize mitochondrial Phe-$tRNA^{Phe}$

It has been postulated that one role of EF-Tu is to induce a uniform conformation in all aa-tRNAs as they enter the A-site of the ribosome [42,43]. EF-Tu_{mt} can form ternary complexes with either *E. coli* Phe-$tRNA^{Phe}$ or mammalian mitochondrial Phe-$tRNA^{Phe}$ and promote the binding of these complexes to the A-site of either prokaryotic or mitochondrial ribosomes [19]. In contrast, *E. coli* EF-Tu

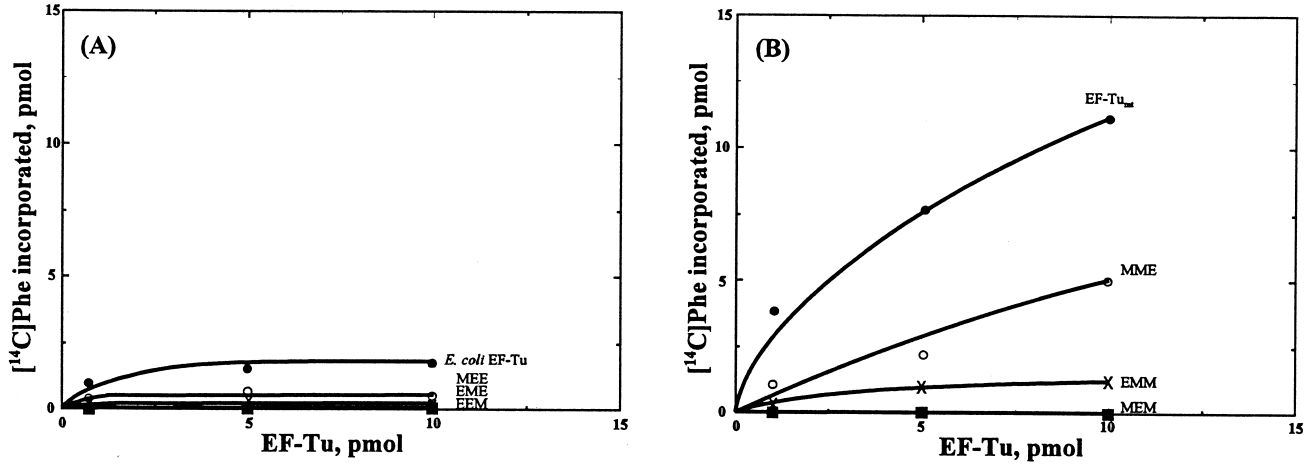

Fig. 5. Ability of wild-type *E. coli* EF-Tu, EF-Tu_{mt} and chimeric proteins to function in translation with mitochondrial Phe-$tRNA^{Phe}$. Coupled aminoacylation/polymerization assays were carried out as described in Section 2. (A) Wild-type *E. coli* EF-Tu and its derivatives. (B) Wild-type EF-Tu_{mt} and its derivatives. The amount of chargeable mitochondrial $tRNA^{Phe}$ was 5 pmol. Blanks representing background amounts of [^{14}C]Phe retained on the nitrocellulose filters in the absence of EF-Tu have been subtracted from each value.

can form ternary complexes with mitochondrial Phe-tRNA^{Phe} but cannot bind it to the A-site of ribosomes [19]. The primary sequences and secondary structures of tRNAs from most organisms are sufficiently conserved that these tRNAs fold into the familiar 'L-shaped' tertiary structure [44–46]. In contrast, variations in the primary and secondary structures in mitochondrial tRNAs lead to a 'boomerang' shaped tertiary structure [47,48]. This altered structure may account for the failure of *E. coli* EF-Tu to promote the binding of mitochondrial aa-tRNAs to the A-site.

All three domains of EF-Tu are involved in the interaction with aa-tRNA. In an effort to assess the roles of each domain of EF-Tu$_{mt}$ in allowing the use of mitochondrial aa-tRNAs, the abilities of the chimeric proteins to carry out polymerization with mitochondrial Phe-tRNA^{Phe} were tested (Fig. 5). To optimize the availability of charged mitochondrial tRNA^{Phe}, coupled aminoacylation/polymerization assays was used. In the presence of mitochondrial Phe-tRNA^{Phe}, none of the chimeric proteins in which any one domain of *E. coli* EF-Tu was replaced with the corresponding domain from EF-Tu$_{mt}$ could effectively catalyze the polymerization of phenylalanine (Fig. 5A). These observations indicate that more than one domain of EF-Tu$_{mt}$ is needed to facilitate binding of mitochondrial Phe-tRNA^{Phe} to the A-site of the ribosome.

Replacement of domain I or II of EF-Tu$_{mt}$ with the corresponding domain from *E. coli* EF-Tu (EMM and MEM, respectively) resulted in constructs unable to support binding of mitochondrial Phe-tRNA^{Phe} to the A-site of ribosomes (Fig. 5B). This observation indicates that domains I and II of EF-Tu$_{mt}$ are vital in inducing the correct conformation of mitochondrial aa-tRNA for ribosome binding.

Finally, the EF-Tu$_{mt}$ derivative (MME) in which domain III was replaced by domain III from *E. coli* EF-Tu was analyzed for its ability to support binding of mitochondrial Phe-tRNA^{Phe} to the A-site of the ribosome (Fig. 5B). MME was approximately 50% as active in polymerization of phenylalanine in the presence of mitochondrial Phe-tRNA^{Phe} as wild-type EF-Tu$_{mt}$. This observation indicates that even though domain III interacts with the mitochondrial tRNA in the ternary complex, it does not play a central role in inducing the structural changes necessary for proper binding to the A-site of the ribosome.

3.6. Summary

The work presented here strongly indicates that the properties of EF-Tu arise from the complex interaction of the three domains of this protein. The binding of guanine nucleotides to *E. coli* EF-Tu domain I is affected by the presence of both domains II and III. Both of these domains act to regulate the efficiency with which domain I binds GDP. Alternatively, the lower affinity for guanine nucleotides characteristic of EF-Tu$_{mt}$ was not increased by the replacement of any one of its three domains by the corresponding domain from *E. coli* EF-Tu.

E. coli EF-Ts stimulated the activity of each of the chimeric enzymes even though it could not stimulate wild-type EF-Tu$_{mt}$. This observation suggests that the lack of activity observed with EF-Tu$_{mt}$ arises from rather subtle conformational differences resulting from interdomain contacts and not from differences in a few critical residues. Domains I and II of EF-Tu$_{mt}$ appear to be required for productive interaction with EF-Ts$_{mt}$ even though domain II is not in direct contact with EF-Ts. The origin of domain III is of less importance.

The unique ability of EF-Tu$_{mt}$ to promote the binding of mitochondrial aa-tRNA to the A-site of the ribosome lies primarily in domains I and II, although for full activity domain III is also important. *E. coli* EF-Tu, regardless of which domains were replaced with the corresponding domains from EF-Tu$_{mt}$ could not induce mitochondrial tRNA into the proper conformation for ribosome binding.

The data provided here argue that the properties of EF-Tu cannot be analyzed solely in the context of the roles and properties of its three domains. Rather, the function of this factor in protein synthesis must be evaluated from the perspective of the whole protein with its three closely integrated physical domains.

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