How does tmRNA move through the ribosome?

Pavel V. Ivanov[a,c], Maria I. Zvereva[a,c], Olga V. Shpanchenko[a], Olga A. Dontsova[a], Alexey A. Bogdanov[a], Galina V. Aglyamova[a,b], Valery I. Lim[b], Yoshika Teraoka[c], Knud H. Nierhaus[c,*]

[a] *Department of Chemistry, M.V. Lomonosov Moscow State University, 119899 Moscow, Russia*
[b] *Engelhardt Institute of Molecular Biology, Russian Academy of Science, Vavilov str. 32, 119991 Moscow, Russia*
[c] *Max-Planck-Institute für Molekulare Genetik, AG Ribosomen, Ihnestrasse 73, D-14195 Berlin, Germany*

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Abstract To test the structure of tmRNA in solution, cross-linking experiments were performed which showed two sets of cross-links in two different domains of tmRNA. Site-directed mutagenesis was used to search for tmRNA nucleotide bases that might form a functional analogue of a codon–anticodon duplex to be recognized by the ribosomal A-site. We demonstrate that nucleotide residues U85 and A86 from tmRNA are significant for tmRNA function and propose that they are involved in formation of a tmRNA element playing a central role in A-site recognition. These data are discussed in the frame of a hypothetical model that suggests a general scheme for the interaction of tmRNA with the ribosome and explains how it moves through the ribosome.

Key words: tmRNA; Ribosome; Translation; Cross-linking; Site-directed mutagenesis

1. Introduction

tmRNA, also known as SsrA RNA, or 10S RNA, is a small stable RNA which is present in all eubacteria as well as in some chloroplasts and mitochondria [1,2]. Its 3′- and 5′-ends are folded into a tRNA-like structure with an amino acid acceptor stem that possesses identity elements of $tRNA^{Ala}$ and enables specific aminoacylation of the tmRNA by alanyl-tRNA synthetase [3]. tmRNA has a short open reading frame in the middle of the molecule surrounded by pseudoknots (the mRNA module) that encodes a degradation signal (tag-peptide) for certain cellular proteases [4]. The combination of properties of both tRNA and mRNA results in an unusual translational mechanism for this molecule known as '*trans*-translation' – switching the translation from a cellular mRNA lacking a stop codon to the coding part of tmRNA, thus adding the tag-peptide to the truncated polypeptide chain [5,6].

Until recently almost nothing was known about how such a bulky molecule with very stable secondary and tertiary structure interacts with the ribosome and how it carries out the *trans*-translation process. In this paper we present data for testing of tmRNA structure by a photoaffinity cross-linking approach. Two sets of cross-links were observed. The first set is within the tRNA-part of tmRNA, whereas the second set represents the connection between U182 and different nucleotide residues in the area of pseudoknots 2 and 3. To reveal the nucleotides potentially involved in A-site recognition by the tmRNA, site-directed mutagenesis was applied. The effects of the mutations were tested in an in vivo system. The mutations in the pre-resume codon are not essential for tmRNA functioning, but substitution of two neighboring nucleotides leads to the phenotype of a tmRNA knockout strain. These data support our hypothesis that describes in general terms how tmRNA can enter and pass through the ribosome.

2. Materials and methods

2.1. Cross-linking and analysis of cross-linked products

Modified tmRNA was synthesized by T7 transcription as described in [7]. After purification, tmRNA was preincubated for 10 min at 37°C under the following ionic conditions: 20 mM HEPES–KOH, pH 7.8 (0°C), 3 mM $MgCl_2$, 150 mM NH_4Cl, 2 mM spermidine, 0.05 mM spermine and 4 mM 2-β-mercaptoethanol. The final concentration of tmRNA was 1 pmol/μl. The reaction mixture was irradiated by mild (>300 nm) UV light, as described in [8], and cross-linked products were analyzed by the combination of RNase H and primer extension techniques, as described in [9,10], in the presence of oligodeoxyribonucleotides, as indicated in the legend to Fig. 4.

2.2. Strains, plasmids and phage

Escherichia coli strains were grown in LB-medium or on LB-agar plates containing 1–1.5% agar/l. Ampicillin and/or chloramphenicol were added to plates or media as needed. Strains K8619 (ssrA::cat), K37 and hybrid phage λimmP22 dis c2-5 were kind gifts from Dr. David Friedman. To generate the plasmid pGEM-ssrA, the promoter and coding sequences of the ssrA gene were amplified by PCR to add *Nco*I and *Sal*I sites at the 5′- and 3′-ends, respectively. This DNA was digested with *Nco*I and *Sal*I and cloned into *Nco*I–*Sal*I digested pGEM (Promega). SsrA mutants were constructed using the QuickChange Site-Directed Mutagenesis kit (Stratagene) or PCR splicing by the overlap extension method [11], and cloned into pGEM using *Nco*I and *Sal*I digestion. Mutant sequences were verified by DNA sequencing.

2.3. Efficiency of plating (EOP)

The EOP of phage λimmP22 dis c2-5 on the wild-type strain K37 and its derivate K8619 with or without mutant plasmids was determined as described in [12].

2.4. In vitro aminoacylation

Full transcripts of tmRNA were alanylated in 20 mM HEPES–KOH, pH 7.6 at 0°C, 10 mM $Mg(CH_3COO)_2$, 150 mM NH_4Cl, 4 mM β-mercaptoethanol, and 3 mM ATP. 50 pmol of tmRNA and 30 pmol of AlaRS were incubated in the presence of 20 mM [^{14}C]Ala (specific activity 350 dpm/pmol) in a volume of 30 μl for 20 min at

*Corresponding author. Fax: (49)-30-8413 1690.
E-mail address: nierhaus@molgen.mpg.de (K.H. Nierhaus).

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37°C. The yield of charging was determined by precipitation with cold trichloroacetic acid.

3. Results and discussion

The central question for understanding the mechanism of tmRNA function is how does it move through the ribosome. We propose here a working hypothesis that describes schematically the major steps of tmRNA–ribosome interactions and tmRNA movement through the ribosome. The results of our recent experiments performed to check the reliability of this hypothesis are also presented.

The presence of four pseudoknots in the secondary structure of *E. coli* tmRNA (Fig. 1) is well documented [13,14]. It is hard to imagine that these pseudoknots have to be unwound during tmRNA movement through the ribosome [15]. We propose the existence of two compactly folded domains (containing pseudoknots) in the tmRNA molecule that interact with specific sites of the ribosome and retain their contacts with the ribosome during translation of the coding region of tmRNA. The first domain (domain 1) is proposed to be essential for tmRNA entering the ribosome. Thus, it should contain an alanylated-tRNA region which interacts with elongation factor (EF)-Tu (and SmpB) on the one hand and an element that structurally mimics a codon–anticodon complex of mRNA with tRNA to be recognized by the A-site of the ribosome on the other. The remaining elements of this domain might be necessary to modulate the shape of the tRNA–mRNA complex in order to fit the intersubunit space of the ribosome and to allow interactions with EF-Tu–GTP. We assume that this domain contains the tRNA-like region, helix 2 and pseudoknot 1. Domain 2 might consist of helix 5 and pseudoknots 2 and 3. It should be mentioned that in different organisms the elements of this domain can vary [16], but spatially they can be folded into a similarly shaped structure. Additional evidence for two independent domains in tmRNA is the existence in some organisms of

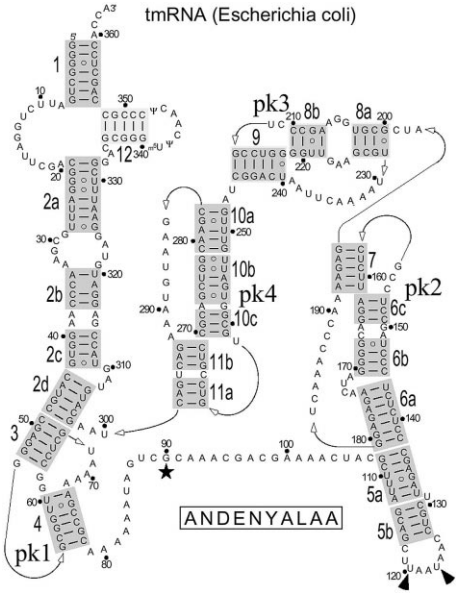

Fig. 1. Secondary structure of tmRNA with numbered helices and pseudoknots (taken from http://psysche.uthct.edu/dbs/tmRDB/tmRDB.html).

tmRNA molecules consisting of two pieces (containing domains 1 and 2 correspondingly) [17]. Domains 1 and 2 are connected with each other by the mRNA module and the pseudoknot 4. The schematic arrangement of tmRNA is shown in Fig. 2. Domains 1 and 2 can be close to each other in solution (Fig. 2A) and form a very compact structure that might be stabilized by interactions with proteins (e.g. SmpB [18], S1 [19], EF-Tu [20]). In this structure, the mRNA single-stranded module should be buried between the two domains.

A compactly folded aminoacylated (aa-)tmRNA enters the ribosomal A-site in a complex with EF-Tu–GTP [20], which is probably similar to that formed by a 'normal' aa-tRNA [21]. When domain 1 of tmRNA is recognized by the A-site, a conformational change take place in the ribosome that triggers GTP hydrolysis. This leads to conformational changes in EF-Tu and possibly also of tmRNA. The alanylated CCA-end of the tmRNA moves into the peptidyl-transferase center of the ribosome and the intersubunit space opens to allow EF-Tu to be released. At this stage domain 2 should occupy its binding site at the ribosome.

In accordance with the known location of the mRNA channel [22] the domain 2 binding site should be situated on the solvent side of the 30S subunit. In this case, the single-stranded coding region of tmRNA will occupy the channel in such a way that helices 5a and 5b (see Fig. 1), which must be unwound in the course of *trans*-translation, would be located at or near the hypothetical mRNA helicase [22].

Next, EF-G–GTP interacts with the ribosome to promote translocation, after which the tRNA-like part of domain 1 as well as the functional analog of the codon–anticodon duplex is at the P-site, and the resume codon of tmRNA (shown by nucleotide residues in Fig. 2B) has entered the A-site due to opening of the mRNA region. Domain 2 remains at its binding site on the ribosome. In the next step, domain 1 moves from the P site into the E site and might leave the decoding area of the ribosome, still being outside of the L1 'mushroom' (Fig. 2C). Domain 2 remains in the ribosome until termination takes place (Fig. 2B,C). The mRNA region moves step by step in the usual way (Fig. 2C). When the stop codon appears at the A-site, canonical termination occurs and the tmRNA leaves the ribosome after dissociation of the latter to subunits.

Two experimental approaches were used to test the above hypothesis.

Photoaffinity cross-linking was used to test the structure of tmRNA in solution. tmRNA with randomly distributed thiouridine residues was subjected to mild UV irradiation. Five cross-linked products were detected (Fig. 3), which were analyzed with the help of RNase H digestions (Fig. 4A) and in some cases with primer extension (Fig. 4B), the commonly used procedures for cross-link site analysis [9,10]. Cross-link 5 is located in the tRNA-region of tmRNA (Fig. 1) and links U16-17 with the tmRNA sequence 325–345. Cross-links 1–3 all contain U182 from the loop of pseudoknot 2 (Fig. 1), linked to three different nucleotides from the region containing pseudoknots 2 and 3. Cross-link 4 consists of combination of two cross-linking products: cross-link 5 with one of the products of 1–3. Neither RNase H nor primer extension worked during our attempts to determine the partners of U182, indicating an extremely compact folding of this domain of the tmRNA that prevents the annealing of oligodeoxyribonucleotides to this region. Thus, the cross-linking data obtained in this work allow us to suggest the existence of two

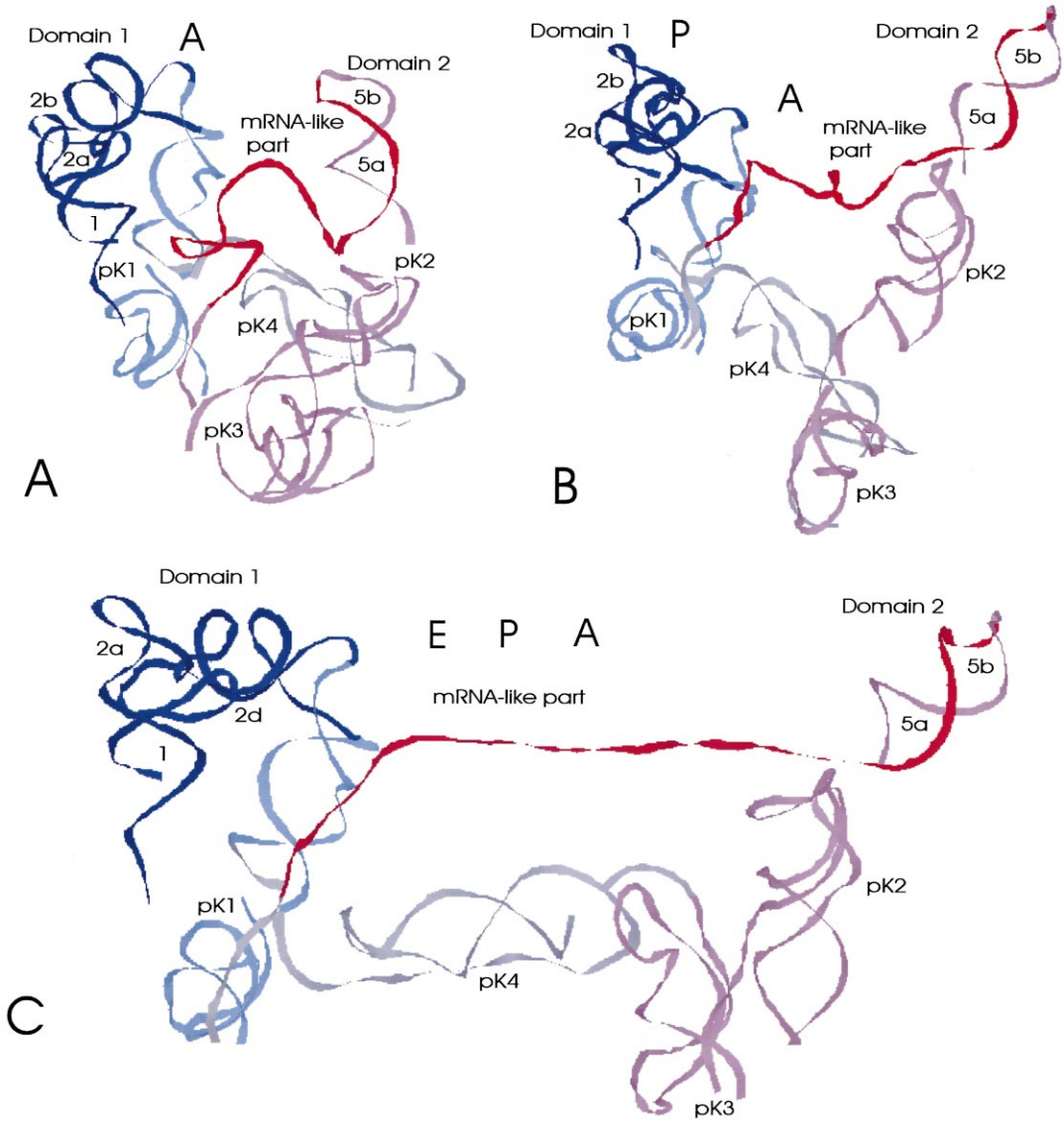

Fig. 2. A model for tmRNA folding. A, P, E ribosomal sites are marked. Numbering of helices and pseudoknots corresponds Fig. 1. A: tmRNA in solution; the tRNA-like structure is black, the rest of the molecule is gray. Domains 1 and 2 are marked. B: tmRNA with the resume codon (shown as nucleotides) at the A-site and the tRNA-like structure of domain 1 at the P-site of the ribosome. C: tmRNA after several steps of translocation. For more explanation, see text.

compactly folded domains in the tmRNA molecule, in agreement with the hypothetical scheme of tmRNA tertiary structure described above (Fig. 2).

Site-directed mutagenesis experiments were performed to reveal those nucleotides of the tmRNA that participate in formation of the tmRNA element potentially involved in recognition by the ribosomal A-site (an element of the domain 1 in the hypothetical model). We assume that this element contains a structure that functionally mimics the codon–anticodon duplex of a canonical aa-tRNA–mRNA complex to be recognized by the decoding center of the A-site [23]. The first candidate for 'codon' in the presumed duplex is the triplet preceding the resume codon, since after the translocation event the resume codon enters the ribosomal A-site, allowing the translational switch to the coding region of tmRNA. The complementary triplet partner (potential 'anticodon') was proposed to be nucleotides 318–320, because this sequence is complementary to the pre-resume triplet and could be placed at the anticodon position of the tRNA-like region, this tmRNA region has been proposed to interact with the decoding site of the ribosome [24]. To test this suggestion, the tmRNA sequence (87)GUC(89) adjacent to the resume-codon GCA was replaced by UAC, GAC or GAG. Similarly, the potential anticodon (318)GAU(320) was changed to GAG or AAG. A plasmid pGEM-ssrA carrying the gene ssrA (or its mutants) with its own promoter and terminator was constructed for in vivo experiments. The mutants were tested for tmRNA activity with the help of phage λimmP22 dis c2-5, since it is known that functional tmRNA is required to support the growth of this phage [12]. No significant effects were detected in any of the mutants (Fig. 5), indicating that the tmRNA element recognized by the A-site has a more complicated structure than that of a simple triplet duplex formed with the help of the pre-resume codon.

The second assumption was that nucleotides (84)AUA(86) might participate in the formation of a codon–anticodon du-

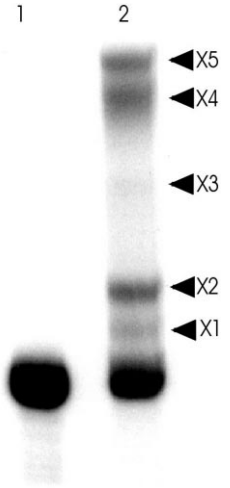

Fig. 3. Gel electrophoretic analysis of the cross-linked products of tmRNA, containing one to two thiouridine residues per molecule, after mild UV irradiation. Lane 1, tmRNA without UV irradiation (non-cross-linked tmRNA used as a control for further analysis); lane 2, tmRNA after UV-irradiation. X1–X5 – cross-linked products.

plex analogue in tmRNA. The trinucleotide sequence (84)AUA(86) is conserved in almost all known tmRNAs and is located close to the resume codon. Further, (84)AU(85) might form Watson–Crick pairs with other, very conserved elements of tmRNA, namely (68)UA(69) or (300)UA(301). Alternatively, the tmRNA element recognized by the ribosomal A-site could be a single-stranded codon that may keep the conformation of the RNA A-form [25]. This function could be preformed by the pre-resume codon in tmRNA (J.F. Curran and V.I. Lim, personal communication), but in this case the neighboring nucleotides, (85)UA(86), should have a significant effect on the conformation of this codon.

Accordingly, we mutated (85)UA(86) to CA, UC, or CC. Dramatic effects were observed: in the case of CA, the efficiency of phage plating was reduced 15 times as compared with the wild type, whereas in the case of UC and CC, the EOP was reduced by as much as four orders of magnitude, similar to the phenotype with a knocked-out tmRNA gene (Fig. 5). To test whether one of the mutations might affect the structure of the tRNA part, the level of aminoacylation was assessed for the lethal mutants. The yield of aminoacylation was 36% for all the tmRNAs, both mutants and wild type, suggesting that our mutants have local functional defects rather than severe effects on the overall structure of domain 1 which is recognized by alanyl-tRNA-synthetase. The results of our in vivo hybrid phage analysis are also in agreement with the data reported by Lee at al. [26] where the mutation A86C seriously damaged the efficiency of *trans*-translation in an in vitro system, although this mutant retained the normal binding capacity to the ribosome. These data indicate that nucleotide residues U85 and A86 of tmRNA are significant for tmRNA function and may be involved in the formation of a tmRNA element playing a central role in A-site recognition.

Previously, we have shown that residues U308 (helix 2) and U268 (pseudoknot 4) outside the tRNA-like part of tmRNA can form a cross-link with EF-Tu–GDP [7], thus demonstrating a new binding site of the EF which is quite different from the canonical interaction of EF-Tu–GTP with aa-tRNA. On the basis of the proposed hypothesis, we can speculate about

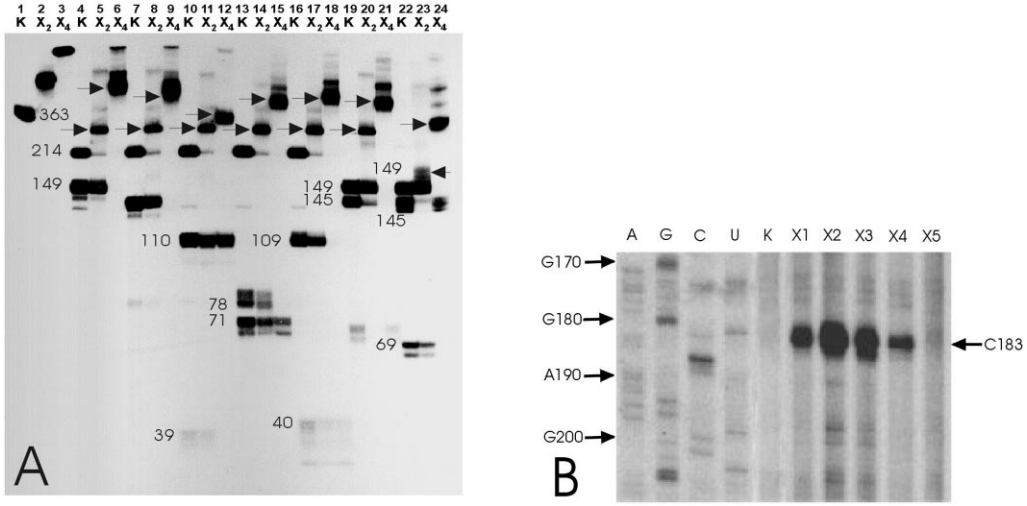

Fig. 4. Examples of the analyses of the cross-linked products. A: RNase H analysis of cross-linked products 2 and 4 by gel electrophoresis after digestion of the RNA by RNase H in the presence of pairs of oligodeoxyribonucleotides. **Lanes 1–3**, without oligodeoxyribonucleotides. **Lanes 4–6**, in the presence of an oligodeoxyribonucleotide complementary to the sequence 200–217 of tmRNA. **Lanes 7–9**, oligodeoxyribonucleotides complementary to 200–217 and 346–363. **Lanes 10–12**, oligodeoxyribonucleotides complementary to 200–217 and 312–325. **Lanes 13–15**, oligodeoxyribonucleotides complementary to 200–217 and 274–291. **Lanes 16–18**, oligodeoxyribonucleotides complementary to 200–217 and 243–260. **Lanes 19–21**, oligodeoxyribonucleotides complementary to 200–217 and 128–145. **Lanes 22–24**, oligodeoxyribonucleotides complementary to 200–217 and 56–73. **Lanes 1, 4, 7, 10, 13, 16, 19, 22**, hydrolysis of the control sample (K in Fig. 3). **Lanes 2, 5, 8, 11, 14, 17, 20, 23**, hydrolysis of cross-link 2 (X2 in Fig. 3) product. **Lanes 3, 6, 9, 12, 15, 18, 21, 24**, hydrolysis of cross-link 4 (X4 in Fig. 3) product. The positions of the cross-links are marked by arrowheads. Numbers at the bands correspond to the length of the fragments. B: Primer extension analysis of the cross-links. An oligodeoxyribonucleotide complementary to region 274–291 in tmRNA was used as primer. A, G, C, U, sequencing lanes. Lane K, control sample (from control lane 1 in Fig. 3). Lanes X1–X5, analysis of the cross-linked products (Fig. 3). The position of the reverse transcriptase stop at position C183, corresponding to the cross-link from U182, is marked by an arrow.

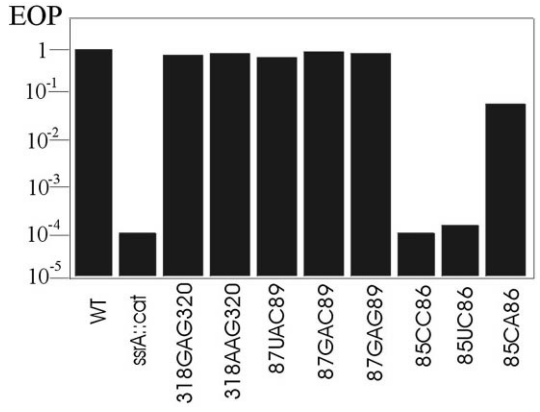

Fig. 5. EOP of phage λimmP22 dis c2-5 on K8619 (ssrA::cat) with a plasmid carrying different mutants of tmRNA. K37 (WT) is a strain with wild-type ssrA.

the functional importance of this unusual complex. (i) The complex might reflect the interaction of EF-Tu with ribosome-bound tmRNA after GTP hydrolysis. GTP hydrolysis causes a significant conformational change in EF-Tu [21], whereby the EF-Tu domains 2 and 3 move in relation to domain 1 by up to 40 Å, and this dramatic conformational change might be exploited by tmRNA to facilitate the opening of its structure, according to the proposed model (Fig. 2A,B). (ii) An alternative explanation is the protection of domain 1 of tmRNA by EF-Tu–GDP, when the tRNA module of the tmRNA leaves the ribosome during recognition of the third codon of the mRNA module.

The experimental data presented here are consistent with the hypothetical scheme proposed in this paper, but clearly the hypothesis needs more experimental testing.

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