Bovine mitochondrial elongation factor Ts (EF-Tsmt) stimulates the activity of Escherichia coli elongation factor Tu (EF-Tu). In contrast, E. coli EF-Ts is unable to stimulate mitochondrial EF-Tu. EF-Tsmt forms a tight complex with E. coli EF-Tu governed by an association constant of 8.6 × 10^{10}. This value is 100-fold stronger than the binding constant for the formation of the E. coli EF-Tu Ts complex. To test which domain of EF-Tsmt is important for its strong binding with EF-Tu, chimeras were made between E. coli EF-Ts and EF-Tsmt. Replacing the N-terminal domain of E. coli EF-Ts with that of EF-Tsmt increases its binding to E. coli EF-Tu 2-3-fold. Replacing the N-terminal domain of EF-Tsmt with the corresponding region of E. coli EF-Ts decreases its binding to E. coli EF-Tu ~4-5-fold. A chimera consisting of the C-terminal half of E. coli EF-Ts and the N-terminal half of EF-Tsmt binds to E. coli EF-Tu as strongly as EF-Tsmt. A chimera in which Subdomain N of the core of EF-Ts is replaced by the corresponding region of EF-Tsmt binds E. coli EF-Tu ~25-fold more tightly than E. coli EF-Ts. Thus, the higher strength of the interaction between EF-Tsmt and EF-Tu can be localized primarily to a single subdomain.

The classical model for the elongation cycle of protein biosynthesis was developed on observations made with Escherichia coli EF-Tu. EF-Tu forms a ternary complex with aminoacyl-tRNA and GTP that promotes the binding of the aminoacyl-tRNA to the A-site of the ribosome. EF-Tu then hydrolyzes the bound GTP and is released from the ribosome as an EF-Tu-GDP complex. A second elongation factor (EF-Ts) promotes the release of GDP, forming an intermediate EF-Tu-GDP complex. In E. coli, GDP binds to the EF-Tu-GTs complex, promoting the release of EF-Ts and the formation of an EF-Tu-GTP complex. A new ternary complex can then form, and the cycle repeats. In Thermus thermophilus, a dimeric complex (EF-Tu-Ts) occurs through the interaction of two EF-Tu molecules with a stable EF-Ts dimer (3). In contrast to the E. coli EF-Tu-Ts complex, the T. thermophilus complex is not dissociated to a significant extent by either GDP or GTP alone (4).

Mitochondrial EF-Tu and EF-Ts have been purified from bovine liver as a tightly associated complex (EF-Tu-Tsmt) (5, 6). The EF-Tu-Tsmt complex differs from the corresponding E. coli complex in that EF-Tu-Tsmt is not readily dissociated by guanine nucleotides (5, 6). Furthermore, no significant amounts of intermediates equivalent to EF-Tu-GTP or EF-Tu-GDP can be detected in the animal mitochondrial system. However, mammalian EF-Tu-Tsmt forms a ternary complex with GTP and aminoacyl-tRNA (7). The basic steps of the bacterial elongation cycle thus appear to be occurring in mammalian mitochondria. However, the equilibrium constants that govern the interaction of EF-Tu with EF-Ts and guanine nucleotides appear to be significantly different.

The cDNAs encoding EF-Tumt and EF-Tsmt have been cloned and sequenced from bovine and human liver (8, 9). Sequence analysis indicates that EF-Tumt has 56% identity to E. coli EF-Tu while EF-Tsmt is less than 30% identical to E. coli EF-Ts. When EF-Tsmt is expressed and purified from E. coli, it forms a 1:1 complex with E. coli EF-Tu (EF-TuEcoTsmt) (9). This heterologous complex is very resistant to dissociation by guanine nucleotides even at high concentrations of GDP or GTP (10). This feature of the heterologous complex is quite reminiscent of the native EF-Tu-Tsmt complex. Thus, it is apparently the nature of EF-Ts that determines the strength of its interaction with EF-Tu. However, it is not clear what features of EF-Tsmt modulate its tight interaction with EF-Tu.

The crystal structures of trypsin-modified E. coli EF-Tu-GDP and of Thermus aquaticus EF-Tu complexed with a nonhydrolyzable GTP analogue have been determined (11, 12). Analysis of these structures indicates that EF-Tu folds into three domains. Domain I encompasses about the first 200 residues and contains the guanine nucleotide binding site. Domains II and III are each ~100 residues long. All three domains are involved in binding aminoacyl-tRNA (13). The structure of the E. coli EF-Tu-Tsmt complex has also been determined (Fig. 1) (14). This structure indicates that EF-Ts consists of 4 structural modules: the N-terminal domain (residues 1–54); the core domain (residues 55–179 and 229–263); the dimerization domain (residues 180–228); and the C-terminal module (residues 264–282). The core domain is further divided into Subdomain N (residues 55–140) and Subdomain C (residues 141–179 and 229–263). The N-terminal domain, Subdomain N, and the C-terminal module interact with Domain I of EF-Tu, whereas Subdomain C interacts with Domain III of EF-Tu. EF-Tsmt binds to EF-Tu more tightly than does E. coli EF-Ts. This work attempts to determine the regions of the EF-Tsmt giving rise to its higher affinity for EF-Tu.
EF-Tu

EF-Ts

N-Terminal

Subdomain N

h1-h3

h4-h7

s1-s3

s4-s6

Subdomain C

h8, h12

Dimerization

h9-h11

FIG. 1. Three-dimensional representation of the structure of E. coli EF-Tu-Ts. The structure of EF-Tu is shown on the left with the domains labeled I, II, and III. EF-Ts is shown on the right, with the N-terminal domain (h1–h3) at the top. Coordinates were kindly provided by Dr. T. Kawashima and are displayed using RASWIN.

MATERIALS AND METHODS

Construction of Deletion Mutants of EF-Ts—Clones encoding His-tagged variants of E. coli EF-Ts and EF-Ts<sub>mt</sub> in pET24(+<sup>1</sup>) were prepared previously (9, 15). To make a mutant of E. coli EF-Ts lacking the N-terminal domain (residues 1 to 53), a fragment of the E. coli EF-Ts gene containing sequences encoding amino acid residues 54–282 was amplified by polymerase chain reaction (PCR) using primer ETsP4 (ccggctcgagcttggacatcgcagc) and ETs<sub>ΔN</sub>(ccggctcgagagactgcttggacatcgcagc). The PCR fragment was digested with NdeI and Xhol and cloned into NdeI- and Xhol-digested pET24(+<sup>1</sup>), giving the construct pETsΔN. To make a mutated form of EF-Ts<sub>mt</sub> lacking the N-terminal domain (residues 1–56), a fragment of EF-Ts<sub>mt</sub> gene containing the sequence encoding amino acid residues 57–283 was amplified by PCR using primer BMC-N (ccggatcccatatgcgtaagaccaaagaaggt) and C-Xhol (ccggatcccatatgtttctc). The PCR fragment was digested with NdeI and Xhol and cloned into NdeI- and Xhol-digested pET24(+<sup>1</sup>), giving the construct pETs<sub>ΔN</sub>ΔN.

Preparation of Chimeras of E. coli and Mitochondrial EF-Ts—To make Chimeras I and II (see Fig. 5), a HindIII site was introduced into the EF-Ts<sub>mt</sub> gene at nucleotide sequence position 342 by site-directed mutagenesis using the Chameloon<sup>TM</sup> Double-Stranded, Site-Directed Mutagenesis Kit (Stratagene). Primer MP15 (cttcctcccatggtagttcggc) and ETsP2 (ccgacgtgtgcattgtttagtgcct) was used for the mutagenesis. The HindIII site is underlined and the mutated residues are shown in boldface. This mutation changes residue Arg-54 to Lys-54. In the human EF-Tsmt gene, the amino acid sequence encoded by the N-terminal domain (residues 1–56), a fragment of the EF-Tsmt gene containing the amino acid residues 57–282 was amplified by PCR using primer Vec-2 (agagcaacatagttgtagttttagtgcct) and MP24 (agagcaacatagttgtagttttagtgcct). The amplified DNA was purified by gel electrophoresis, digested with NdeI and Xhol, and used to replace the NdeI to HindIII fragment of EF-Ts<sub>mt</sub> using standard methods. To make Chimeras II, a PCR fragment of E. coli EF-Ts encompassing amino acid residues 52 to the C terminus was amplified using primer ETsP3 (ccgacgtgtgcattgtttagtgcct) and ETsP4 (ccgagctcagtcagttttagtgcct). The E. coli EF-Ts sequence is underlined and the restriction enzyme sites are boldface. To make Chimeras I, the PCR fragment was digested with NdeI and HindIII and used to replace the NdeI to HindIII fragment of EF-Ts<sub>mt</sub> using standard methods. To make Chimeras II, the PCR fragment was amplified from 100 ng of plasmid DNA derived from the EF-Tsmt gene, while the sequence underlined is derived from the E. coli EF-Ts<sub>mt</sub> gene.

Chimeras III and IV were prepared using chimeric primers and two rounds of PCR. This strategy, which is outlined in Fig. 2, permits the preparation of chimeric proteins at any position without the need to introduce new restriction sites. To make Chimera III, a portion of EF-Ts<sub>mt</sub> gene from the N terminus to amino acid residue 162 in the mature form of this factor (9) was amplified by PCR using primer Vec-2 (taggggaattgtgagcggataac), derived from the pET24(+<sup>1</sup>) vector, and primer MP25 (agagcaacatagttgtagttttagtgcct). This primer is in itself a chimeric piece of DNA. The sequence shown in boldface is derived from the EF-Ts<sub>mt</sub> gene, while the underlined sequence is derived from the E. coli EF-Ts gene. DNA was amplified from 100 ng of plasmid DNA carrying the EF-Ts<sub>mt</sub> gene in a 100-μl reaction mixture containing 100 pmol of each primer and 2.5 units of Taq polymerase. PCR was carried out for 20 cycles (94 °C for 1 min, 55 °C for 2 min, and 72 °C for 2 min).

The DNA fragment from this amplification reaction was purified by gel electrophoresis. A chimeric EF-Ts fragment was then amplified by using primer ETsP4 and the PCR fragment prepared above as primers with the E. coli EF-Ts gene in pET24(+<sup>1</sup>) as the template and the PCR fragment conditions described above. This second DNA fragment was purified by gel electrophoresis, digested with NdeI and Xhol, and cloned into pET24(+<sup>1</sup>) to make Chimeras III.

To make Chimera IV, a PCR fragment of the E. coli EF-Ts gene in pET24(+<sup>1</sup>) from the N terminus to amino acid residue 148 was amplified by primer Vec-2 and MP24 (taggggaattgtgagcggataac). This primer is a chimeric in which the underlined sequence is derived from the E. coli EF-Ts gene while the sequence in boldface is derived from the EF-Ts<sub>mt</sub> gene. The PCR fragment was amplified by gel electrophoresis, digested with NdeI and Xhol, and cloned into...
EF-Tu was examined as described under "Materials and Methods." Reaction mixtures contained 7.3 μg of expressed E. coli EF-Tu (−80 pmol of active factor) and the indicated amounts of E. coli EF-Ts (EF-TuEco) or EF-Tsmt. Blanks representing the amount of GDP exchange carried out by EF-Tu alone during this incubation period (−7 pmol) have been subtracted from each value. A GDP binding could be detected in the absence of EF-Tu, indicating that the preparations of EF-Ts used here were free of EF-Tu. B, stimulation of the activity of E. coli EF-Tu in poly(U)-directed polymerization by E. coli EF-Ts (EF-TuEco) and EF-Tsmt. Reaction mixtures contained 1 pmol of expressed E. coli EF-Tu and the indicated amount of EF-Ts. Blanks representing the amount of polymerization catalyzed by EF-Tu alone (−7 pmol) have been subtracted from each value. C, stimulation of the activity of EF-Tsmt in polymerization by E. coli EF-Ts (EF-TuEco) and EF-Tsmt. Reaction mixtures contained 3 pmol of expressed EF-Tu and the indicated amount of EF-Ts. Blanks representing the amount of polymerization obtained with EF-Tu alone (−2.3 pmol) have been subtracted from each value.

**Expression and Purification of EF-Tu and EF-Ts**—The His-tagged forms of E. coli EF-Tu and EF-Tsmt were expressed and purified as described previously (15). E. coli EF-Ts, Chimera I, and Chimera IV were expressed as described previously (15) and purified under two conditions. In the first set of conditions, 10 mM Mg²⁺ was included in the buffers. In the second set of conditions, 10 μM GDP was also included in the preparation buffers. Expression of EF-Tsmt and Chimeras II, III, and V was carried out as described (9), and these proteins were purified under native and denaturing conditions. Under native conditions, 10 mM Mg²⁺ was included in the buffers, but no GDP was added. When cell extracts were prepared under native conditions, EF-Tsmt was isolated as 1:1 complex with E. coli EF-Tu. To purify EF-Tsmt, Chimera II, and Chimera III free of E. coli EF-Tu, the EF-TuEco-EF-Tsmt complexes were denatured. The His-tagged forms of EF-Tsmt and the chimeras were purified through a nickel-nitrilotriacetic acid column and renatured (10). The protein concentrations were determined by the Micro-Bradford method (Bio-Rad). Samples (−10 μg each) were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Blue (16).

**Binding Constant Measurements**—To determine the binding constants of EF-Tsmt, Chimeras III and V to E. coli EF-Tu, 1:1 complexes of EF-TuEco ∼ EF-Tsmt, EF-TuEco-Chimera III, or EF-TuEco-Chimera V (0.2 to 0.4 μM) were incubated in 500-μl reactions containing 10 μM or 20 μM [3H]GDP (200 cpm/pmol) in Buffer A (50 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 1 mM dithiothreitol, and 0.1 mM NH₄Cl) at 0°C for 90 min. The amount of EF-TuEco-GDP present at equilibrium was determined using a nitrocellulose filter binding assay (17). The amount of active EF-Tu in the complex was estimated to be ~50% based on its ability to bind GDP. To determine the binding constants of E. coli EF-Tu-EF-Tsmt, EF-TuEco-Chimera I and Chimera II to E. coli EF-Tu, EF-TuEco-[3H]GDP (0.5 μM, 50 pmol), [3H]GDP (500 cpm/pmol) (5 or 10 μM) and EF-Ts or its chimeras (1–3 μM, 100–300 pmol) were incubated in a reaction mixture (100 μl) at 20°C for 30 min in the buffer indicated above. The amount of EF-TuGDP at equilibrium was measured by the nitrocellulose filter binding assay (17). K_ds for the reaction

EF-Tu ∙ GDP + EF-Ts ⇄ EF-Tu ∙ Ts ∙ GDP 

(Eq. 1)

K_ds = [EF-Tu ∙ Ts ∙ GDP] / [EF-Tu ∙ GDP][EF-Ts] 

(Eq. 3)

K_ds[GDP] is the binding constant of E. coli EF-Tu to GDP (3.3 × 10⁸ M⁻¹) (19). The E. coli EF-Ts used for these experiments was ~50% active based on its ability to bind GDP. EF-Ts was estimated to be fully active based on the percentage of EF-Tsmt that could bind EF-Tu.

**RESULTS**

Interaction of E. coli and Mitochondrial EF-Ts with E. coli and Mitochondrial EF-Tu—When mitochondrial EF-Tsmt is expressed in E. coli as a His-tagged protein, it forms a 1:1 complex with E. coli EF-Tu (9). Free EF-Tsmt can be purified by denaturing this complex and then allowing renaturation of the EF-Tsmt (10). E. coli EF-Ts has also been overexpressed in E. coli and purified as a His-tagged protein (15). When no GDP is added to the isolation buffers, a small amount of E. coli EF-Tu co-purifies with the E. coli EF-Ts following chromatography on nickel-nitrilotriacetic acid resin. E. coli EF-Ts was prepared free of E. coli EF-Tu by using buffers containing GDP and Mg²⁺.

The abilities of EF-Tsmt and E. coli EF-Ts to stimulate GDP exchange and poly(U)-directed polymerization with E. coli EF-Tu were tested (Fig. 3, A and B). The purified EF-Tsmt stimulates guanine nucleotide exchange with E. coli EF-Tu and also stimulates the poly(U)-directed polymerization of phenylalanine (Fig. 3A). However, substantially higher levels of EF-Tsmt are required to achieve the same degree of stimulation observed with E. coli EF-Ts. Nearly 10-fold higher concentrations of EF-Tsmt are required to promote the same amount of nucleotide exchange obtained with E. coli EF-Ts (Fig. 3A). The activity of EF-Tsmt in stimulating poly(U)-directed polymerization...
tion with *E. coli* EF-Tu is ~25% of that obtained with *E. coli* EF-Ts (Fig. 3B).

EF-Tsmt stimulates the activities of both EF-Tu mt and *E. coli* EF-Tu in poly(U)-directed polymerization (Fig. 3A, B, and C). *E. coli* EF-Ts cannot stimulate the activity of EF-Tu mt (Fig. 3C). This observation indicates that *E. coli* EF-Ts may be unable to bind EF-Tu mt or that it binds to EF-Tu mt much more weakly than GDP does. Alignment of the primary sequence of *E. coli* EF-Tu and EF-Tu mt indicates that these two factors are 56% identical. In addition, all of the residues in *E. coli* EF-Tu that are in contact with EF-Ts in the crystal structure are identical or are conservative replacements in EF-Tu mt. Thus, the failure of *E. coli* EF-Ts to stimulate the mitochondrial factor in translation is surprising.

As indicated above, higher levels of EF-Tsmt are required to produce the same degree of stimulation of *E. coli* EF-Tu observed with low levels of *E. coli* EF-Ts. Previous results have suggested that EF-Tsmt binds to *E. coli* EF-Tu more tightly than does *E. coli* EF-Ts (10). This idea is based on the observation that the heterologous complex EF-Tu Eco EF-Tsmt is not readily dissociated by guanine nucleotides while the homologous *E. coli* complex is. Thus, the lower activity of EF-Tsmt probably arises from its slow release from EF-Tu which reduces the rate of ternary complex formation. To test this idea, we have determined the approximate equilibrium association constant for the binding of EF-Tsmt to *E. coli* EF-Tu and have compared this value with that obtained with the homologous factor.

To determine the association constant for the binding of EF-Tsmt to *E. coli* EF-Tu, the $K_{obs}$ of the following reaction was measured as described under "Materials and Methods."

$$E. coli\ EF-Tu \cdot GDP + EF-Tsmt \rightleftharpoons EF-TuEco \cdot Tsmt + GDP \quad (\text{Eq. 4})$$

The binding constant ($K_p$) of EF-Tsmt to *E. coli* EF-Tu was calculated from $K_{obs}$, and the binding constant of *E. coli* EF-Tu to GDP ($3.3 \times 10^8 \text{ M}^{-1}$) (Table I) as described by Miller and Weissbach (19). The value obtained ($8.6 \times 10^{10}$) indicates that EF-Tsmt binds to *E. coli* EF-Tu quite tightly. The $K_{obs}$ for *E. coli* EF-Ts was also measured, and the corresponding binding constant ($K_p$) was calculated (Table I). The $K_p$ of *E. coli* EF-Ts determined here ($9 \times 10^8 \text{ M}^{-1}$) is quite similar to the value of $5 \times 10^8 \text{ M}^{-1}$ obtained from the literature (19). Comparison of the $K_p$ of *E. coli* EF-Ts with the $K_p$ of EF-Tsmt indicates that EF-Tsmt binds to *E. coli* EF-Tu ~100 times more tightly than does *E. coli* EF-Ts.

**Role of the N-terminal Domain of EF-Tsmt—Analysis of the protease sensitivity of EF-Ts (20) and the x-ray structure of the *E. coli* EF-TuTs complex (14) shows that the N-terminal region of *E. coli* EF-Ts folds into an independent domain (Fig. 1). This region is essential for the ability of *E. coli* EF-Ts to stimulate guanine nucleotide exchange with EF-Tu (20). Sequence alignment indicates that there is significant homology between *E. coli* EF-Ts and EF-Tsmt in the N-terminal domain and it is likely that it will fold in a similar three-dimensional structure. It was, therefore, of interest to determine whether the N-terminal domain of EF-Tsmt was also important for its binding to EF-Tu or whether other interactions of this factor could compensate for the loss of this domain. To examine this question, N-terminal deletion mutants of *E. coli* EF-Ts and EF-Tsmt were constructed (Fig. 5). The EF-Ts deletion mutants were tested for their abilities to bind *E. coli* EF-Tu and to stimulate the activities of *E. coli* EF-Tu and EF-Tsmt in polymerization. The N-terminal deletion mutant of *E. coli* EF-Ts is unable to stimulate the activity of EF-Tu in guanine nucleotide exchange or in poly(U)-directed polymerization (data not shown). This observation is in agreement with previous results showing that a proteolytic derivative of *E. coli* EF-Ts lacking the N-terminal domain is unable to stimulate the activity of EF-Tu (20). The N-terminal deletion mutant of EF-Tsmt is unable to bind *E. coli* EF-Tu (data not shown). It is also inactive in stimulating the activity of either *E. coli* EF-Tu or EF-Tsmt (data not shown). These data indicate that the N-terminal domain of EF-Tsmt, like that of *E. coli* EF-Ts, is important for its function in protein synthesis. These data, while indicating that the N-terminal domain is important for the interaction of EF-Tsmt with EF-Tu, do not provide any insight into whether this region plays a role in the stronger affinity for EF-Tu observed with EF-Tsmt.

**Predicted Secondary Structure of EF-Tsmt and Analysis of Chimeric Proteins between *E. coli* and Mitochondrial EF-Ts—In the crystal structure of the *E. coli* EF-TuTs complex (Fig. 1) (14), the N-terminal domain and Subdomain N of the core interact with Domain I of *E. coli* EF-Tu. Subdomain C of the core interacts with Domain III of *E. coli* EF-Tu. As indicated above, EF-Tsmt binds to *E. coli* EF-Tu more tightly than does *E. coli* EF-Ts. It has not yet been possible to make direct measurements of the binding constant of EF-Tsmt for EF-Tu (data not shown). However, it is clear that guanine nucleotides cannot dissociate the EF-TuTsmt complex (6). Thus, it is likely that EF-Tsmt will

---

**Table I**

<table>
<thead>
<tr>
<th>EF-Ts used</th>
<th>$K_{obs}$</th>
<th>$K_p$ ($\text{M}^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> EF-Ts</td>
<td>2.7 ± 0.4</td>
<td>8.9 ± 1.3 x 10^8</td>
</tr>
<tr>
<td>EF-Tsmt</td>
<td>260 ± 29</td>
<td>8.6 ± 1.0 x 10^10</td>
</tr>
<tr>
<td>Chimera I</td>
<td>6.8 ± 1.9</td>
<td>2.3 ± 0.6 x 10^9</td>
</tr>
<tr>
<td>Chimera II</td>
<td>59 ± 0.2</td>
<td>1.9 ± 0.2 x 10^10</td>
</tr>
<tr>
<td>Chimera III</td>
<td>3.08 ± 0.62</td>
<td>1.0 ± 0.2 x 10^11</td>
</tr>
<tr>
<td>Chimera V</td>
<td>67 ± 12</td>
<td>2.2 ± 0.4 x 10^10</td>
</tr>
</tbody>
</table>

---

**Fig. 4.** Secondary structure of EF-Ts. The secondary structure of *E. coli* EF-Ts is based on the crystal structure (14). The secondary structure of EF-Tsmt is based on multiple sequence alignments using the Pileup program in GCG and the Clustalw program followed by manual adjustment of the alignment. aa, amino acids.
also have a high affinity for EF-Tu\textsubscript{mt}. To help determine which region(s) might be important for the strong interaction of EF-Ts\textsubscript{mt} with EF-Tu, an analysis of the possible structure of EF-Ts was carried out using the 3-dimensional structure of \textit{E. coli} EF-Ts\textsubscript{Eco} and several secondary structure prediction programs as a guide (Fig. 4). The overall lengths of \textit{E. coli} EF-Ts and the mature form of EF-Ts\textsubscript{mt} are the same (9). However, the two factors appear to have several significant differences in their overall structure. EF-Ts\textsubscript{mt} aligns well with \textit{E. coli} EF-Ts and the N-terminal domain, and this region of the protein most likely folds into 3 helices as observed for the \textit{E. coli} factor. The first 2 strands of $\beta$-sheet in Subdomain N and helices h4 and h5 are also predicted to be present in EF-Ts\textsubscript{mt}. However, Subdomain N is interrupted by an insertion of $\sim$20 amino acids. The precise position of this insertion is difficult to predict since the two proteins show very little primary sequence homology in this region making the alignment difficult. The remainder of Subdomain N including helices h6 and h7 and the final strand of the $\beta$-sheet (s3) are all predicted to be present. Subdomain C is predicted to begin with $\beta$-strand s4 which is followed by an insertion of $\sim$12 residues before strands s5 and s6 and helices h8 and h12 are found. The dimerization domain (helices h9, h10, and h11) which is involved in contacts between two EF-Ts molecules in the crystal structure of the EF-Tu-EF-Ts complex is largely missing from EF-Ts\textsubscript{mt}. Only portions of what may be helix h10 appear to be present. Finally, the C-terminal module (h13) present in \textit{E. coli} EF-Ts is missing in EF-Ts\textsubscript{mt}.

The secondary structural analysis described above provided a good tool for the analysis of what regions of EF-Ts\textsubscript{mt} are important for the tight binding to EF-Tu observed with EF-Ts\textsubscript{mt}. Based on this analysis, a series of chimeric proteins consisting of portions of \textit{E. coli} EF-Ts and EF-Ts\textsubscript{mt} were constructed (Fig. 5). Chimeras were first constructed by exchanging the N-terminal domains (h1 through h3) of \textit{E. coli} EF-Ts and EF-Ts\textsubscript{mt} (Fig. 5, Chimeras I and II). These two chimeric proteins were expressed in \textit{E. coli} as His-tagged proteins and purified by chromatography on nickel-nitriiotriacetic acid columns. In addition, His-tagged forms of EF-Ts\textsubscript{mt} and \textit{E. coli} EF-Ts were also expressed and purified. All of these proteins were initially purified from cell extracts in buffers without GDP but containing Mg$^{2+}$. The purified proteins were analyzed by SDS-PAGE followed by Coomassie Blue staining (Fig. 6). Under these conditions, EF-Ts\textsubscript{mt} is purified as a 1:1 complex with \textit{E. coli} EF-Tu (Fig. 6, lane 1). In contrast, \textit{E. coli} EF-Ts binds to \textit{E. coli} EF-Tu much less tightly, and only $\sim$1 mol of EF-Tu is present for every 20 mol of \textit{E. coli} EF-Ts (Fig. 6, lane 2). The weaker binding of \textit{E. coli} EF-Ts prevents it from competing effectively for EF-Tu with the guanine nucleotides and aminoacyl-tRNA present in the extract. Chimera I which carries the N-terminal domain of EF-Ts\textsubscript{mt} binds \textit{E. coli} EF-Tu $\sim$2–3-fold better than \textit{E. coli} EF-Ts does (Fig. 6, lane 3). It is isolated with a higher ratio of EF-Tu present in the preparations ($\sim$1:10) than observed with \textit{E. coli} EF-Ts. This observation suggests that the N-terminal domain has a small effect on increasing the affinity of EF-Ts for EF-Tu. Chimera II binds to \textit{E. coli} EF-Tu much more tightly than does \textit{E. coli} EF-Ts but it somewhat less tightly than EF-Ts\textsubscript{mt} (Fig. 6, lane 4).

To obtain more quantitative measurements of the affinity of these chimeras for \textit{E. coli} EF-Tu, equilibrium association constants were determined as described above for the normal proteins. For these experiments, \textit{E. coli} EF-Ts\textsubscript{Eco} and Chimera I were prepared free of EF-Tu by the use of buffers containing GDP during the preparation of the factors. EF-Ts\textsubscript{mt} and Chimera II were prepared by the denaturation of the EF-Tu\textsubscript{Eco}/EF-Ts\textsubscript{mt} complex followed by renaturation of the EF-Ts\textsubscript{mt} (10). Analysis of these preparations on SDS-PAGE indicated that they were free of EF-Tu (data not shown). As indicated in Table I, replacing the N-terminal domain of \textit{E. coli} EF-Ts with that of EF-Ts\textsubscript{mt} (Chimera I) increases the binding constant for EF-Tu $\sim$2–3-fold. This observation suggests that this region of EF-Ts\textsubscript{mt} has a small effect on the strength of the interaction with EF-Tu. In the complementary construct (Chimera II), replacing the N-terminal domain of EF-Ts\textsubscript{mt} with that of \textit{E. coli} EF-Ts decreases the binding constant of EF-Ts\textsubscript{mt} to \textit{E. coli} EF-Tu $\sim$4–5-fold. Chimera II which is predominantly derived from EF-Ts\textsubscript{mt} still binds EF-Tu $\sim$20-fold more tightly than does \textit{E. coli} EF-Ts. These observations indicate that the strength of the interaction observed with EF-Ts\textsubscript{mt} is governed primarily by sequences from h4 to the C terminus with a small contribution from sequences in the N-terminal domain.

To localize the region giving EF-Ts\textsubscript{mt} its stronger affinity for EF-Tu more closely, two more chimeras were prepared (Fig. 5, Chimeras III and IV). The preparation of these chimeras was based on the observation that the N-terminal half (the N-terminal domain and Subdomain N, h1 through s3) and the C-terminal half (from s4 to the C terminus) of \textit{E. coli} EF-Ts fold somewhat independently in the crystal structure of the \textit{E. coli} EF-Tu-EF-Ts complex (Fig. 1). However, the three-stranded $\beta$-sheet structure in Subdomain N forms an interface with the three-stranded sheet in Subdomain C (Fig. 1) and these chime-
The determinants for the strong interaction between EF-Tsmt and EF-Tu alone during this incubation period (−7 pmol) have been subtracted from each value.

This observation suggests that it has folded correctly and that sequences within Subdomain N of EF-Tsmt are primarily responsible for the stronger interaction of this factor with EF-Tu. To confirm this idea, an additional chimera was prepared (Chimera V, Fig. 5) in which Subdomain N of E. coli EF-Ts was replaced with that of EF-Tsmt. When Chimera V is prepared from E. coli, it co-purifies with considerable amounts of EF-Tu as does EF-Tsmt (Fig. 6, lane 7). This chimera is longer than the others and migrates on SDS-PAGE at a higher molecular weight than the other chimeras, due to the insertion present in Subdomain N of EF-Tsmt. The association constant for the binding of Chimera V to EF-Tu (Table I) was 25-fold higher than that of E. coli EF-Ts. This observation agrees with the idea that sequences in Subdomain N of EF-Tsmt are primarily responsible for the stronger interaction of this factor with EF-Tu. Since there is an insertion of ~20 amino acids in this region, contacts between one or more of these residues may be occurring between EF-Tsmt and Domain I of EF-Tu.

The reciprocal construct (Chimera IV, Fig. 5) was unable to bind E. coli EF-Tu (Fig. 6, lane 6) and probably folds incorrectly. This observation suggests that the interface between Subdomain N and Subdomain C does not form correctly in this construct. Chimera IV is expressed well in E. coli and does not appear to form inclusion bodies or to be readily degraded. Hence, it is reasonable to suggest that Chimera IV has significant structural defects. This is consistent with the idea that sequences in Subdomain N of EF-Tsmt are primarily responsible for the stronger affinity of this factor for EF-Tu.

These data also indicate that the C-terminal half of EF-Tsmt. The association constant for the binding of Chimera III to EF-Tu (Table I) indicated that this chimera binds to EF-Tu as strongly as does the native EF-Tsmt. This observation indicates that all of the stronger binding between EF-Tsmt and EF-Tu arises from the N-terminal domain and Subdomain N of EF-Tsmt. This region of EF-Ts is in contact with Domain I of EF-Tu. These data also indicate that the C-terminal half of EF-Tsmt does not contribute significantly to the ability of EF-Tsmt to bind EF-Tu more tightly than E. coli EF-Ts.

The reciprocal construct (Chimera IV, Fig. 5) in which Subdomain N of EF-Tsmt was replaced that in EF-Tsmt, has about one-half of the activity. This region of EF-Ts is in contact with Domain I of EF-Tu. Since there is an insertion of ~20 amino acids in this region, contacts between one or more of these residues may be occurring between EF-Tsmt and Domain I of EF-Tu.

**FIG. 7. Stimulation of guanine nucleotide exchange with E. coli EF-Tu.** Nucleotide binding assays were carried out as described under “Materials and Methods” and contained 7.3 μg of expressed E. coli EF-Tu (~80 pmol of active factor) and the indicated amount of E. coli EF-Ts, Chimera I or Chimera II (A) and EF-Tsmt or Chimera III (B). For Chimera V, the EF-Tsmt-Chimera V complex (3–9 pmol) was used directly in the GDP exchange assay. The amount of GDP binding observed with this complex alone was very small (~1 pmol). Blanks representing the amount of GDP exchange carried out by EF-Tu alone (~7 pmol) have been subtracted from each value.

**FIG. 8. Stimulation of the activities of E. coli EF-Tu and EF-Tsmt in polymerization by the chimeras.** A, reaction mixtures contained 1 pmol of expressed E. coli EF-Tu and the indicated amounts of EF-Ts or chimera. Blanks representing the amount of polymerization catalyzed by EF-Tu alone (~4 pmol) have been subtracted from each value. B, reaction mixtures contained 3 pmol of expressed EF-Tsmt and the indicated amount of EF-Ts. Blanks representing the amount of polymerization catalyzed by EF-Tu alone (~2–3 pmol) have been subtracted from each value.
observed with E. coli EF-Ts (Fig. 7A). This chimera has 3–4-fold higher activity than that seen with EF-Tsmt. The activity of Chimera III in stimulating GDP exchange is about the same as that observed with EF-Tsmt (Fig. 7B). This chimera has the entire N-terminal half of EF-Tsmt and binds to EF-Tu as tightly as EF-Tsmt. Chimera IV is not active in stimulating GDP exchange as would be expected from its apparent inability to bind EF-Tu. The activity of Chimera V is slightly higher than that of EF-Tsmt, but significantly lower than E. coli EF-Ts. Overall, these results indicate that EF-Tsmt and the chimeras that bind to EF-Tu more tightly have lower activities in promoting guanine nucleotide exchange.

The activities of the chimeras in stimulating the activity of E. coli EF-Tu in polymerization were also tested (Fig. 8A). In this assay, as in the GDP exchange assay, EF-Tsmt is less active than E. coli EF-Ts (Figs. 3 and 8A). The activity of Chimera I is very similar to that of E. coli EF-Ts. The activity of Chimera II is only slightly lower than that of E. coli EF-Ts and much higher than that of EF-Tsmt. This assay is probably somewhat less sensitive to changes in the affinity of EF-Ts for EF-Tu, since the formation of the EF-Tu-Ts complex is coupled to the subsequent very favorable formation of the ternary complex. This coupling might tend to offset the stronger interaction between EF-Tu and EF-Ts to some extent. The activity of Chimera III is about the same as that observed with EF-Tsmt. Since this chimera has the same affinity for EF-Tu as does EF-Tsmt, this result is to be expected. These data further indicate that replacing the N-terminal domain of EF-Tsmt (Chimera II) increases the activity of EF-Tsmt in stimulating the activity of E. coli EF-Tu, while replacing the C-terminal half of EF-Tsmt does not appear to affect the activity of EF-Tsmt.

As shown in Fig. 3C, E. coli EF-Tu is not able to stimulate the activity of EF-Tsmt in polymerization. The activities of the chimeras in stimulating EF-Tsmt in polymerization were tested (Fig. 8B). Replacing the N-terminal domain of E. coli EF-Ts with the corresponding region of EF-Tsmt (Chimera I) does not restore activity, indicating that the interaction between Domain I of EF-Tu and the N-terminal domain of EF-Ts is not responsible for the lack of activity observed with E. coli EF-Ts. In agreement with this idea is the observation that Chimera II, in which the N-terminal domain of EF-Tsmt is replaced by the corresponding region from E. coli EF-Ts, is quite comparable with that of EF-Tsmt (Fig. 8B). Chimera III, in which the C-terminal half of EF-Tsmt has been replaced by that from E. coli EF-Ts, has very little or no activity in stimulating EF-Tsmt in polymerization. Chimera V could not be tested in this assay since it could not be refolded into an active conformation following denaturation of the EF-Tu-EcoEF-Ts mt-Chimera V complex. These data indicate that E. coli EF-Ts cannot stimulate the activity of EF-Tsmt because of a failure of the C-terminal half of this factor to interact correctly with Domain III of EF-Tsmt. This observation is surprising since residues in EF-Tu making contact with Subdomain-C of EF-Ts in the crystal structure have been conserved in the mitochondrial factor.

**DISCUSSION**

The N-terminal Domain and Subdomain N Determine the Tight Binding of EF-Tsmt to E. coli EF-Tu—The data presented here show that EF-Tsmt binds to E. coli EF-Tu ~100-fold more tightly than E. coli EF-Ts. Analysis of the chimeric proteins indicates that sequences in Subdomain N of the core of EF-Ts are primarily responsible for the difference in the tightness of binding observed while the N-terminal domain also makes a small contribution. Six residues in the N-terminal domain of E. coli EF-Ts make contact with residues in Domain I of E. coli EF-Tu (14). Some of these residues are conserved in EF-Tsmt while others are primarily conservative replacements. These latter residues in EF-Tsmt may account for the small effect on binding contributed by the N-terminal domain.

In the interactions between Domain I of E. coli EF-Tu and Subdomain N of the core of E. coli EF-Ts, four residues (Asp-80, Phe-81, Ile-125, and Gly-126) of E. coli EF-Ts are directly involved. The only residue in EF-Tsmt that is different in this group is the conservative replacement of Leu-151 for the corresponding residue Ile-125 in E. coli EF-Tu. Since Ile-125 of E. coli EF-Ts makes a backbone contact with EF-Tu, it is unlikely that Leu-151 of EF-Tsmt contributes to the stronger interaction observed between EF-Tsmt and EF-Tu. D80A and F81A mutants of E. coli EF-Ts bind to EF-Tu much more weakly than does wild type EF-Ts. However, the corresponding D84A and F85A mutants of EF-Tsmt still bind to E. coli EF-Tu as tightly as does the wild-type factor (15). These results suggest that Asp-84 and Phe-85 of EF-Tsmt do not contribute to the stronger interaction of EF-Tsmt with E. coli EF-Tu. However, as indicated in Fig. 4, Subdomain N of the core of EF-Tsmt has an insertion of ~20 residues. Although the exact position of this insertion is difficult to assess, it is likely that these residues account for the stronger interaction between EF-Tsmt and EF-Tu.

Decreased Tightness of Binding between EF-Tsmt Increases Its Ability to Stimulate the Activity of EF-Tu—Although EF-Tsmt interacts with E. coli EF-Tu very well, it is significantly less active than E. coli EF-Ts in stimulating the activity of E. coli EF-Tu. One explanation for the lower activity observed is that the strong binding actually inhibits the activity of EF-Tsmt. The reaction of E. coli EF-Ts in stimulating guanine nucleotide exchange can be described as

\[
EF-Tu \cdot GDP + EF-Ts \rightleftharpoons [EF-Tu \cdot Ts \cdot GDP] 
\rightleftharpoons EF-Tu \cdot Ts + GDP \quad (Eq. 5)
\]

E. coli EF-Ts is recycled by the dissociation of the EF-Tu-Ts complex by GDP or GTP. When EF-Tsmt forms a tight complex with E. coli EF-Tu, the dissociation of the complex by GDP or GTP is very slow, making the turnover number of EF-Tsmt in the reaction low. The small turnover number may explain the low activity of EF-Tsmt in stimulating GDP exchange. In the polymerization assay, the availability of Phe-tRNA probably pulls the reaction toward the formation of the ternary complex and increases the turnover of EF-Tsmt, which results in the somewhat higher relative activity of EF-Tsmt in the polymerization assay. In agreement with this idea is the observation that the chimeras that bind EF-Tu less tightly generally have higher activities than those that bind EF-Tu more tightly.

Interactions of E. coli EF-Ts and Chimeras with EF-Tsmt—Despite the 56% identity in sequence between E. coli EF-Ts and EF-Tsmt, E. coli EF-Ts is unable to stimulate the activity of EF-Tsmt in polymerization. This result suggests that E. coli EF-Ts does not interact very well with EF-Tsmt or that the interaction occurring fails to result in effective nucleotide exchange. Unfortunately, the poor binding of guanine nucleotides to EF-Tsmt does not allow a direct measure of the exchange reaction. Since Chimera II is able to stimulate the activity of EF-Tsmt, the N-terminal domain of E. coli EF-Ts probably interacts with EF-Tsmt quite well. The low activities of Chimeras I and III with EF-Tsmt suggest that the C-terminal half of E. coli EF-Ts does not form a good interaction with Domain III of EF-Tsmt. This observation is unexpected since the residues in EF-Tu making contact with Subdomain C of EF-Ts in the crystal structure have been conserved in EF-Tsmt. On the other hand, the alignment of the sequences of EF-Ts from a number of organisms shows that the C-terminal half of EF-Ts is not as conserved as the N-terminal half. E. coli EF-Ts has an extra α-helix (h13) at the C-terminal terminus compared with
EF-Ts\textsubscript{mt}. In addition, \textit{E. coli} EF-Ts has a dimerization domain that is not present in EF-Ts\textsubscript{mt}. These structural differences may lead to the inability of \textit{E. coli} EF-Ts to stimulate the activity of EF-Tu\textsubscript{mt}.

REFERENCES
Role of Domains in *Escherichia coli* and Mammalian Mitochondrial Elongation Factor Ts in the Interaction with Elongation Factor Tu
Yuelin Zhang, Vyvyan Sun and Linda L. Spremulli

doi: 10.1074/jbc.272.35.21956

Access the most updated version of this article at [http://www.jbc.org/content/272/35/21956](http://www.jbc.org/content/272/35/21956)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 20 references, 5 of which can be accessed free at [http://www.jbc.org/content/272/35/21956.full.html#ref-list-1](http://www.jbc.org/content/272/35/21956.full.html#ref-list-1)