Complex of Transfer-messenger RNA and Elongation Factor Tu

UNEXPECTED MODES OF INTERACTION*  

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Transfer-messenger RNA (tmRNA) is a stable RNA in bacteria of 360 ± 40 nucleotides that can be charged  
with alanine and can function as both tRNA and mRNA. Ribosomes that are stalled either in a coding region  
of mRNA or at the 3′ end of an mRNA fragment lacking a stop codon are rescued by replacing their mRNA for  
tmRNA. Here we demonstrate that the interaction of tmRNA with the elongation factor Tu shows unexpected  
features. Deacylated tmRNA can form a complex with either EF-Tu-GDP or EF-Tu-GTP, the association  
constants are about one order of magnitude smaller than that of an Ala-tRNA-EF-Tu-GTP complex. tmRNA as well  
as Ala-tmRNA can be efficiently cross-linked with EF-Tu-GDP using a zero-length cross-link. The efficiency of  
cross-linking in the case of deacylated tmRNA does not depend on an intact CCA-3′ end and is about the same,  
regardless whether protein mixtures such as the post-ribosomal supernatant (S100 enzymes) or purified  
EF-Tu are present. Two cross-linking sites with EF-Tu-GDP have been identified that are located outside  
the tRNA part of tmRNA, indicating an unusual interaction of tmRNA with EF-Tu-GDP.

Transfer-messenger RNA (tmRNA or 10 Sa RNA) is a small, stable RNA of 360 ± 40 nucleotides. The tmRNA is encoded by  
the ssrA gene (1). A knock-out of this gene has little effect on the growth of Escherichia coli cells at 37 °C but suppresses  
growth at elevated temperatures (2). Additionally, this knock-out strain is outcompeted by wild-type strains in a culture (3).  
The gene is present in all kingdoms of the bacterial domain, including the alpha-proteobacteria (4, 5). This molecule is of  
special interest because it can function as both a tRNA and an mRNA. The 5′ and 3′ ends of the molecule can be folded into a  
tRNA-like structure with an amino acid-acceptor stem and a  
TΨC-stem/loop that contains two modifications universally  
conserved in tRNAs (6). The tmRNA contains an identity element  
for the alanyl-tRNA synthetase and can be charged with alanine in vivo and in vitro (7). In addition, tmRNA has a  
messenger RNA sequence that encodes a decapeptide in E. coli (8, 9), which is a recognition signal for some proteases (10).  
The main function of tmRNA in the cell seems to be the rescue of ribosomes that are arrested by truncated mRNA  
lacking their stop codon (8, 9). These ribosomes carry a peptidyl-tRNA at the P site and either a truncated codon or no codon  
at all at the A site. The alanine-charged tmRNA enters the ribosomal A site, and the alanine residue is transferred to the  
growing peptide. After translocation, translation switches from  
the truncated mRNA to the messenger part of tmRNA. After  
reading the codon sequence on tmRNA, the ribosome terminates at the stop codon encoded in tmRNA by the usual termination  
motion. This process has two consequences: (i) the ribosomes arrested with truncated mRNA are rescued and  
recycled for protein synthesis, and (ii) the truncated proteins are tagged with a signal peptide that is recognized by specific  
proteases. Recently, it was also demonstrated that stalling of a ribosome at rare codons can trigger tagging of polypeptides  
despite the presence of a substantial region of non-translated  
3′-mRNA (11).

Aminoacyl-tRNAs enter the ribosome as a complex with the elongation factor Tu (EF-Tu) in the presence of GTP (12).  
Thermus thermophilus EF-Tu complexed with GTP protects Ala-tmRNA from spontaneous hydrolysis (13), as EF-Tu-GTP  
does with aminoacyl-tRNA. EF-Tu is therefore believed to be involved in the transport of Ala-tmRNA to the ribosome. An  
additional protein, the RNA-binding SmpB protein, binds with high affinity to tmRNA and is important for the tagging  
functions of tmRNA on the ribosome (14). Furthermore, the ribosomal protein S1 can interact with tmRNA (15), and three further  
proteins have been identified in the tmRNA-SmpB complex, the role of which is still unknown in relation to tmRNA function  
(16). Thus far, the interaction of EF-Tu with tmRNA has not been directly demonstrated.

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be cross-linked to tmRNA in E. coli cell-free S100 extract (S100 enzymes). Surprisingly, decyaeylated tmRNA can form complexes with EF-Tu-GDP, and this unusual complex was characterized by cross-linking and footprinting experiments.

EXPERIMENTAL PROCEDURES

**Transcription and Annotation of tmRNA**—The plasmid containing the tmRNA gene from E. coli under the control of a T7 promoter was a kind gift of Dr. Brosius. tmRNA transcript was synthesized using an in vitro T7 transcription system (22, 32) in the presence of [α-32P]UTP using linearized plasmid as the DNA template. For the formation of sU-modified tmRNA transcripts, UTP was supplemented with 4-thio-UTP at different molar ratios (from 1:1 to 1:100). The level of 4-thiouridine incorporation was estimated using the T2 fingerprint technique (17). For example, one or two 4-thiouridine residues per molecule were incorporated at an sU/R ratio of 1:100, and tmRNA transcripts were purified as described for mRNAs (23). To create truncated tmRNAs, the plasmid was linearized with Ncol or XcmI yielding tmRNAs that lacked the CA-3’ end or the ACCA-3’ end, respectively.

Full transcripts of tmRNA were alanylated in 20 mM HEPES-KOH, pH 7.6, at 0 °C, 10 mM Mg(ascite), 150 mM NH4Cl, 4 mM β-mercaptoethanol, and 3 mM ATP. 50 pmol of tmRNA and 30 pmol of AlaRS were incubated in the presence of 20 μM [35S]Ala (specific activity 350 dpm/pmol) for 30 μm for 20 min at 37 °C. The charging yield was determined by precipitation with cold trichloroacetic acid. The plasmid containing the His-tagged AlaRS was a kind gift from Dr. Paul Schimmel (The Scripps Research Institute, La Jolla, CA).

**Complex Formation in the Presence of S100 Enzymes or EF-Tu for Cross-linking and Footprinting**—E. coli trNA-free S100 extract (S100 enzymes) was kindly provided by Dr. M. Dabrowski (MPI für Molekular Genetik). EF-Tu His-tagged at the C terminus was isolated from enzymes) was kindly provided by Dr. M. Dabrowski (MPI für Molekular Genetik). EF-Tu his-tagged at the C terminus was isolated from

**Association Constants**—Association constants (K_a) for tmRNA-EF-Tu-GDP/GTP complexes were measured by filtration of the complexes through a 0.45-μm nitrocellulose membrane (Millipore). This method was a modification of that described previously (30). EF-Tu-GDP was incubated in a multiple of 3.33 μl per 33.33 μl of complete reaction buffer containing 60 mM HEPES-KOH, pH 7.6, at 4 °C, 10 mM Mg(ascite), 150 mM NH4Cl, and 1 μM dithiothreitol, and (in the complex of GTP) 5 μM phosphoenolpyruvate, 30 μM γ-[32P]GTP (specific activity 3500 dpm/pmol) and 0.1 μM pyruvate kinase. After incubation at 37 °C for 10 min, the volume unit was increased to 10 μl to maintain the ionic conditions, and up to 80 pmol of tmRNA was added. After 20 min at 0 °C, 10 μl was spotted onto a nitrocellulose filter pre-soaked in reaction buffer and washed with 100 μl and subsequently three times with each 3 ml of reaction buffer. The filter was resolved in 5 ml of Filter-Count (Packard, Groningen, The Netherlands) and counted. The difference, cpm (binary complex EF-Tu/GDP formed) and cpm (ternary complex in a 10-μl volume unit) — cpm (ternary complex in a 1-μl volume unit), gives the amount of ternary complex formed. The S100 enzymes were added to 30 pmol of the reaction mixture that was irradiated by mild (330 nm) UV light for 5 min as described previously (25). Half of this sample was treated with proteinase K (1.4 μg/ml final concentration) for 15 min at 37 °C, and both aliquots were loaded onto the same gel. Cross-linked and non-cross-linked tmRNAs were separated by 7.5% SDS-polyacrylamide gel electrophoresis (26) for 4 h at 100 V.

**Analysis of the Cross-linked Protein**—The gel region containing the tmRNA-protein cross-link (Fig. 1, line 2) was cut out and incubated in 300 μl of a buffer containing 20 mM Tris-HCl, pH 7.8, at 0 °C, 0.1% (v/v) SDS with 5 μl of RNAse T1 (10 μg/ml) for 20 min at 55 °C. The same volume of a buffer containing 0.1% SDS, 0.1% (v/v) 2-mercaptoethanol, 900 mM sodium acetate, and 5 μl of RNAse T1 (10 μg/ml) was added. The mixture was then overnight at 4 °C. The proteins were precipitated by ethanol (5 volumes), and after a low speed centrifugation the pellet was dissolved in 10 μl of ultrapure water and 1 μl of RNAse T1 (10 μg/ml) was added. This mixture was then incubated for 20 min at 55 °C, diluted by an equal volume of loading buffer (250 mM Tris-HCl, pH 6.8, at 8 °C, 8% (v/v) SDS, 40% glycerol, 400 mM dithiothreitol, and 0.04% bromphenol blue), and loaded onto a 7.5% protein gel (26). Protein bands were visualized by staining in a solution containing 0.1% (w/v) Cooamassie Blue, 0.5% (v/v) acetic acid, 20% (v/v) methanol for 2 h. Destaining was achieved by washing the gel with 30% (w/v) methanol.

**Tryptic Digestion and MALDI-Mass Spectrometry**—Cross-linked protein-proteins were digested in gel according to the method described in a previous study (27) using 100 ng of sequencing grade modified trypsin (Roche Molecular Biochemicals, Germany) per gel piece containing a band. The eluted peptides were purified on C-18 ZipTip micro columns (Millipore, Bedford, MA) and analyzed by matrix-assisted laser desorption/ionization mass spectrometric analysis (MALDI-MS) in the presence of n-cyano-4-hydroxy-cinnamic acid as matrix. MALDI-MS analysis was performed on a Reflex-II instrument (Bruker Daltonik, Bremen, Germany) reflectron mode. Samples for MALDI-MS were prepared by the dried droplet method on stainless steel targets precovered with thin matrix films. The determined peptide masses were submitted to the web-based database search programs, MS-Fit and Profound, to identify the protein (see Table II below).

**Footprinting Analysis**—Conditions of the modification reactions for footprinting analysis were taken from Moazed and Noller (28). For modification with CMCT (1-cyclohexyl-3-(2-morpholinooethyl)carboimide metho-toluene sulfonate), 25 μl of a 42 mg/ml CMCT solution was added to either 25 μl of tmRNA-EF-Tu-GDP complex mixture (2 pmol/μl) obtained as described above (but without an energy regeneration system) or to 25 μl of untreated control tmRNA in solution. This mixture was incubated at 0 °C for 10 min, followed by immediate quenching of the modifying reagent with the appropriate stop solution (28). The modification positions were determined by a primer-extension (29) using primers complementary to the nucleotides 55–73, 149–217, or 345–363 of tmRNA.

**Determination of the Positions of Cross-links**—The cross-linked proteins were extracted from the gel as described above (under “Analysis of the Cross-linked Protein”) and shaken overnight at 4 °C. Samples were precipitated by ethanol (5 volumes), and after a low speed centrifugation the pellet was dissolved in 10 μl of ultrapure water and treated with fresh protease K (1.4 μg/ml in the reaction mixture) for 15 min at 37 °C. The extracted RNA was purified in a 8% polyacrylamide-urea gel, and extracted in 5 μl of Milli-Q water (Millipore) and analyzed by primer extension (29) using the primers described in the preceding paragraph.

**Preparation of tmRNA Containing the Photo-reagent 4-Thio-uridine (sU)—tmRNA containing statistically two randomly distributed sU residues was synthesized by in vitro transcription using T7 RNA polymerase. The level of sU incorporation was controlled by RNase T2-fingerprints (25). The T7-transcript tmRNA could be charged with [14C]alanine up to a maximal 40%. A similar value (20–40%) has been found by other groups (6, 14, 30) and might indicate a conformational heterogeneity. The alanine residue could be efficiently attached to the binary complex is adsorbed to the filter, whereas the ternary complex goes through. The procedure of gel-shift experiments for measuring the formation of ternary complexes was as described previously (44).

**RESULTS**

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The formation of tmRNA-EF-Tu complexes in the presence of either GTP or GDP has been examined by using nitrocellulose filtration methods (30). Indeed, binding was observed, and the association constants, \( K_a \), for either complex could be determined. Controls with Phe-tRNA were performed and, furthermore, the association constants with Phe- and Ala-tRNA were determined using band-shift assays.

Both constants with deacylated tmRNA (in the presence of GDP or GTP) were about 1 \( \times 10^6 \) M\(^{-1} \), for the canonical ternary complexes Ala-tRNA-EF-Tu-GTP or that with Phe-tRNA up to 10 to 20 times higher values were observed (Table II). It follows that even EF-Tu-GDP can form a ternary complex with deacylated tmRNA. In contrast, a comparable complex even with a large excess of deacylated tRNA \(^{bulk} \) (40-fold over the concentration of tmRNA) could not be detected with the applied nitrocellulose filtration technique, in agreement with literature data underscored the specificity of the complex observed with deacylated tmRNA.

An intact CCA end is a prerequisite for aminocaylation of tRNA. Therefore, a rigorous test whether or not deacylated tmRNA can form a complex with EF-Tu-GDP would be an analysis of the question: Is an intact CCA-3′ end of the tmRNA truly a prerequisite for complex formation? To address this question, tmRNA was transcribed lacking either the CA-3′ or the ACCA-3′ ends, and cross-linking experiments were performed with tRNA-free S100 enzymes as shown in Fig. 1A. The cross-linked products were isolated, and the protein species was analyzed by gel-electrophoresis after digestion of RNA with RNase T1. The protein observed was indeed co-migrating with the non-irradiated sample (Fig. 1A; lane 1 contains the non-irradiated sample). This mobility shift was due to the formation of a complex of tmRNA with one or more proteins, because treatment with proteinase K cancelled the shift (lane 3).

**Analysis of the Cross-linked Complexes**—For identification of the cross-linked protein(s(s) the complex was purified from the S100-derived proteins that co-migrated with the tmRNA during gel electrophoresis. The complex seen in lane 2 of Fig. 1A was extracted from the gel and subjected to a second electrophoresis step under the same conditions, after the RNA had been digested with T1 RNase. Cross-linked proteins were expected to change their mobility upon the loss of the RNA moiety. Only one protein band was clearly seen in the gel after this treatment. The gel section containing the band of the cross-linked protein was isolated and subjected to tryptic digestion, and the resulting peptides were analyzed by MALDI (Table I). A database search concerning the peptide masses revealed that all major peptides could be assigned as fragments of EF-Tu. The identified peptides covered 65% of the protein’s sequence. It follows that EF-Tu is the major protein component of the cross-linked complex.

**Complexes of tmRNA with Isolated EF-Tu-GTP/GDP**—An intriguing observation from the experiments described above is the high yield of cross-linked tmRNA in the presence of EF-Tu-GTP and in the absence of alanylation. Under the conditions used here, a significant amount of transcribed tmRNA could not be alanylated in the presence of S100 enzymes, because alanine was not added to the reaction mixture. Therefore, it is possible that EF-Tu-GTP can form complexes with deacylated tmRNA.
Unexpected Interactions between tmRNA and EF-Tu

**TABLE I**

Assignment table of the tryptic peptides of the cross-linked protein

| Measured | Calculated value of [M + H]^+ | Deviation | Sequence coverage |
|----------|-----------------------------|-----------|------------------|
| m/z      | ppm                         |           |                  |
| 776.39   | -22.07                      | 320       | 325              |
| 837.49   | -7.36                       | 118       | 124              |
| 1027.60  | 6.50                        | 271       | 280              |
| 1100.67  | -0.58                       | 383       | 394              |
| 1201.57  | 27.92                       | 254       | 263              |
| 1233.62  | 5.67                        | 326       | 334              |
| 1376.67  | 28.55                       | 46        | 57               |
| 1703.81  | 22.02                       | 250       | 263              |
| 1728.84  | -1.30                       | 305       | 319              |
| 1789.93  | -6.16                       | 359       | 374              |
| 1795.96  | -14.44                      | 9         | 25               |
| 1796.94  | -21.08                      | 359       | 374              |
| 1803.89  | 1.99                        | 60        | 75               |
| 1812.92  | -6.18                       | 359       | 374              |
| 1962.02  | -3.42                       | 189       | 205              |
| 1964.96  | 7.56                        | 156       | 172              |
| 2117.16  | -0.69                       | 206       | 224              |
| 2515.13  | -38.52                      | 250       | 270              |
| 2546.24  | 1.97                        | 335       | 358              |
| 2729.35  | 2.58                        | 91        | 117              |
| 2745.34  | 0.48                        | 91        | 117              |
| 3080.73  | 3.33                        | 9         | 38               |

\(^a\) Met-ox, oxidized methionine; Cys-am, acrylamide-modified cysteine.

**TABLE II**

Association constants for EF-Tu and tmRNA and Ala- and Phe-tRNA

For nitrocellulose, binding constants were determined with nitrocellulose filtration method (see “Experimental Procedures”). For gel-shift, shift experiments were performed as described previously (44). For Phe-tRNA, note that the constants are usually smaller (up to one order of magnitude) when measured via gel shift as compared to nitrocellulose filter measurement (45).

| Complex                      | Association constants ($K_a$) |
|------------------------------|-------------------------------|
|                              | Nitrocellulose  | gel-shift  |
| tmRNA-EF-Tu GDP              | $1.0 \times 10^6$ | $1.0 \times 10^6$ |
| tmRNA-EF-Tu GTP              | $1.4 \times 10^6$ | $1.4 \times 10^6$ |
| Ala-tRNA-EF-Tu GTP           | $1.1 \times 10^7$ | $1.1 \times 10^7$ |
| Phe-tRNA-EF-Tu GTP           | $2.9 \times 10^7$ | $0.7 \times 10^7$ |

**TABLE III**

Cross-link experiment with EF-TuGDP and [14C]Ala-[32P]tmRNA

For details see text.

| Excised band from the gel | Ratio $[^{13}C]/[^{14}C]$ | Ratio |
|--------------------------|---------------------------|-------|
| Cross-linked complex (cf. upper band in Fig. 1A) | 1268/467 | 2.7:1 |
| Free Ala-tRNA (cf. lower band in Fig. 1A) | 786/323 | 2.4:1 |

results are shown in Fig. 2. Specific stops of the reverse transcriptase were observed at the positions C269 and C270 (Fig. 2A, lane X) and at A309 (Fig. 2B, lane X). Therefore, the corresponding cross-linking sites are U268 and U308, respectively. The bands seen in lane K (Fig. 2A) are probably internal cross-linked sites, if they are stronger than the corresponding band in the X lane (cross-linked tmRNA). The bands corresponding to cross-link sites did not show a band at the same position in the control lane. Bases of the tmRNA that revealed altered reactivity against the modifying reagent CMCT upon complex formation with EF-TuGDP were identified in footprinting experiments. CMCT reacts specifically with N3 of the uracil base and N1 of the guanine base. The most obvious changes in the reactivity of the nucleotide bases were detected in the helix 2 of tmRNA. The results are shown in Fig. 3. The reactivity of the base G315 is strongly decreased whereas that of U311 is only weakly reduced corresponding to the control lane were only found in the regions shown in panels A and B. K, control eluted from the non cross-linked tmRNA in lane 3 of Fig. 1A (lower band). X, cross-linked tmRNA eluted from the upper band in lane 2 of Fig. 1A. The reverse transcription arrests corresponding to cross-links are indicated. The primer for reversed-transcription reaction was complementary to the region 345–363 of tmRNA.

**FIG. 2.** Primer extension analysis of the region around nucleotide C270 (A) and G310 (B) after cross-linking tmRNA with EF-TuGDP. The whole molecule was scanned, but changes with respect to the control lane were only found in the regions shown in panels A and B. K, control eluted from the non cross-linked tmRNA in lane 3 of Fig. 1A (lower band). X, cross-linked tmRNA eluted from the upper band in lane 2 of Fig. 1A. The reverse transcription arrests corresponding to cross-links are indicated. The primer for reversed-transcription reaction was complementary to the region 345–363 of tmRNA.
FIG. 3. Footprint analysis of tmRNA in the complex with EF-Tu-GDP after the reaction with CMCT. Reverse transcription arrests corresponding to the nucleotides with enhanced or reduced accessibility are marked with solid or open triangles, respectively. The primer for the reversed-transcription reaction was the same as in Fig. 2. Lane 1, control with non-modified tmRNA in solution; lane 2, control with non-modified tmRNA in the complex with EF-Tu-GDP; lane 3, tmRNA modified by CMCT in the complex with EF-Tu-GDP; lane 4, tmRNA modified by CMCT in solution.

The cross-linking and footprinting data presented here were seen reproducibly in several independent experiments.

DISCUSSION

The elongation factor EF-Tu within ternary complex aminoacyl-tRNA-EF-Tu-GTP increases the affinity of an aminoacyl-tRNA (aa-tRNA) to the ribosomal A site by at least two orders of magnitude (34). EF-Tu interacts with the short arm of an aa-tRNA that comprises the acceptor stem and the T loop-stem structure (35). Hydrolysis protection of alanylated tmRNA by EF-Tu indicates that this factor also interacts with alanylated tmRNA at the end of the acceptor stem (13).

A productive interaction of a ternary complex aa-tRNA-EF-Tu-GTP with the A site results in GTP cleavage and, in most cases, an incorporation of the aminoacyl residue into the nascent polypeptide chain. Such a productive interaction has to fulfill a requirement that cannot be accomplished by the tmRNA; a cognate or at least a near-cognate anticodon is a prerequisite, whereas a non-cognate ternary complex cannot productively interact with the A site. However, tmRNA lacks an anticodon, yet one of its features is that, in the absence of a codon-anticodon interaction, its alanyl residue is incorporated into the nascent polypeptide chain. It follows that either the EF-Tu interaction with tmRNA differs in important aspects from that with aa-tRNA and/or additional factors are involved in the productive interaction of tmRNA with the A site. Therefore, we set out to determine molecular partner(s) of tmRNA by applying a cross-linking approach.

sU is a well known cross-linking reagent used in studies of RNA-RNA and RNA-protein interactions (17). This reagent can be easily incorporated into RNA molecules by in vitro T7-transcription, and incorporation of sU residues does not significantly affect the structure of the RNA molecules nor their biological activity (36). Also, mild UV irradiation used for the activation of sU does not destroy RNA, proteins, or their activity. For example, it is known that substitution of 20% of U by sU in 5 S rRNA does not affect its ability to be reconstituted into active 50 S ribosomal particles (25). In our case, tmRNA with about 5 ± 2 sU residues per molecule was used to avoid multiple cross-linking.

tmRNA-protein complex formation in S100 extract was demonstrated by hand-mobility shift analysis. The high efficiency of the cross-linking reaction, with about 20% of the input tmRNA, indicated that a specific complex is formed in the S100 fraction with a significant affinity. The cross-linked protein was found to be EF-Tu. Under the conditions of complex formation, alanine was not added, so the amount of charged tmRNA present was negligible. This means that only uncharged tmRNA participated in the cross-linking to EF-Tu. The tmRNA without the 3′-terminal CA or ACCA could be cross-linked as efficiently as intact transcribed tmRNA, confirming the observation that alanylation of tmRNA is not required for complex formation. The specific complex tmRNA-EF-Tu formed in a mixture of tmRNA and S100 enzymes; surprisingly, the formation of this complex did not depend on the presence of GTP, i.e. the same yield of cross-linked products was obtained in the presence of GDP. At this point we did not know whether the presence of EF-Tu is sufficient for the complex formation or whether there is another protein such as SmPB that helps the formation of the complex of deacylated tmRNA with EF-Tu. The protein SmPB has been reported to interact directly with tmRNA (14). However, we did not identify this protein in the cross-linked complex. Finally, control experiments demonstrated that purified EF-Tu-GDP is sufficient for complex formation, as shown with both cross-linking experiments and determination of binding constants that were similar in the presence of GDP or GTP (Table II).

The specificity of our assays is demonstrated by the fact that, with deacylated tRNA and EF-Tu-GTP or with aminoacyl-tRNA and EF-Tu-GDP, no complexes were observed. This contrasts with deacylated tmRNA and EF-Tu-GDP either with or without an intact CCA-3′ end. This raises the question, what about the specificity of the tmRNA complexes? Could it be that this complex is an artifact only involving an inactive conformer of deacylated tmRNA that has no physiological relevance? The answer is no, if we accept that the alanylated form represents an active conformer of tmRNA. As described under “Results,” Ala-tmRNA can participate in cross-link formation with EF-Tu-GDP in the same way as deacylated tmRNA. That the cross-link (i) occurs in high yields of 20% of the input tmRNA, (ii) involves distinct nucleotides, and (iii) can be demonstrated also with Ala-tmRNA strongly indicates the physiological relevance of the complex. We further note that the complexes described here have been formed under near in vivo conditions (see Ref. 37 for discussion).

Another point concerns the use of transcribed tmRNA: the two modifications found in native tmRNAs (m5U342 and V347) are lacking but have minor effects upon the overall stability of tmRNA and might destabilize the acceptor stem (6). At present, it is not known whether the absence of these modifications influences the formation of this unusual complex with EF-Tu.

It is conceivable that EF-Tu-GTP delivers Ala-tmRNA to the A site, because it protects the Ala-tmRNA from hydrolysis as an aminoacyl-tRNA (13). However, the existence of a stable EF-Tu-GDP complex with tmRNA was unexpected and required closer inspection. In particular, we assessed possible contacts of tmRNA with EF-Tu in tmRNA-EF-Tu-GDP complexes using a combination of chemical footprinting and photoaffinity cross-linking techniques. Footprinting data identified one region of tmRNA protected from chemical modifications in the complex. The protected nucleotide bases (U311, G315) belong to helix 2 (nomenclature follows that of Refs. 38 and 39). This helix is the most conserved element of (U311, G315) belong to helix 2 (nomenclature follows that of Refs. 38 and 39). This helix is the most conserved element of tmRNAs (40), thus indicating the significance of its role, which is still to be identified. The cross-link to EF-Tu takes place at U308, which is within a three-nucleotide bulge (Fig. 4), and this arrangement might be related with the increased reactivity toward CMCT observed at U308 upon complex formation (Fig. 3). This nucleotide is conserved in ~90% of known tmRNAs, and the next nucleotide, G307, is completely conserved in all known sequences (40). It follows that the interaction of EF-Tu-GDP with deacylated tmRNA outside the tRNA module at helix 2 (Fig. 4) is strikingly
different to that of EF-Tu-GTP in a canonical ternary complex with aminocarboxyl-tRNA.

Nucleotide residues of tmRNA protected by EF-Tu-GDP from chemical modification are near or at the ACCGA sequence during the decoding of the mRNA module of tmRNA. EF-Tu has left the ribosome calling for protection by a EF-Tu-GDP/GTP complex as long as the translation of the tmRNA continues. It is noteworthy that eukaryotic eEF-1α can form a stable complex with deacylated tRNA that might be involved in transporting the deacylated tRNA from the E site to the corresponding synthetase (43).

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Fig. 4. Secondary structure model of the tmRNA according to Ref. 46. Conserved helices are shadowed. Large black circles indicate bases conserved in ~90% of the sequences, and small circles indicate ~75% conservation. X, cross-link sites. Solid and open arrowheads show sites of increased and reduced accessibility for modification with CMCT in the complex tmRNA-EF-Tu-GDP, respectively.
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