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One SmpB molecule accompanies tmRNA during its passage through the ribosomes

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Abstract **tmRNA and SmpB are the main participants of *trans*-translation, a process which rescues the ribosome blocked during translation of non-stop mRNA. While a one-to-one stoichiometry of tmRNA to the ribosome is generally accepted, the number of SmpB molecules in the complex is still under question. We have isolated tmRNA–ribosome complexes blocked at different steps of the tmRNA path through the ribosome and analyzed the stoichiometry of the complexes. Ribosome, tmRNA and SmpB were found in equimolar amount in the tmRNA–ribosome complexes stopped at the position of the 2nd, 4th, 5th or the 11th codons of the coding part of the tmRNA.**

Keywords: Translation; RNA–protein interaction; *Trans*-translation; tmRNA; SmpB

1. Introduction

Trans-translation is an important protein synthesis quality control mechanism in prokaryotes [1]. It recycles ribosomes arrested during the translation of non-stop mRNAs and directs the problematic mRNA and corresponding protein product for degradation. Along with the common participants of the translation process two additional players, namely tmRNA (transfer-messenger RNA, or 10Sa RNA, or SsrA RNA) and SmpB (small protein B), are required for *trans*-translation. tmRNA combines the features of both tRNA and mRNA. Alanylated tmRNA recognizes the arrested ribosome and enters its empty A site. After the transfer of the growing peptide to the CCA-end of the tRNA-like domain (TLD) of tmRNA, the translation switches to its mRNA-like domain (MLD). Ribosomes terminate at the in-frame stop-codon of a short internal reading frame within the tmRNA. The chimeric protein synthesized from the problematic mRNA has at its C terminus a tmRNA-encoded 11 amino acid long tag-peptide, directing it for degradation by a number of cellular proteases.

The second indispensable factor involved in *trans*-translation is small protein B. Since its discovery [2], many reports have shown that SmpB is essential for *trans*-translation. Deletion of >80% of the *smpB* gene in the W3110 strain of *Escherichia coli* results in the same phenotype as observed in *ssrA*::kan cells: both strains are temperature-sensitive, fail to support induction of the lytic development of Mu c-ts lysogens, have a defect in μimmP22 plating and fail to add the degradation tag to the proteins synthesized from damaged mRNAs. SmpB stimulates alanylation of tmRNA [3–5] and protects the Ala-tmRNA product against enzymatic deacylation [3], via binding to tmRNA, SmpB protects it from degradation in the cell [4,6]. The SmpB protein is required for tmRNA association with stalled ribosomes possessing an empty A site [2,4].

At the ribosomal A site the unstructured C-terminal tail of the protein is involved in the accommodation of tmRNA on the ribosome, before transpeptidation [7,8]. Direct hydroxyl radical probing data suggest that the C-terminal tail of SmpB recognizes the decoding center of the 30S subunit and the mRNA path [9]. It was shown using a reconstituted in vitro system that the addition of SmpB and tmRNA stimulates ribosome-dependent GTPase activity of EF-Tu independently of template mRNA [10], suggesting that SmpB compensates for the lack of a codon–anticodon interaction during *trans*-translation initiation. This suggestion agrees with the results of an X-ray study of tmRNA TLD in complex with SmpB [11,12].

It was shown that two SmpB molecules bind the ribosome during the initiation of *trans*-translation [13,14]. An alternative study, however, suggests that only one molecule of SmpB, in complex with tmRNA, interacts with the ribosome [15].

Earlier, we have studied in vivo the formation of two tmRNA–ribosome complexes corresponding to late steps of the passage of tmRNA through a stalled ribosome. The ribosomes were stopped at the 4th or the 11th codons of the coding part of tmRNA. Based on gel staining technique, we estimated that at least one SmpB molecule remained bound to these tmRNA–ribosome complexes [16]. Here, we have prepared a number of tmRNA–ribosomal complexes: blocked after the first translocation of MLD translation (stop codon at the 2nd position, leading to a two amino acid-tag), two complexes stopped at the middle part of MLD (4th and 5th codons, leading to a 4 and 5 amino acid-tag, respectively) and at the very end of MLD (11th codon, leading to a 11 amino acid-tag).

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Abbreviations: tmRNA, transfer-messenger RNA; SmpB, small protein B

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We used these complexes for more precise determination of the stoichiometry of SmpB to the ribosome within the stalled tmRNA–ribosome complexes with the help of Western blot analysis using antibodies against SmpB and the ribosomal protein S3, as internal control.

2. Materials and methods

2.1. Plasmid construction

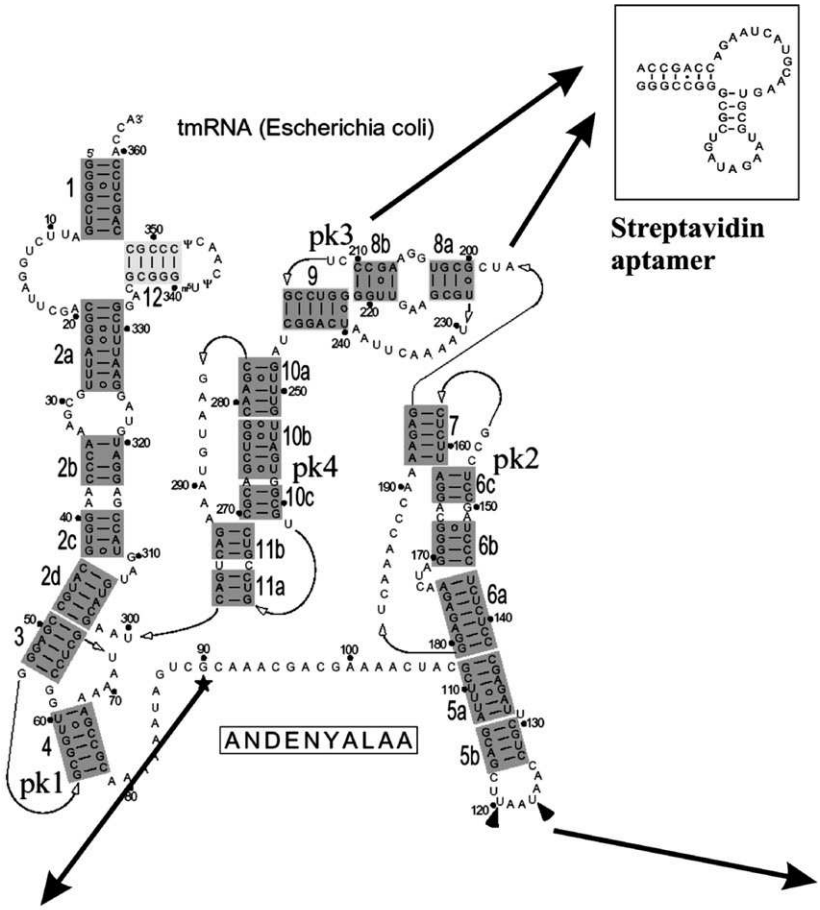
pGEM-stra-2 and pGEM-stra-5 plasmids coding for tmRNA-2 and tmRNA-5, respectively, with mutated termination signals (stop codons in positions 2 and 5 of the tag sequence) were constructed using the QuikChange site-directed mutagenesis kit (Stratagene) on the base of the plasmid pGEM-stra [16]. Mutant sequences were verified by DNA sequencing.

2.2. Complex preparation and affinity purification

tmRNA–ribosome complexes were isolated from *E. coli* SKZ-1 cells [16] carrying the appropriate plasmids and purified on Streptavidin-Sepharose (GE Healthcare) as described [16].

2.3. RNA content of the complex

The RNA fraction of the complex was analyzed by electrophoresis following phenol extraction [17] using a 6% polyacrylamide 7 M urea denaturing gel (19:1 (w/w) monomer to bisacrylamide). The RNA was stained with ethidium bromide (0.004%). The gels were photographed using the program "Gel" (utility for capture video iuVCR 4.10.0.327), and the amount of RNA in each band was estimated using the ImageQuant program 5.0.Ink.



		Stop Stop
Wild Type	GCA AAC GAC GAA AAC UAC GCU UUA GCA GCU **UAA UAA**	
	Pro Stop	
tmRNA-2	**CCA UGA A**AC GAA AAC UAC GCU UUA GCA GCU UAA UAA	
	Pro Stop	
tmRNA-4	GCA **GAC CCA UGA A**AC UAC GCU UUA GCA GCU UAA UAA	
	Pro Stop	
tmRNA-5	GCA AAC **GAC CCA UGA A**AC GCU UUA GCA GCU UAA UAA	
		Pro Stop
tmRNA-11	GCA AAC GAC GAA AAC UAC GCU UUA **GAC CCA UGA A**AA	

Fig. 1. Secondary structure of tmRNA with numbered helices and pseudoknots (adapted from psyche.uthct.edu/dbs/tmRDB/tmRDB.html). pk3 (nucleotides U212–A239) was substituted with an aptamer to streptavidin (inset). Mutations in the mRNA-like part of tmRNA are underlined and shown in bold.

2.4. Protein content of the complex

After trichloroacetic acid precipitation, proteins were separated in 17.5% Tricine–SDS–PAGE [18]. The semi-dry method was applied to transfer proteins onto a Hybond-P membrane (GE Healthcare) for subsequent Western blotting. Proteins were visualized using polyclonal antibodies directed against the ribosomal protein S3 and the SmpB, horse-radish peroxidase conjugated secondary antibody (Imtek Ltd.) and ECL kit (GE Healthcare) according to the manufacturer's instructions. The intensities of the bands corresponding to SmpB and S3 were determined using the ImageQuant program 5.0.Ink.

3. Results

3.1. tmRNA mutants

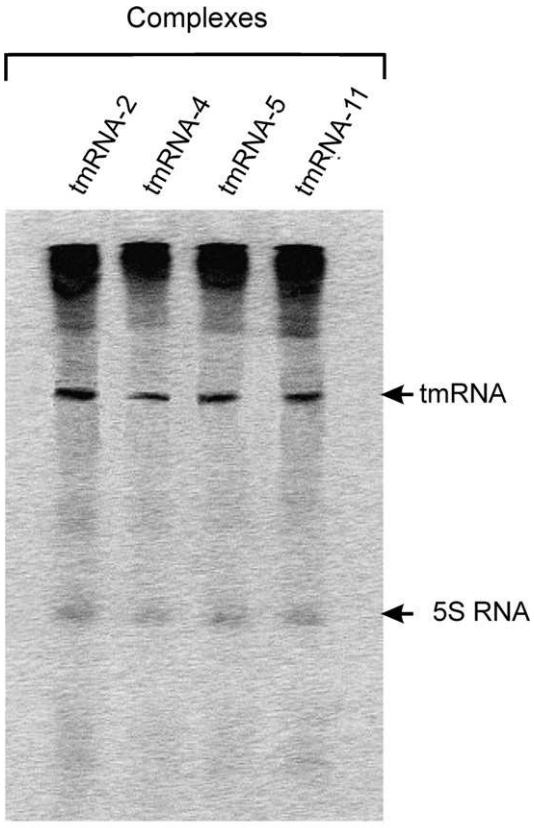
Earlier [16], we created a set of plasmids coding for mutant tmRNAs containing (1) a streptavidin aptamer for purification by affinity chromatography and (2) the UGA stop codon at the 4th or the 11th codons of the MLD, within the weakest termination context (tmRNA-4 and tmRNA-11). Expression of these mutant tmRNAs in the *E. coli* strain SKZ1 harboring thermosensitive RF2 allowed isolation of the ribosome complexes containing mutant tmRNAs with the streptavidin tag affinity method. In the present work, we used the same site-directed mutagenesis approach to place a UGA stop codon (with a weak termination context) at the position of the 2nd or 5th codon of MLD (tmRNA-2 and tmRNA-5). As a result, we have a set of four mutant tmRNAs that allow tmRNA–ribosome complexes from early to very late stages of MLD translation to be investigated (Fig. 1).

3.2. The RNA content of tmRNA–ribosome complexes

tmRNA–ribosome complexes were isolated exactly as described previously [16]. RNA molecules were extracted from the isolated tmRNA–ribosome complexes and analyzed by gel electrophoresis in denaturing conditions as shown in Fig. 2. All four complexes were obtained in good yield and found to contain tmRNA, 16S, 23S, and 5S ribosomal RNAs and tRNAs (Fig. 2). The intensities of the 5S rRNA and tmRNA bands densities were measured. The results of three independent experiments indicate that the ratio of intensity from the band of tmRNA to that one of 5S rRNA was about 3:1 for all four tmRNA–ribosome complexes (table in Fig. 2). The signal from ethidium bromide stained RNA is proportional to the length of the RNA. *E. coli* tmRNA has 363 nucleotides, which is about three times more than the length of 5S rRNA (120 nucleotides). Thus, the molar ratio of ribosome to tmRNA is about 1:1 in all studied complexes.

3.3. Analysis of the protein content of tmRNA–ribosome complexes

To estimate the molar ratio of ribosome:SmpB in the complexes, we performed quantitative Western blot analysis. For that purpose, increasing amounts of purified SmpB-6His protein (Fig. 3, lanes 1–5) and total proteins from the 30S ribosomal subunit (Fig. 3, lanes 6–10) were applied to the Tricine–SDS–PAGE, transferred to Hybond-P membrane and detected using antibodies against the SmpB protein and ribosomal protein S3. Comparison of the intensities of the bands corresponding to SmpB or S3 in the ribosomal complexes (Fig. 3, lanes tmRNA-2, tmRNA-4, tmRNA-5 and tmRNA-11) with the intensity of the reference lanes allowed the amount of each of the two proteins in the ribosomal complexes to be determined with accuracy. An example of such analysis performed for the tmRNA-2 complex is presented in Fig. 3. The results of three independent experiments indicate that the stoichiometry of SmpB to the ribosome was 1 to 1 for all four tmRNA–ribosome complexes studied (table in Fig. 3).

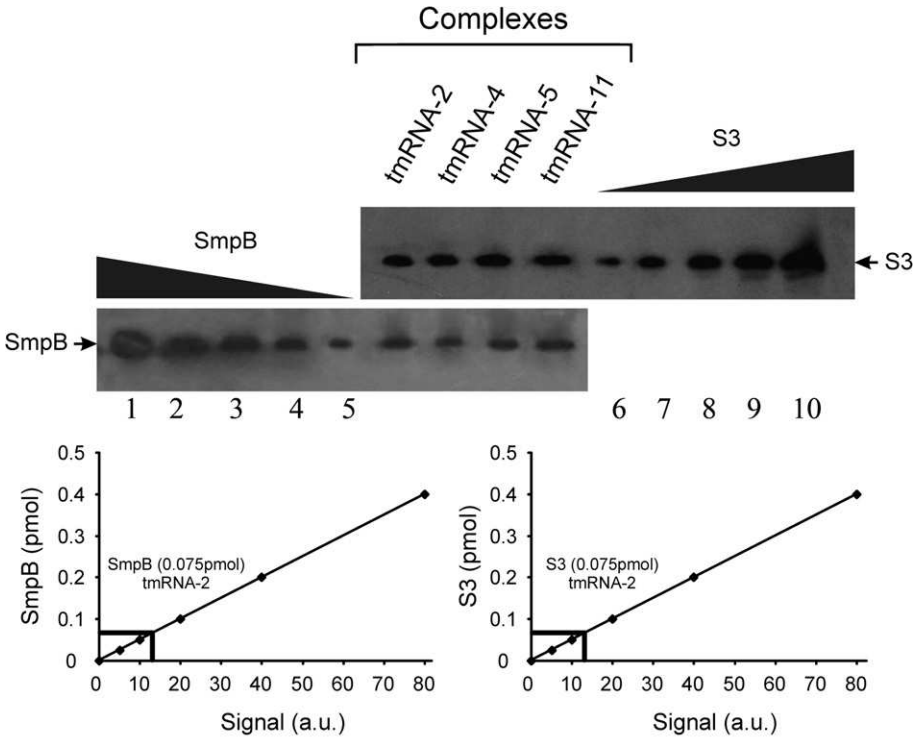


Complex	tmRNA/5S RNA
tmRNA-2	3.1 ± 0.1
tmRNA-4	3.0 ± 0.2
tmRNA-5	2.7 ± 0.1
tmRNA-11	2.9 ± 0.1

Fig. 2. Analysis of the RNA content of the tmRNA–ribosome complexes. Electrophoretic separation of RNA isolated from the complexes with tmRNA-2, tmRNA-4, tmRNA-5 and tmRNA-11 purified by affinity chromatography in 6% denaturing PAGE. The positions of the RNAs are marked with the arrows. The ratio of the intensity from the band of tmRNA to that one of 5S rRNA (tmRNA/5S RNA) was measured and the results of three independent experiments are summarized in the table.

4. Discussion

In spite of intensive studies, there is still uncertainty about the stoichiometry between SmpB and the tmRNA in the ribosome complex during all the steps of *trans*-translation. An analysis performed in solution, outside the ribosome, detects three independent SmpB binding sites per tmRNA molecule



Complex	Ratio SmpB/S3
tmRNA-2	1.00 ± 0.05
tmRNA-4	0.94 ± 0.09
tmRNA-5	1.06 ± 0.05
tmRNA-11	1.03 ± 0.08

Fig. 3. SmpB:ribosome ratio measurements in tmRNA–ribosome complexes. Western blot of the SmpB and ribosomal protein S3 in the protein fractions isolated from the complexes with tmRNA-2, tmRNA-4, tmRNA-5 and tmRNA-11. Lanes 1, 2, 3, 4 and 5 – 0.4, 0.2, 0.1, 0.05 and 0.025 pmol of purified SmpB-6His, respectively. Lanes 6, 7, 8, 9 and 10 – 0.025, 0.05, 0.1, 0.2 and 0.4 pmol of TP30 (proteins isolated from small ribosomal subunit). The two graphs represent the calibration curves derived for the purified SmpB and S3 proteins, the Western blot signals are in arbitrary units (a.u.). The experimental densities of the band for SmpB or S3 from the isolated complex containing tmRNA-2 were applied to the corresponding calibration curve and in this way the amounts of SmpB or S3 in the ribosomal complex were determined. The ratio of the SmpB to S3 was estimated for all complexes and the results of three independent determinations are summarized in the table.

[19]. In the early stages of *trans*-translation, before accommodation, structural [20] and biochemical [13] data indicate that two molecules of SmpB can interact with the stalled 70S ribosome. However, a recent study carried out under different salt concentrations showed that at 300 mM ammonium sulfate concentration, SmpB does not pre-bind the stalled ribosomes and a 1:1:1 complex of SmpB-tmRNA-EF-Tu (GTP) binds the stalled ribosomes to trigger *trans*-translation [15].

Earlier, we have developed an elegant system which allows *trans*-translation to be blocked at the desired step of the passage of the MLD through the ribosome and to isolate the various tmRNA–ribosome complexes via affinity chromatography [16]. In this report, we have purified set of ribosomal complexes stalled at different stages of *trans*-translation (the 2nd, 4th, 5th or the 11th codons of the ORF). Stalling *trans*-translation at the 2nd codon of the ORF corresponds to a complex in which the TLD of tmRNA is, in principle, located within the ribosomal E site. Inserting an inefficient termination codon at the 2nd codon of the ORF leads to two changes in the sequence of tmRNA, U88A and G90C, a region that is essential for tmRNA activity and resume codon selection [21,22]. The two mutations introduced in tmRNA-2, however, do not alter the resume codon and only slightly reduce tagging activity in vitro [22]. The tmRNA-4 and tmRNA-5 variants correspond to the middle position within the tag reading frame of tmRNA. The tmRNA-11 reflects the natural termination step.

Thus, this set of the tmRNA–ribosome complexes provided us the possibility to follow the fate of SmpB protein during the translation of the ORF from the early (TLD is at the E site) to the very late (termination) stages. All four of the tmRNA–ribosome complexes were isolated in vivo at yields sufficient for the subsequent analysis. Their RNA contents indicate the presence of the three ribosomal RNAs, as well as tmRNA and the tRNAs. Scanning the stained RNA gels and quantitation of the signals corresponding to tmRNA in comparison with the 5S rRNA, used as an internal standard, indicate that a ratio of the ribosomes with tmRNA is 1:1 in all the complexes.

Since protein staining depends on the protein sequence, the comparison of the stained bands corresponding to different proteins does not allow a precise measurement of the protein

stoichiometry in all the four ribosomal complexes. In this report, we used Western blots with antibodies against either SmpB or the ribosomal protein S3, as an internal control. Calibration curves were obtained separately for purified SmpB-6His and used for the determination of the precise amounts of SmpB present within the four ribosomal complexes. In parallel, S3 calibration curves were obtained and molar amounts of that ribosomal protein were determined in these ribosomal complexes, as internal controls. These data indicate that SmpB binds to each of the four complexes that are stalled at different codon positions in MLD in a one to one ratio with the stalled ribosomes. Thus, in the course of *trans*-translation, tmRNA and SmpB are present in equimolar amounts in the ribosomal complexes. These data are in agreement with the fact that SmpB:tmRNA ratio in the cell is ~1:1 and the results of Sundermaier and Karzai [15], although we cannot exclude the presence of the second SmpB molecule in the ribosomal complexes at the early pre-accommodation stage of *trans*-translation.

According to current Cryo-EM model for tmRNA on the ribosome in the pre-accommodation complex SmpB occupies the part of decoding centre on the 30S subunit [14] which later should be taken by normal codon–anticodon interactions. We have found that only one SmpB molecule is present in the complexes from the moment when translation of ORF is started till the termination step and most probably SmpB interacts with one of its binding site on the tmRNA. We can speculate that SmpB occupies its canonical site on the tmRNA [11,23].

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