Finding the unknowns in \textit{trans}-translation

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Abstract

Ribosomes stalled on problematic mRNAs can be rescued by a mechanism called trans-translation. This mechanism employs a dual transfer-messenger RNA molecule (tmRNA) together with a helper protein (SmpB).

In this work we have used an in vitro translation system with pure components to further clarify the roles of tmRNA and SmpB in trans-translation.

We found that SmpB binds ribosomes in vivo and in vitro independently of tmRNA presence and is essential for tmRNA binding and trans-peptidation. We show that two SmpB molecules can bind per ribosome, that SmpB does not leave the ribosome after trans-peptidation and that SmpB pre-bound to the ribosome can trigger trans-translation.

We demonstrated that the rate of trans-transfer of a peptide from the P-site tRNA to Ala-tmRNA and the efficiency by which Ala-tmRNA competes with peptide release factors decrease with increasing the mRNA length downstream from the P site of the ribosome. We showed that trans-translation is strongly stimulated by ReIL cleavage of A-site mRNA. We concluded that tmRNA action in vivo must always be preceded by mRNA truncation.

We showed that rapid release of truncated mRNAs from the ribosome requires translocation of the peptidyl-tmRNA into the ribosomal P site, which is strictly EF-G dependent. mRNA release is slowed down by strong Shine and Dalgarno like sequences upstream the A site and by long 3′-extensions downstream from the P-site codon.

Footprinting was used to monitor SmpB binding to tmRNA, ribosomes and subunits and to study tmRNA interactions with the ribosome at distinct trans-translation stages. We confirmed that two SmpB molecules bind per ribosome and interact with nucleotides below the L7/L12-stalk on the 50S subunit and near the subunit interface on the 30S. We showed that tmRNA is mostly in complex with SmpB in vivo and during trans-translation. Specific cleavage patterns of tmRNA were observed at different stages of trans-translation, but the overall tmRNA conformation seems to be maintained during the whole process.

Keywords: trans-translation, tmRNA, SmpB, kinetics, footprinting

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In memoriam

This work is dedicated to the memory of my aunt Dasha and my grandfather Peyko.
LIST OF PAPERS


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<td>aa-tRNA</td>
<td>Aminoacyl-transfer ribonucleic acid</td>
</tr>
<tr>
<td>AlaRS</td>
<td>Alanyl-transfer ribonucleic acid synthetase</td>
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<tr>
<td>Cryo EM</td>
<td>Cryo electron microscopy</td>
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<td>DC</td>
<td>Decoding center</td>
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<td>EF-G</td>
<td>Elongation factor G</td>
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<td>Elongation factor Tu</td>
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<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>MLD</td>
<td>Messenger ribonucleic acid like domain</td>
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<td>NMR</td>
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<td>ORF</td>
<td>Open reading frame</td>
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<td>PK</td>
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<td>Release factor</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RRF</td>
<td>Ribosome recycling factor</td>
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<td>Shine and Dalgarno</td>
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<td>Small protein B</td>
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<td>Transfer ribonucleic acid like domain</td>
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<td>tmRNA</td>
<td>Transfer-messenger ribonucleic acid</td>
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<td>tmRNP</td>
<td>Transfer-messenger ribonucleic acid-protein</td>
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INTRODUCTION

The ribosome and the mechanism of translation

Translation of the genetic information from nucleotide sequences (mRNAs) to amino acid sequences (proteins) takes place on the ribosome – a large ribonucleoprotein complex that consists of two subunits with distinct functions. Bacterial ribosomes (70S) are composed of a small subunit (30S) containing 16S rRNA and around 20 proteins, and a large subunit (50S) which contains 23S rRNA, 5S rRNA, and more than 30 proteins [1]. In addition to the ribosome, the translation process requires around 45 tRNA molecules, 20 aminoacyl-tRNA synthetases that acylate specifically these tRNAs with particular amino acids and a number of translation factors participating at different steps of the translation cycle.

The ribosome has three tRNA binding sites: the A (aminoacyl or acceptor) site, which accepts the incoming aminoacylated tRNA in complex with EF-Tu•GTP; the P (peptidyl) site, where the tRNA carrying the nascent peptide chain is bound; and the E (exit) site, where the deacylated tRNA transiently moves before it leaves the ribosome.

The mRNA and the anticodon stem-loops of A- and P-site tRNAs bind to the small ribosomal subunit, where the genetic information is translated in the decoding center (DC) by verifying the correct base pairing between the mRNA codon and the anticodon of the A-site tRNA. The peptide bond formation between the amino acid on the A-site bound tRNA and the peptide on the P-site bound tRNA is catalyzed by the peptidyl transferase center (PTC) on the large ribosomal subunit.

Crystal structures of both ribosomal subunits as well as the whole ribosome have been recently solved ([1] and reviewed in [2, 3]). Detailed structures (at 2.4 to 3.3 Å resolution) were published for the 50S subunit [4, 5] and for the 30S subunit [6, 7]. The atomic structure of the entire 70S ribosome solved at 5.5 Å resolution has been reported in 2001 [1].
Knowledge of the ribosome structure provides an enormous amount of information about details of protein-RNA interactions in each subunit, as well as details of the interactions of the ribosomal protein and RNA components with translation factors and RNA molecules (mRNA, and tRNA). The information provided by structural studies is now used as a basis for designing functional assays leading to a deeper understanding of the mechanisms of translation and the roles played by different factors.

The translation process can be divided into initiation, elongation, termination and ribosome recycling (Fig. 1). The formation of the initiation complex is considered to be the rate-limiting step of translation. During initiation the 30S subunit, in complex with IF3, binds a messenger RNA, IF1, IF2•GTP and initiator tRNA (fMet-tRNA\(^{f\text{Met}}\)), forming a 30S pre-initiation complex, which rapidly recruits the 50S subunit in the formation of a 70S initiation complex [8, 9].

Translation continues with cycles of elongation of the polypeptide chain. During the elongation step, the mRNA codons are displayed sequentially in the A site of the ribosome and aminoacyl-tRNAs (aa-tRNAs) with complementary anticodons are selected. Elongation factor Tu (EF-Tu) delivers aa-tRNAs to the A site where tRNA recognition and selection takes place [10]. After peptide bond formation elongation factor G (EF-G) translocates the newly formed peptidyl-tRNA from the A site to the P site and, concomitantly, the deacylated tRNA from the P site into the E site from which it rapidly dissociates. Translocation of tRNAs is accompanied by translocation of mRNA, enabling the ribosome to slide along the mRNA to the next codon. (Fig. 1)

The translation process is terminated by the dissociation of the ribosome•mRNA•tRNA•nascent peptide chain complex [3]. When an mRNA stop codon enters the A site, release factors “class I” RF1 and RF2 (in bacteria) recognize the stop codon - UAA for both RF1/RF2, UAG for RF1 and UGA for RF2 and activate hydrolysis of peptidyl-tRNA to release the nascent peptide chain (reviewed in [11]). A “class II” release factor - RF3, a ribosome-dependent GTPase, accelerates the dissociation of RF1 or RF2 and their recycling between ribosomes [12-14].

After the peptide chain has been released, the ribosome is left with the mRNA and a deacylated tRNA bound to the P site. This stable complex is disassembled to prepare the ribosome for a new round of protein synthesis by two factors: ribosome recycling factor (RRF) and elongation factor G (EF-G) [15, 16] (see Fig. 1).
**Figure 1.** The translation model.
Proper termination can not occur on messengers lacking stop codons. The ribosome gets stalled at the end of such mRNAs. The ribosomal stalling results in two major problems: firstly, depletion of the pool of ribosomes available for translations and, secondly, the partially synthesized polypeptide can be toxic for the cell.

These two problems can be solved in the cell by a quality-control rescue process called trans-translation [17, 18] which employs a transfer-messenger RNA molecule (tmRNA) and its protein cofactor SmpB.

**tmRNA and the mechanism of trans-translation**

**Discovery of tmRNA and trans-translation**

tmRNA, known also as SsrA RNA and 10Sa RNA is a unique dual RNA molecule which functions both as tRNA [19-21] and mRNA [19, 20].

It was discovered by Suzanne Lee in David Apirions group [22] as a small stable RNA in *Escherichia coli* with unknown function. This RNA species was first named 10Sa RNA [23] because of its sedimentation speed of 10S. The 10Sa RNA was estimated to be present in about one to ten molar ratio to the amount of ribosomes in *E. coli* cells grown on minimal media [22].

Transfer-messenger RNA was found to be encoded by the ssrA gene [24-26] and its disruption caused a reduction in the rate of cell growth, especially at higher temperature [20, 27]. Even though tmRNA-deletion mutants were viable it was noticed that they fail to support growth of hybrid phages λimmP22. This allowed a more robust phenotypical identification of *E. coli* strains lacking functional tmRNA [28].

It was, however, not until 1996, almost 20 years after tmRNA was first discovered, when Robert Sauers group [17] proposed a model for tmRNA action. This seminal work was preceded by other studies demonstrating that tmRNA possesses tRNA properties and contains an internal open reading frame. The tRNA properties of SsrA were discovered first. In 1994 two groups, working in parallel with different organisms (*E. coli, B. subtilis* and *Mycoplasma capricolum*) discovered that the 5’ and 3’ ends of tmRNA fold into a structure resembling a tRNA molecule [20, 21]. Komine and co-workers found that this tRNA-like structure of *E. coli* 10Sa was
very similar to a part of tRNAAla and that purified 10Sa RNA can be charged with alanine in vitro [20]. Ushida et al. discovered that 10Sa analogs in two Gram-positive bacteria (*B. subtilis* and *M. capricolum*) also fold in a tRNA-like structure, aminoacylatable with alanine [21].

An internal open reading frame (ORF) was identified in the *ssrA* gene when it was sequenced in 1989 [24]. However, there was no proof until 1995 that this ORF was translated. In 1995 Tu et al. [19] observed that when murine interleukin-6 (IL-6) was overexpressed in *Escherichia coli*, a subpopulation of its truncated forms had the same C-terminal modification. This modification was a peptide tag, consisting of 11 amino acids (AANDENYALAA) attached to the incomplete IL-6 variants. It was found that this sequence (except for the first alanine) corresponded to the ORF of 10Sa RNA and a *ssrA* disruption mutant in *E. coli* didn’t produce tagged IL-6 forms [19].

By finding that the C-terminal amino acids of the *ssrA*-encoded peptide tag are recognized by the periplasmic protease Tsp, Keiler and co-workers discovered the last piece of information necessary to solve the tmRNA function mystery. They put together the pieces of the puzzle and came up with a model for *trans*-translation [17]. Interestingly, the name *trans*-translation was given by Atkins and Gesteland [18] when commenting the original paper [17].

**Current model for trans-translation**

After the establishment of the general principles of *trans*-translation in 1996 many important questions concerning the detailed mechanism of this process remained unanswered. A number of studies has demonstrated that *trans*-translation has many features in common with the normal translation but also clarified the requirement for additional non-canonical translation factors.

It was established that tmRNA aminoacylation with alanine is prerequisite for its binding to the ribosome [29]. Moreover, EF-Tu•GTP forms a ternary complex with the tRNA-like part of Ala-tmRNA and delivers it to the ribosome as in the case of canonical tRNAs [30]. Further, Small protein B (SmpB) was shown to be indispensable for *trans*-translation and in particular for tmRNA binding to the ribosomal A site [31].

The current model for the sequence of events in *trans*-translation is the following: Ala-tmRNA in complex with SmpB and EF-Tu•GTP enters the A site of the stalled ribosome and then the nascent polypeptide is transferred from the P-site tRNA to the Ala-tmRNA. tmRNA now bearing the polypeptide is translocated to the
P site. At this stage the problematic mRNA on which the ribosome is stalled must leave and the translation resumes on the tmRNA ORF. When the ribosome reaches the stop codon of the tmRNA ORF the tagged polypeptide is released by the action of release factors and directed for degradation (Fig. 2). Thereafter, the ribosome can be recycled back to a new round of initiation by ribosomal recycling factor RRF and elongation factor EF-G [15, 16].

**Figure 2.** The *trans*-translation model, adopted from paper IV with changes.
The last and extremely important event in trans-translation is the degradation of the released tagged polypeptide. Keiler and co-workers found that the tmRNA encoded tag was recognized by the Tsp protease [17]. Later on ClpXP was identified as the major cytoplasmic protease [32, 33] that is responsible for the degradation of tmRNA-tagged proteins but other proteases were also found to be involved in this process. Those are the ClpAP [32], localized in the cytoplasm and the membrane-localized FtsH [34].

**ssrA phenotypes in different species**

*ssrA* genes have been found in all sequenced so far bacterial genomes [35-37] but have not been identified in the nuclear genomes of Archaea and Eukarya.

The *ssrA* gene is essential for some pathogenic bacteria like: *Mycoplasma genitalium*, *Mycoplasma pneumoniae* [38] and *Neisseria gonorrhoeae* [39]. *S. typhimurium* mutants lacking the SsrA gene are viable but not virulent [40].

In *E. coli* tmRNA-deletion mutants are viable but they fail to support the growth of *Salmonella* phage P22 [40-43] and the hybrid phage λimmP22 constructed from coliphage λ and phage P22 [42]. The selective inhibition of P22 (Sip) phenotype was first described for a mutation called *sipB391* [44] and only later was found to be due to a deletion in the 3'- end of *ssrA* [43, 45]. Using the discovery that growth of variants of λimmP22 with mutations in the C1 gene is supported by *E. coli* lacking tmRNA activity [45, 46] the pair of λimmP22 phages having either wild-type or mutated C1 could be used as a straightforward and specific test to determine whether an *E. coli* strain is expressing active tmRNA or not [28].

Even though the disruption of the *ssrA* gene in *E. coli* and *B. subtilis* is not lethal, it slows down the growth rate under stress conditions [20, 47]. A reduction in motility on semisolid agar was observed for the *E. coli ssrA* deletion mutants [20]. Reduced growth rates at high temperature were seen for both *E. coli* and *B. subtilis* ssrA mutants and in the case of *B. subtilis* tmRNA was also shown to help the cells to adapt under other stresses, such as high concentrations of ethanol or cadmium chloride [47]. Under stress conditions *B. subtilis* growth rate depended on the expression level of tmRNA. The transcription of *ssrA* increased under different stressful conditions up to 10-fold, suggesting that it is a stress-response gene. These observations suggest that alanyl-tmRNA participates in stress adaptation in bacteria probably via recycling stalled ribosomes using trans-translation [47].
The presence of a functional trans-translation system is important for survival of other bacterial species under adverse conditions as well. Experiments in *Synechocystis* showed that in the absence of tmRNA activity this cyanobacterial species cannot survive exposure to low concentrations of antibiotics inhibiting the protein synthesis, whereas growth of a wild-type *Synechocystis* strain is unaffected by low concentrations of these inhibitors [48]. Pretreatment of *ssrA* mutant *Synechocystis* with chloramphenicol led to a 50% reduction in translation relative to the level of translation observed after pretreatment of wild-type *Synechocystis* with the same antibiotic. These results implied that trans-translation is required for efficient translation in the presence of protein synthesis inhibitors, perhaps by rescuing ribosomes stalled by the inhibitor.

Altogether the evidence suggests that tmRNA is important for the survival of the cell under abnormal conditions and gives it an advantage in adaptation. During various stresses there is a stronger need for tmRNA since its function is to rescue stalled ribosomes and to tag abortive polypeptides for degradation. In addition, quality control mediated by tmRNA might ensure that the proteins allowing growth under stress are produced and the consequences of translational errors that are predicted to increase are corrected [49].

**ssrA genes in different species**

In most cases both tmRNA domains are encoded by the same gene and expressed as a single molecule. The 5’ and 3’ of this single RNA molecule basepair to form the tRNA-like domain and the mRNA part is placed in the inner part of the molecule usually flanked by pseudoknot structures.

However, independent circular gene permutation events have led to the appearance of two-piece tmRNA, that effectively breaks open the mRNA domain loop, in alphaproteobacteria and cyanobacteria [36, 50] as well as in *Dechloromonas aromatica* and close relatives [51]. In the permuted genes the segment normally at the 3’ end of tmRNA genes is instead found upstream of the segment usually at the 5’ end. Convergent evolution to a similar form in three separate bacterial lineages suggests that loop-opening benefits tmRNA function by solving topological problems of moving the whole one-piece tmRNA molecule through the ribosome [51].

A degenerated homologue of tmRNA, lacking the ORF was discovered in the mitochondrial genome of *Reclinomonas americana* [36]. However, because no tag-like sequence was found, it is unclear whether it donates its charging alanine and it is very unlikely that it has retained the tagging function.
tmRNA genes were found in the genomes from representatives of all three plastid lineages [52, 53]. The *N. olivacea* plastid has the only genome identified up-to-date, containing two copies of ssrA. Plastid tmRNAs show loss of pseudoknots as well as the tmRNA sequence from a bacterial endosymbiont of insect cells, *Tremblaya princeps*. Pseudoknots may optimize tmRNA function in free-living bacteria but might become dispensable the endosymbiots which have reduced selective pressure for fast growth [53].

**tmRNA expression and maturation**

Mature tmRNA in *E. coli* encompassing 363 nucleotides is formed from a much bigger precursor, a pre-tmRNA (457 nucleotides), originally called p10Sa [54]. The pre-tmRNA is processed both at the 5’ and 3’ ends.

The endonuclease RNAse P removes the 7-nucleotide leader sequence to generate the mature 5’ end [20] as it does for canonical tRNAs [55].

Several enzymes were found to participate in the processing of the pre-tmRNA 3’ end – RNAse E, III, PH, T and BN [54, 56, 57]. Recently it was discovered that the large multiprotein ribonucleolytic “degradosome” [58-60] complex and in particular the RNAse E present in it cleave the pre-tmRNA and generate the mature tmRNA 3’ terminus [61]. However, cleavage by RNAse E is not sufficient to generate the mature 3’ CCA end in tRNAs. Taken together these data suggest that other RNases might have a role in the 3’-end processing of pre-tmRNA but the precise mechanism of the formation of the mature CCA end is still unclear [28].

Little is known about the tmRNA transcriptional regulation. However it was observed that tmRNA synthesis is increased during stress in *B. subtilis* [47] or at particular points of the cell cycle in *Caulobacter crescentus* [62]. These observations are consistent with the idea that tmRNA is involved in cellular adaptation and participates in gene-expression control.

**tmRNA structure and functional interactions**

**tmRNA structure**

The size of tmRNA in bacteria varies between ~260 and 430 nucleotides depending on the species. The secondary structure was modeled on the basis of studies applying chemical and enzymatic probing [63], UV-induced intramolecular cross-linking [64] and NMR [65-67], combined with phylogenetic analysis [35, 68,
69]. In vivo probing of the tmRNA structure with lead(II) confirmed the model based on in vitro work [70].

The 5’ and 3’ termini of tmRNA are folded into a structure mimicking a tRNA molecule and form the tRNA like domain (TLD). In addition to the TLD the tmRNA molecule contains an internal ORF (an mRNA like domain, MLD), several RNA helices and in most cases (PK1-PK4) with RNA single strands in between [65] (Fig. 3).

**Figure 3.** Model for the secondary structure of tmRNA from E. coli, adopted from the tmRNA web-site (www.indiana.edu/~tmrna/).
The TLD includes the T-arm, the D-loop and the acceptor stem of the cloverleaf structure of a generic tRNA molecule. The TLD mimics the corresponding features of tRNA^{Ala} and can be aminoa- cylated by Ala-tRNA synthetase with alanine [20, 21]. The accep- tor stem and the T-arm of the TLD posses the determinants recognized by Ala-tRNA synthetase, namely the G:U wobble pair [20, 21] which is critical for the aminoaacylation with alanine [71], two pseudouridines and one 5-methyluridine in the T-loop in corre- sponding to canonical tRNAs positions [72]. However, even though all natural tmRNAs are charged with alanine and the G:U wobble pair is conserved it was shown that the amino acid identity of tmRNA can be switched without affecting the tagging function of tmRNA [73].

Some other nucleotides in the TLD, important for the amino acylation and proper folding, were identified by mutational studies and footprinting techniques. Nucleotides 16-20 and 334-335 in the acceptor stem were found to participate in functional interactions with the ribosome [74]. Most probably these interactions are medi- ated by SmpB since in other studies it was seen that nucleotides 330-335 are protected by SmpB [75, 76]. It was proposed that SmpB compensates for the lack of a proper anticodon in tmRNA and mediates the interaction of the TLD with the ribosome [77-79].

The TLD is connected to the mRNA like domain (MLD) by a pseudoknot-rich domain consisting of four pseudoknots (PK1- PK4) in most tmRNAs.

The MLD in all known bacterial and plastid tmRNAs consists of a short open reading frame (ORF) starting with a “resume” codon and ending with two stop codons. The ORF encodes a 9 to 28 amino acid long (ANDENYALAA for *E. coli*) peptide tag, with con- served C-terminal hydrophobic/aromatic character that is recog- nized by cellular proteases [17, 32, 34]. The mechanism of tmRNA switching from tRNA to mRNA mode of action is poorly understood and has been the subject of several studies [80-82].

Important determinants for defining the resume codon have been found by Williams et al. [82]. The mode of determining the tmRNA reading frame is unique since tmRNA TLD doesn’t have an anticodon so the first translated codon is not specified by a par- ticular tRNA anticodon as it is in all other studied ORFs [82]. Wil- liams and co-workers found that the resume-positioning segment is located between PK1 and the resume codon (nt 90-92 in *E. coli*). Adenosine 86 located 4 nt upstream of the resume codon was found to be essential for *trans*-translation and is universally con-
served among bacterial tmRNAs. Mutations of A86 seriously decreased or completely abolished the tmRNA-dependent tagging even though they had little or no effect on tmRNA charging and association with the ribosome [80-82]. This nucleotide and the neighboring U85 may participate in ribosomal A site recognition by the tmRNA [80]. Recently it was suggested that the -1 codon plays the role of a codon:anticodon duplex, which is recognized by the decoding center of the 30S subunit [83]. Lim and Garber came up with a rule regarding which triplets should not be used as the –1 triplet and their analysis of the tmRNA database confirms that all –1 triplets found so far obey this rule [83].

It was shown that altering of the tag coding sequence did not abolish tmRNA-mediated tagging but affected proteolysis when the C-terminal hydrophobic residues were substituted by charged amino acids [17].

The internal open reading frame in the MLD of tmRNA is surrounded by four pseudoknot (PK) structures (PK1 – PK4) connected by single stranded RNA stretches and helices. PK1 is situated upstream with respect to the ORF and is connected to the TLD via a long stem. The 3’ end of the ORF is flanked by PK2-4 where PK-4 is linked back to the stem connecting the MLD with the TLD.

Nameki and co-workers found that three of the four pseudoknots can be substituted by single stranded RNA and are interchangeable [84] without any significant effect on tmRNA functions and that only PK1 was important for tmRNA aminoacylation and tagging [84]. However, a recent study by Wower and Zwieb suggests that PK2, PK3, and PK4 are important for the proper overall folding of the tmRNA. PK2 and the abutting helix 5 are particularly important for the binding of ribosomal protein S1 to tmRNA and at least one pseudoknot, optimally PK4, is required for tmRNA tagging [85]. The obvious discrepancy between these results and the ones obtained by Nameki et al. [84] could be due to the insensitivity of the in vitro assay used by the later group.

**SmpB function and structure**

The gene encoding Small protein B (SmpB) in *E. coli* was identified by D. Apirions group in 1991 and is situated immediately upstream of ssrA [86]. This close proximity is not the case for all bacterial genomes. In *N. gonorrhoeae*, for example, the two genes are situated on the opposite sides of the chromosome (reviewed in [28]).

A study by Karzai et al. in 1999 first identified SmpB as a protein participating in trans-translation [41]. *E. coli* mutant strains lacking SmpB expressed the same phenotype as tmRNA-depleted
strains. Purified SmpB was shown to bind specifically and with high affinity to SsrA RNA and to be required for stable association of SsrA with ribosomes in vivo [41].

The structure and function of SmpB have been extensively studied in parallel with research focusing on the tmRNA. SmpB has no other known role besides being a co-factor of tmRNA in trans-translation. Two studies in 2002 using in vitro techniques and ssrA and/or smpB deletion strains have proven that SmpB is indispensable for trans-translation [31, 87].

In one of these SmpB was shown to mediate tmRNA – ribosome binding and rescue the tmRNA from degradation in the cell as well as to enhance the aminoacylation by alanyl-tRNA synthetase in vitro [87]. A well-defined translation system consisting of purified components was used to assay the function of SmpB protein in the trans-translation process [31]. The results confirmed that SmpB protein enhances aminoacylation of tmRNA and that SmpB together with tmRNA are sufficient to complete the trans-translation. The presence of SmpB was a prerequisite for tmRNA binding to the ribosomal A site [31].

The structure of SmpB in solution was solved by NMR methods for A. aeolicus [88] and T. thermophilus [89]. In both cases the core of the protein consisted of an antiparallel β-barrel structure which in the case of A. aeolicus contained six β-barrel sheets and for T. thermophilus – eight. In both proteins three α-helices were present packed outside the barrel core. The last 20 C-terminal residues were unstructured and flexible in solution. An oligonucleotide binding (OB) fold was identified embedded in the β-barrel structure of SmpB, although the overall structure appeared to be unique. This motif was also found in other RNA-binding proteins associated with translation, such as: IF1, ribosomal protein S17 and the N-terminal domain of aspartyl-tRNA synthetase [88].

Recently a unique function has been assigned to the C-terminal tail of SmpB [90]. Deletion of just half the tail abolished trans-translation, while the affinity of SmpB for either ribosomes or tmRNA and its stimulatory effect on alanylation of tmRNA were not significantly affected. This indicates that SmpB may have a more complex role than just providing a link between tmRNA and the ribosome. Jacob et al. propose that the tail adopts an α-helical structure during trans-translation. They also suggest that even though the SmpB tail does not appear to interact with tmRNA outside of the ribosome, it does so inside the ribosome [90].

The precise mechanism of SmpB action still remains unknown. It would be interesting to see which elements of the ribosome the C-terminus of SmpB eventually contacts and if the contacts are
different at the different stages of trans-translation. Until recently it wasn’t even known whether SmpB remains bound to the ribosome during the whole trans-translation process. We have investigated this question in paper IV and we found that SmpB is in complex with tmRNA and the ribosome throughout all trans-translation steps. This result was also confirmed by a recent in vivo study [91].

We have also seen that SmpB binds the ribosome and the ribosomal subunits with high affinity even in the absence of tmRNA (papers 1 and 5).

**tmRNA and SmpB interactions with each other and with the ribosome**

A variety of techniques has been employed to study tmRNA-SmpB interactions, including binding assays [41, 75, 76, 87, 90], UV-induced cross-linking [76], enzymatic and chemical probing [75, 76], and X-ray crystallography [77].

In the pioneering study by Karzai et al. the interaction of SmpB protein with SsrA RNA was investigated using a gel-mobility shift assay [41]. Half-maximal binding was reached at a free SmpB concentration of ~20 nM. It was concluded that SmpB binds tmRNA specifically since 400-fold higher molar concentrations of yeast tRNA was required to outcompete it [41]. Barends et al. [75] used a gel-mobility shift assay to measure the SmpB affinity to full length tmRNA, as well as δtmRNA containing only the TLD and tRNAAla. They found similar $K_d$ values for all 3 species around ~50nM. In 2002 Hanawa-Suetsugu et al. reported a $K_d$ of 30nM [87] for the tmRNA-SmpB interaction and Wower et al. have estimated a value for $K_d$ ~100 nM [76] for the affinity of SmpB binding to T arms derived from the *E. coli* tRNAAla and from *A. aeolicus* tmRNA.

Finally, in a recent study employing a different technique, nitrocellulose filter binding assay, the $K_d$ for SmpB-tmRNA binding was found to be 0.34 ± 0.18 nM [90] indicating a much higher affinity of SmpB for tmRNA than those reported previously [41, 75, 76]. The reason for this discrepancy is most probably the non-equilibrium nature of the techniques gel retardation and indirect assays used for quantification of the binding and completely different buffer conditions used by different groups.

SmpB in complex with TLD of tmRNA from *A. aeolicus* was crystallized and the structure was solved at high resolution (3.2 Å) [77]. SmpB formed a one-to-one complex with TLD. The protein-binding surface was formed by elements inserted in its oligonucleotide-binding (OB) fold and consisted of a hydrophobic core sur-
rounded by positively charged residues. It interacted with the D-loop (nt 15–18) and connector loops of tmRNA (nts 318–319). Nucleotide pairs A19-G317 and C20-G316 from the H5 stem were also found to be involved in interaction with SmpB as well as G324 from the T-stem and nts 328-329 from the T-loop (Fig. 4). These interactions between SmpB and TLD are consistent with the results of footprinting studies showing that nucleotides 330-335 of full length tmRNA are protected by SmpB [75, 76] (see also paper IV).

Figure 4. A 3D-model of a complex between SmpB, EF-Tu•GTP and tRNA like domain (TLD) of tmRNA (green). Nucleotides of TLD protected from lead (II) cleavage (Present investigation, paper IV) are shown in red (G333-G335) and pink (U16, U17). The model was derived by combining the crystal structure of SmpB•TLD complex (PDB file 1V6P) with a crystal structure of EF-Tu•GTP in complex with Phe-tRNA (PDB file 1TTT) by aligning T-loop of tRNA and TLD, adopted from paper IV.

tmRNA functional interactions with Ala-RS, EF-Tu and SmpB

In addition to SmpB [41], two other proteins, EF-Tu [30, 92] and alanyl-tRNA synthetase [20, 21], can also bind to the TLD [75].

The interaction between tmRNA and AlaRS was reported even before it was found that tmRNA binds EF-Tu. The finding that 3’
and 5’ termini of tmRNA fold into a structure resembling the acceptor stem of tRNA$^{\text{Ala}}$, led to in vitro assays showing that tmRNA can be charged by alanyl-tRNA synthetase [20, 21]. tmRNA aminoacylation with alanine is indispensable for its binding to the ribosome [29]. 10Sa was found in the cell mainly associated with ribosomes (though not with polysomes) and it dissociated upon splitting ribosomes into subunits [29, 93]. Mutations annulling the aminoacylation abolished the tmRNA mediated tagging as well as the ribosome binding [29]. However altering the amino acid identity from alanine to histidine didn’t abolish trans-translation [73] confirming the importance of aminoacylation but not the one of the alanine amino acid itself. Moreover, peptides tagged by His-tmRNA had histidine as the first amino acid of the degradation signal, once again demonstrating that the first amino acid is not coded by the internal ORF in tmRNA but originates from the amino acid with which tmRNA was acylated [73].

The similarity between canonical tRNAs and TLD of tmRNA led to the assumption that EF-Tu might interact with the TLD as it does with tRNAs. This assumption was checked by Rudinger-Thirion et al. 1999 who found that EF-Tu indeed binds tmRNA [30]. Biochemical [94] and structural studies [95, 96] showed that EF-Tu interacts with the 3’ end of tRNAs which is similar to the one of tmRNA.

Effects of EF-Tu and SmpB on tmRNA alanylation

The effect of SmpB and EF-Tu on tmRNA alanylation by AlaRS is one of the most contradictory topics in the current literature.

The first study on the SmpB function in trans-translation by Karzai and co-workers didn’t show any effect of SmpB on tmRNA aminoacylation [41]. However, the subsequent studies clearly demonstrated a stimulatory role of SmpB in tmRNA alanylation. Barends et al. [92] found that $k_{\text{cat}}/K_{M}$ value for AlaRS for the canonical tRNA is 75 times higher than the one measured for tmRNA. The $K_{d'}$ value for Ala-tmRNA binding to EF-Tu•GTP was about 30-fold higher than that for Ala-tRNA$^{\text{Ala}}$ binding. In this study an enhancing effect of EF-Tu on the aminoacylation, and on the yield of Ala-tmRNA was observed [92]. The substantially lower value for $k_{\text{cat}}/K_{M}$ and the higher $K_{d'}$ for EF-Tu•GTP binding implicated the presence of an additional factor necessary for efficient charging of tmRNA and its binding to the ribosome. This factor could be the helper protein SmpB, which was confirmed one year later by the same group [75]. The researchers demonstrated the simultaneous and functional binding of EF-Tu, SmpB and AlaRS to the TLD of tmRNA assayed by RNase T1 footprinting in combination with gel mobility-shift. The EF-Tu was shown to bind to tmRNA at the same
position as to canonical tRNAs [75]. Barends et al. saw a synergistic effect on the enhancement of aminoacylation of tmRNA by EF-Tu and SmpB [75].

These stimulatory effects of EF-Tu and SmpB on tmRNA alanylation were also confirmed by two other groups in 2002 [31, 87] and recently by Jacob et al., who used both full length and truncated SmpB variants [90]. SmpB increased both the rate of the tmRNA alanylation and the yield of Ala-tmRNA.

tmRNA interaction with ribosomal protein S1

Another tmRNA interacting protein that has been extensively studied is the ribosomal protein S1. Wower et al. 2000 [97] found that tmRNA could be cross-linked to the 30S ribosomal subunit and specifically to the S1 protein. S1 interactions with tmRNA gave a different cross-linking pattern on and off the ribosome. Binding and cross-linking studies using different tmRNA variants suggested that PK2, PK3 and PK4 are involved in S1 binding and that the later doesn’t depend on tmRNA aminoacylation [97, 98]. Another study using different structural probes showed that S1 binds to PK2 which undergoes a significant conformational change [99]. Gel mobility-shift experiments suggested that more than one molecule of S1 could bind per tmRNA molecule [99].

The cryo EM structure for the tmRNA entry into the ribosome by Valle et al. was solved both in the presence and in the absence of S1 [100]. A difference between the two was observed in the tmRNA region encompassing the 5’ end of the ORF and a portion of PK1. This region was visible in the absence of S1 and unstructured when S1 was present in the complex.

The observed difference taken together with the evidence of S1 binding to pseudoknots [97-99] suggest that S1 may play a role in the transition from tRNA mode to mRNA mode of the tmRNA [99] by its unwinding activity [78, 97]. This role would be consistent with prior biochemical evidence showing that S1 can disrupt the secondary structure of certain pyrimidine-containing polynucleotides [101].

Whatever is the role of S1 in trans-translation it is clear from the cryo EM structure that the presence of S1 is not a prerequisite for tmRNA binding to the ribosome and a recent study shows that it is dispensable for trans-translation [102].

Thus, the results from the studies on S1 protein show that while this protein binds to tmRNA and the ribosome its presence is dispensable for trans-translation, although it might eventually facilitate the tmRNA movement through the ribosome by unwinding structural elements in the pseudoknot regions.
Other factors, interacting with tmRNA

SmpB [41], EF-Tu [30, 92] and S1 [98] are directly involved in the trans-translation process. AlaRS [20, 21] charges the TLD with alanine, while RNase P participates in tmRNA processing [20, 56] together with RNase E, III and other RNases [54, 56, 57].

In addition to these factors with defined interactions with tmRNA phosphoribosyl pyrophosphate synthetase (PrsA), RNase R (VacB) and a protein of unknown function (YfbG) were identified to form a stable contact with the tmRNA•SmpB complex by Karzai & Sauer [98].

PrsA is an enzyme required for de novo synthesis of nucleotides, tryptophan, and histidine [103-105]. The growth phenotypes of temperature-sensitive prsA mutants are suppressed by wild-type ssrA but not by ssrA mutants [106], suggesting an interaction of some type between these two genes but its exact nature is still unknown [98].

Earlier studies indicated that ssrA mutations in Salmonella typhimurium result in up-regulation of rnr [40]. Karzai and Sauer noticed that the level of SsrA tagging of endogenous proteins increased and also that the pattern of tagging was altered in an rnr mutant. They speculate on the basis of this result that RNase R plays some role in degradation of the mRNA that is replaced on the stalled ribosomes by SsrA RNA during trans-translation and that some mRNAs are more susceptible to RNase R degradation than others [98].

Structure of the ribosome rescue complex

The first step of trans-translation, the tmRNA entry into the ribosomal A site, was studied by cryo-electron microscopy [100]. tmRNA•SmpB•EF-Tu complex in the presence of GTP was reacted with ribosomes containing a short mRNA and fMet-tRNA^{Met} in the P-site and trapped there due to the presence of the antibiotic kirromycin (Fig. 5). Kirromycin allowed GTP hydrolysis by EF-Tu but prevented peptidyl bond formation and stalled the tmRNA•SmpB•EF-Tu•GDP complex in the A site as it does in the case of canonical ternary complexes tRNA•F-Tu•GDP. Based on the cryo-electron map, the TLD and EF-Tu were placed in the ribosomal A site similarly to canonical tRNAs in complex with EF-Tu. The SmpB is assigned a position bridging the elbow and lower portion of the TLD and helices H69, H71 and H89 of the 23S rRNA, in the vicinity of the GTPase-associated center (GAC) of the 50S ribosomal subunit. The rest of the tmRNA molecule (the MLD and the pseudoknots) leave the inter-subunit space at the base of L12/L11.
stalk, bend around the beak of the 30S subunit and form an arc on the solvent site placing the MLD in the vicinity of the mRNA channel entrance.

It is proposed that the SmpB-mediated interaction stimulates the GTPase activation by EF-Tu compensating for the absence of codon-anticodon interactions. Valle and co-workers [100] saw only one copy of SmpB in their complex. This deviates from other studies where the SmpB•tmRNA stoichiometry was estimated to be 2:1 or even 3:1 [76] as well as from our results (paper 1) showing a ratio of 2:1 in SmpB•70S complexes. The orientation of SmpB towards the 50S in the cryo-EM study disagrees also with the model of tmRNA•SmpB arrangement in the A site of the ribosome proposed by Gutmann et al. in 2003 [77], based on their SmpB•TLD crystal structure. In the latter model SmpB was oriented towards the decoding site of the 30S subunit [77] (Fig. 6). This model was built by fitting the structure of SmpB•TLD into the model of 70S ribosome with A-site bound tRNA obtained at 5.5 Å resolution [1]. Due to the 90ºC rotation of the TΨC-arm SmpB is oriented towards the decoding center (DC) of the small subunit and the H5-stem together with the remaining tmRNA extend towards the L7/L12 stalk of the 50S ribosomal subunit. However, the discrepancies between these two models may be due to the fact that the cryo-EM structure represents an earlier stage of trans-translation – the entry of the tmRNP complex into the stalled ribosome, whereas the crystal structure corresponds to tmRNA•SmpB position after its accommodation into the A site on 50S subunit when EF-Tu•GDP has already dissociated. In both models the TLD has an increased angle between the acceptor and “anticodon” arms compared to the one in tRNAs. The elbow angle is ~110º in the cryo EM structure and 120º in the crystal structure.

The cryo EM model and the crystal structure cast more light on the positioning of tmRNA and SmpB on the ribosome in the initial stages of trans-translation. However it still remains unclear how the bulky tmRNA molecule moves through the ribosome.

The results of Valle et al. [100] support the idea that the unwinding of helix 5 allows the movement of the ORF through the ribosome. A large change in the conformation of the rest of tmRNA is not required since the pseudo-knots (PK2-4) remain outside the head of the 30S subunit without forming extensive contacts with it. Thus, PK2-4 could easily move while the ORF and TLD progress inside the ribosome in the course of trans-translation. In this model S1 is assigned a role in unwinding part of the single-stranded region, thus allowing correct presentation of the ORF [100].
Figure 5. Model for tmRNA, EF-Tu, and SmpB in the cryo-EM map of 70S ribosome in complex with tmRNA, adopted from [100].

Figure 6. Crystal structure of the transfer-RNA domain of transfer-messenger RNA in complex with SmpB, adopted from [77].
A different model for the tmRNA movement through the ribosome is presented by P. Haebel, S. Gutmann and N. Ban [77, 79]. They suggest that tmRNA must undergo significant conformational changes to avoid steric clashes with the ribosome. After tmRNA has received the nascent polypeptide the TLD has to translocate to the ribosomal P site, switch from tRNA to mRNA mode of action and place the internal ORF into the decoding center of 30S. Later, the TLD has to translocate to the E site. The assumption is that the TLD somehow pulls the pseudoknots through the ribosomal inter-subunit space and during these movements SmpB could transiently dissociate to allow the TLD higher flexibility.

A third model describing how tmRNA moves through the ribosome was proposed by Ivanov et al. [80] and is in agreement with Valles suggestion that PK2-PK4 remain outside the 30S. Recently it was confirmed that at least PK3 is situated outside the ribosome and SmpB remains in complex with tmRNA and the ribosome through the whole trans-translation process [91].

We have addressed the question of tmRNA movement through the ribosome by footprinting tmRNA (paper IV) as well as ribosomal RNA (work in process) in different stages of trans-translation. Our results support the idea that there are no major changes in the tmRNA structure in the course of trans-translation and that SmpB remains bound to the TLD.

Causes for ribosomal stalling and the roles of tmRNAs

Causes for ribosomal stalling

Originally, it was observed that tmRNA targets ribosomes stalled at the very 3′-end of truncated mRNAs lacking an in-frame stop codon [17, 19]. After this seminal discovery a number of subsequent studies identified tmRNA-mediated tagging of apparently full-length proteins as well as tagging of non-completed proteins translated from apparently full-length mRNAs. The implication of these studies was that tmRNA can target any ribosome stalled on mRNA and that it is the ribosome stalling, not mRNA truncation that represents the signal for tmRNA targeting.

Ribosome stalling is a relatively rare event but it is potentially detrimental for the cell. Ribosomes can stall on an mRNA for various reasons (see Fig. 7):

I) in case of an mRNA lacking a termination codon [17]
II) if the ribosome reaches a cluster of rare codons [107]
III) due to amino acid or charged tRNA starvation for the specific codon which the ribosome has reached

IV) on stop codons [108-112] due to depletion or low level of ribosomal release factors or as a part of cell cycle [62] or metabolic regulation [108]

V) in the case of readthrough due to nonsense suppressors [113] or miscoding drugs [114]

VI) stalling is possible if the ribosome reaches a stable secondary structure on the translated mRNA [49]

Tagging at rare codons

Roche and Sauer observed that trans-translation takes place at rare arginine codons (AGA and CGA), seemingly within intact mRNAs [107]. A major determinant for the tmRNA-dependent tagging in this case was the level of the cognate tRNA in the cell. At wild-type levels of tRNA trans-translation was observed if two or more consecutive rare codons were present in an mRNA. In this case tagging could be aborted if the respective tRNA-isoacceptor was overexpressed. On the contrary upon depletion of cognate tRNA tmRNA-dependent tagging occurred even if the mRNA contained single rare codons. These data suggests that ribosomal stalling and trans-translation at rare codons occurs due to starvation for cognate tRNA.

Tagging at stop codons

It was discovered that tagging by tmRNA can occur also at the termination codons of seemingly intact mRNAs [74, 108, 110, 112, 115] and that tmRNA competes with the ribosomal release factor RF2 on weak stop codons (UGA) [109]. The identity of the codons preceding the stop codon was also shown to be important determinant for the probability of tagging occurrence [111].

Endogenous E. coli, B. subtilis or bacteriophage full size proteins carrying tmRNA-encoded tags were identified [108, 109, 116]. In B. subtilis the stress-inducible proteins PerR and GsiB, were found to be tagged at higher temperature at positions corresponding to full size proteins [116]. Tagging of these stress-related proteins probably is used as a means to regulate their expression.
Figure 7. Involvement of tmRNA during protein synthesis, adopted from [49].

(1) A 70S ribosome is stalled at the 3' end of an incomplete message; (2) A translating ribosome encounters a cluster of rare codons when translating an intact message; (3) A translating ribosome reaches the termination codon of an intact message; (4) Translational 'readthrough' at the termination codon in the presence of suppressor tRNAs; (5) A stable secondary structure within an intact message might be sufficient to trigger tagging.

Endogenous E. coli, B. subtilis or bacteriophage full size proteins, carrying tmRNA-encoded tags were identified [108, 109, 111, 116]. In B. subtilis the stress-inducible proteins PerR and GsiB were found to be tagged at higher temperature at positions corresponding to full size proteins [116]. Tagging of these stress-related proteins is probably used as a way to regulate their expression.

The E. coli YbeL, GalE and ribokinase, together with λcI repressor were found to have the tmRNA-encoded degradation signal added after the normal C-terminus of the protein [108]. The ribokinase has a poorly efficient opal stop codon (UGA), leading to translational recoding events. In addition, the stop codon is preceded by two rare arginine codons. Tagging was observed at both rare arginine codons [111], as well as at the opal stop codon [109, 111]. The substitution of each of the rare codons with the most common arginine codon didn’t abolish tagging but it was greatly reduced. A large reduction of tagging was also observed when changing the inefficient opal stop codon to a more efficient ochre stop codon (UGAc to UAAu). Interestingly, the substitution of a particular arginine codon or the stop codon also reduced the tag-
ging that occurred upstream of the substituted codon positions [111]. This observation is consistent with the sequestration of rare tRNA isoacceptors theory [107] (see above).

Since translation terminates at UGA by the action of translational release factor 2 (RF2) its impact on trans-translation was assayed by using a temperature-sensitive (Ts) RF2 allele. tmRNA-mediated tagging of ribokinase was inversely correlated with RF2 activity, implying a dynamic competition between termination of translation and SsrA tagging [109].

It was discovered that the efficiency of trans-translation versus termination was dependent not only on the identity of the stop codon and the preceding codons but on the C-terminal amino acid sequence as well. In particular C-terminal proline residue has been shown to induce trans-translation at the stop codon [110]. This was first observed with the E. coli YbeL protein in which the stop-codon is originally preceded by a proline codon. However, substituting proline for the C-terminal residue of thioredoxin resulted in tagging at the stop codon of this protein as well [110]. Further experiments showed that changing the identity of the stop codon (as was seen for ribokinase previously) and the levels of translational release factors in the cell could alter the tagging efficiency. The frequency of trans-translation depended greatly on the kind of proline analogs that were incorporated into the protein. These results suggested that the structure of the nascent polypeptide and in particular the chemical or conformational properties of the C-terminal residues can affect the rate of translation termination and hence are a major determinant of SsrA tagging of YbeL [110].

A C-terminal sequence that strongly induces trans-translation at stop codons was identified in LacI C, a variant of the Lac I (Lac repressor) lacking the last C-terminal amino acid [112]. Lac I was earlier reported to be tagged at its stop codon [115]. In this later work a LESG sequence located naturally one amino acid upstream of the LacI C terminus was reported to induce efficient C-terminal tagging of LacI itself and of a version of a non-related protein – the cyclic-AMP receptor (CRP), when fused to its C terminus. Here no difference was seen in tagging levels when altering the stop codon. Further experiments showed that alteration of the amino acid sequence but not the nucleotide sequence of the C-terminal portion eliminated the tagging. Probably this mechanism for inducing trans-translation differs from the one applied in the cases described above [109, 110].

The above mentioned observations imply that ribosomes become targets for trans-translation because of stalling on an ineffi-
ciently translated mRNA sequence, or due to inefficient termination.

**tmRNA-mediated tagging due to readthrough on stop codons and frameshifting**

Readthrough and frameshifting normally occur at a low frequency in the cell. They are more often observed on weak stops. As a result, extended peptides, or peptides with altered amino acid composition, which can be potentially toxic, are translated. The competition between tmRNA-tagging and termination on weak translation termination sites, however, could limit the formation of anomalous proteins resulting from readthrough or frameshifting events that occur frequently at these sites [109]. In this case the primary function for tmRNA would be to tag these proteins for degradation as a protein translation quality control. Surprisingly, experiments have shown that in certain cases tmRNA bearing a modified mRNA sequence also rescues efficiently the observed phenotype [114].

Readthrough frequency can be increased by suppressor tRNAs or miscoding drugs. In both cases augmented protein tagging was observed in *E. coli* [113, 114]. SsrA cells were more sensitive both to miscoding drugs (kanamycin and streptomycin) and to suppressor tRNAs. However, it was observed that in the presence of modified tmRNA encoding a non-degradable tag, the growth rate in the case of inhibition with low concentrations of kanamycin and streptomycin was almost restored to the value measured for the wt cells [114]. These data indicate that the primary function of tmRNA is ribosomal rescue and not the tagging for degradation of the anomalous polypeptide as it was suggested in the original trans-translation model by Keiler et al. [17].

**Gene-regulation via trans-translation**

An obvious question arises after identifying so many proteins normally tagged at positions corresponding to their stop codons. Why are these proteins directed for proteolysis in the cell?

The tmRNA mediated tagging of the Lac repressor (Lac I) was proposed to be involved in the cellular adaptation to lactose availability by supporting a rapid induction of the Lac operon expression [115]. The binding of LacI to the lac operators leads to transcription and translation of truncated lacI mRNAs that are, in turn, recognized by the SsrA system. In addition to LacI, the tagging of ribokinase and GalE [108, 109, 115] suggests tmRNA involvement in the regulation of carbon source metabolism which is in agree-
ment with in vivo data showing poor recovery after carbon starvation of tmRNA-deficient strains [27].

These observations are consistent with tmRNA being involved in regulated degradation of specific cellular proteins. The tagging and proteolysis of these tmRNA natural targets appears to play a role in cellular adaptation. Another example is the above mentioned increased tagging of two stress-related proteins in *B. subtilis* at higher temperature [116].

The proposed function of tmRNA in gene-regulation was reinforced by other observations as well. SsrA dependent tagging of the C-terminus of truncated forms of Mu phage repressor was shown to be the mechanism that controls the stability of Mu lysogens [117, 118].


AIMS

Our general aim was to determine the mechanism of action of tmRNA and its helper protein SmpB in trans-translation by using our defined in vitro translation system. The present study aims at answering the following questions:

I. What is the localization of tmRNA and SmpB in the cell?

II. What is the stoicheometry of tmRNA•SmpB•ribosomes in the functional complexes and where does SmpB bind on the ribosome?

III. What is the major determinant for efficient trans-translation?

IV. When does the original mRNA leave during trans-translation?

V. How does tmRNA move through the ribosome in the course of the trans-translation process?
RESULTS AND DISCUSSION

Small protein B on the ribosome triggers trans-translation

In vivo localization of SmpB and tmRNA

In this study, we have monitored by western and northern blotting the localization of endogenous SmpB and tmRNA in vivo. Cell lysates from *E. coli* strains lacking *ssrA*, *smpB* or both and the parental strain were fractionated by successive centrifugations to obtain S30 (total fraction), S100 (soluble fraction) and P100 (crude ribosome) extracts.

tmRNA was predominantly bound to 70S ribosomes in the crude ribosome P100 fraction in the wt strain but not in the ΔsmpB strain. Compared to wild-type cells, the total tmRNA expression in the ΔsmpB strain was decreased and the tmRNA half life was 3 times shorter. The concentration of free tmRNA was the same in both strains meaning that the absence of SmpB leads to a reduction of the level of ribosome-bound tmRNA, which can also explain the reduced half-life of tmRNA in the ΔsmpB strain since the ribosome binding protects tmRNA from nucleolytic degradation.

SmpB expression was also reduced in the ΔssrA strain compared to the parental strain. Both in the wt and the ΔssrA strain SmpB was found in the ribosomal fraction. Moreover, it remained associated with 70S ribosomes after their purification through sucrose density gradient which means that endogenous SmpB is associated in vivo with 70S ribosomes, independently of the presence of tmRNA. We also showed that SmpB was undetectable in both the ΔssrA and wild-type S100 fractions, although the latter contained tmRNA. The absence of SmpB in the S100 fraction from the ΔssrA strain indicated that there were no SmpB•tRNAs complexes in the soluble fraction despite the fact that SmpB has some affinity to *E. coli* tRNAs [41]. This implies that SmpB has a high and specific affinity towards 70S ribosomes.
A molar ratio of 2:1 was determined for SmpB ribosome binding from in vitro titration experiments, suggesting that one ribosome binds two SmpB molecules and showing that the purified His-tagged SmpB protein was 100% efficient in ribosome binding.

We showed also that SmpB pre-bound to ribosomes can recruit tmRNA and that the binding of tmRNA to ribosomes depends on the presence of SmpB.

**SmpB is essential for the first step of trans-translation while EF-Tu stimulates it**

The function of SmpB during trans-translation was studied in our in vitro system for messenger RNA translation assembled from components prepared to high purity [14]. Ribosomes from ΔsmpB ΔssrA cells were used to translate an mRNA encoding the tetrapeptide fMet-Phe-Thr-Ile, and truncated after the last sense codon of the open reading frame. Translation of this mRNA resulted in ribosomal complexes (RCs), containing fMet-Phe-Thr-Ile-tRNA \textsubscript{Ile} in the P site and a codon-less A site. The RCs were separated from the other components of the translation mixture by gel filtration. These complexes were used as substrate for a trans-transfer reaction to Ala-tmRNA in the presence/absence of EF-Tu•GTP and SmpB.

The rate of trans-transfer was very high with both EF-Tu•GTP and SmpB, intermediate with only SmpB and virtually zero with only EF-Tu•GTP present (Fig. 8).

Our data showing that SmpB is essential to initiate trans-translation are in line with previous reports [31, 87]. The presence of EF-Tu•GTP, however, was not required but stimulated greatly the reaction (Fig. 8). We conclude therefore, that in order to reach physiologically significant values for the efficiency of the trans-transfer reaction and the whole trans-translation process, EF-Tu is required for proper delivery of tmRNA to the stalled ribosomes. In the absence of EF-Tu, the plateau level of alanine incorporation into the stalled peptide chain is significantly reduced (Fig. 8) (also observed elsewhere [75]), possibly because EF-Tu stabilizes the acyl-ester bond of alanyl-tmRNA\textsubscript{Ala}, thus increasing the amount of available aminoacylated tmRNA.
Ribosomes in complex with SmpB recruit tmRNA to trigger trans-translation

The cellular localization of SmpB on the ribosomes suggested that ribosome-bound SmpB can recruit SmpB-free tmRNA to initiate trans-translation. To test this hypothesis we compared the extent of trans-transfer from ribosomal complexes, containing pre-bound SmpB to the added Ala-tmRNA with the extent of trans-transfer from SmpB-free ribosomal complexes to the added SmpB-Ala-tmRNA complex. The extent of trans-transfer was identical in both cases meaning that recruitment of tmRNA to ribosomes could occur either by association of SmpB-free alanyltmRNA to ribosomes in complex with SmpB or by association of SmpB-Ala-tmRNA complex to SmpB-free ribosomes.

Quantitative immunoblots showed that two molecules of SmpB had remained stably bound per RCs during the purification which confirms the result of our titration experiment mentioned above.

SmpB does not recycle

To study if a small amount of SmpB can catalyze trans-transfer by recycling between ribosomal complexes, we designed an ex-
periment where SmpB, at varying concentrations, was pre-bound to RCs. The result of this experiment (Fig. 9) demonstrated that two SmpB molecules were required for each trans-transfer reaction and suggests that no recycling of SmpB occurred during the incubation time of 15 min which is much longer than the time of the trans-transfer reaction itself.

This result is consistent with a relative amount of one molecule of tmRNA per two molecules of SmpB and ten ribosomes in the cell (M. Hallier and B. Felden unpublished data) [22]. Here we have demonstrated that one ribosome can accommodate two SmpB molecules and our in vitro recycling experiment suggests that actually two SmpB molecules are required for the trans-transfer event to occur (Fig 7).

Until now, SmpB has been thought of as a factor stimulating the aminoacylation of tmRNA and, together with EF-Tu, allowing the binding of alanyl-tmRNA to the ribosome [31, 41, 87]. Our findings that the SmpB protein is found associated in vivo with ribosomes, independently of the presence of tmRNA and that SmpB prebound to a ribosome can efficiently recruit an alanyl-tmRNA, demonstrate that trans-translation can be initiated in a different way than originally thought. We suggest that this novel route to trigger trans-translation, at least under some conditions, could be the preferred one in vivo.

Ribosome rescue by tmRNA requires truncated mRNAs

In this work, we have addressed the following question: what is the major determinant for efficient trans-translation?

Trans-transfer rate decreases with increasing mRNA downstream length

To study how the rate of trans-transfer of a short peptide to the 3' end of alanylated tmRNA depends on the length of the mRNA sequence downstream from the P-site codon, ribosomal complexes were prepared by translating two sets of mRNAs; one with a weak (Bar), and one with a strong (XR7) Shine and Dalgarno sequence. The mRNAs encoded fMet-Phe-Thr-Ile, and were extended by zero to thirty bases downstream from the last sense (Ile) codon. The RCs (prepared as described in paper I) were used as substrate in the trans-transfer reaction to Ala-tmRNA in the presence of SmpB and EF-Tu•GTP.
The kinetic data for the time course of the trans-transfer reaction were similar for both groups of mRNA, and showed that when the mRNA continued between zero and six bases downstream from the P-site codon, the rate of trans-transfer was very fast - about 20 s\(^{-1}\) (Table I). However, longer mRNA extensions past the P-site codon inhibited the rate of trans-transfer to a value of about 1 s\(^{-1}\) when the length of the extension was five codons, and to a value approaching zero when the length exceeded ten codons (Table I).

The similarity of the trans-transfer rates for RCs with mRNAs containing either strong or weak SD sequence showed that there was no interference between a putative, stable interaction between the SD:antiSD sequences and the trans-transfer reaction. However, it cannot be ruled out that such an interaction can interfere with subsequent steps in trans-translation.

**Table I.** Rate (k\(_c\)) and normalized efficiency of trans-transfer (R) for mRNAs (XR7 or Bar) with different number (N) of nucleotides downstream from the P-site codon (AUU). R is k\(_{cat}/K_m\) for tmRNA divided by k\(_{cat}/K_m\) for RF1.

<table>
<thead>
<tr>
<th>Nucleotides after the last sense codon AUU:</th>
<th>N</th>
<th>k(_c) (s(^{-1}))</th>
<th>(R)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>XR7</td>
<td>Bar</td>
<td>XR7</td>
</tr>
<tr>
<td>–</td>
<td>0</td>
<td>19</td>
<td>13</td>
</tr>
<tr>
<td>U</td>
<td>1</td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td>UAG</td>
<td>3</td>
<td>23</td>
<td>20.5</td>
</tr>
<tr>
<td>UAG UAC</td>
<td>6</td>
<td>25</td>
<td>23</td>
</tr>
<tr>
<td>UAG UAC AAG</td>
<td>9</td>
<td>15</td>
<td>8.5</td>
</tr>
<tr>
<td>UAG UAC AAG CUU</td>
<td>12</td>
<td>7.8</td>
<td>6</td>
</tr>
<tr>
<td>UAG UAC AAG CUU CAC</td>
<td>15</td>
<td>1.2</td>
<td>1.5</td>
</tr>
<tr>
<td>UAG UAC AAG CUU CAC CCG</td>
<td>18</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*tmRNA in competition with a class-I release factor*

The *in vivo* efficiency of tmRNA targeting of ribosomes that are stalled at slowly translated sense codons or at inefficient stop codons will depend on its ability to compete with ternary complexes containing EF-Tu\(^\ast\)GTP and aa-tRNA for sense codons or with class-I release factors for termination codons. The outcome of such a competition will depend on the concentrations and the effective association rate constants k\(_{cat}/K_m\) for the competitors. To address this question, we compared the efficiency of RF1 induced peptide release and the efficiency of trans-transfer to Ala-tmRNA on ribosomal complexes containing fMet-Phe-Thr-Ile-tRNA\(^{Ile}\) in the
P site, a UAG stop codon in the A site and mRNAs extending with varying lengths downstream from the stop codon. The highest tmRNA competition efficiency was obtained when the mRNA was truncated immediately after the stop codon. It dropped rapidly for mRNAs with successively longer downstream extensions, nearing zero when the number of downstream nucleotides exceeded 18 (Table I).

The efficiency of termination by RF1 was independent on the length of the mRNA after the stop codon, suggesting that the \( k_{cat}/K_m \)-value for RF1 is the same, and equal to \( 6 \times 10^7 \) M\(^{-1}\)s\(^{-1} \) [119] for all the ratios measured in the competition experiments (Table I). Thus the \( k_{cat}/K_m \) for the trans-transfer reaction is about \( 3 \times 10^7 \) M\(^{-1}\)s\(^{-1} \), and similar to \( k_{cat}/K_m \) for the RF1 dependent peptide release, when the mRNA had been truncated immediately after the UAG codon. However, when the mRNA extended four codons or more downstream from the P site, the \( k_{cat}/K_m \)-value for trans-transfer was reduced twenty-fold or more. This implies that the trans-transfer reaction is expected to compete efficiently with elongation and termination when mRNAs are truncated close to the P-site codon, but it would be very inefficient in competition when the ribosome is stalled far from the 3’ end of mRNA.

\textbf{RelE cleavage of mRNA activates the trans-transfer reaction}

The observation that the bacterial toxin RelE specifically cleaves mRNAs in the A site of the ribosome, suggests that RelE inhibition of protein synthesis should create ribosomes with truncated mRNAs that can be rapidly targeted by tmRNA [120]. This prediction was confirmed by comparing the trans-transfer rates for ribosomal complexes with “full length” mRNAs containing UAG stop codon in the A site, before and after RelE treatment. Non-treated RCs were very poor targets for tmRNA while complexes pretreated with RelE were targeted very efficiently by tmRNA with trans-transfer rates of about 25 s\(^{-1} \). We also used 5’-labeled full length mRNA to test whether ribosomes or ribosome bound tmRNA could induce mRNA cleavage in stalled ribosomal complexes. No such cleavage was observed even after an extended incubation. The fact that RelE cleaves mRNAs in the A site of the ribosome [120] suggests a functional link between the toxin and tmRNA in stress response. The latter has already been established \textit{in vivo} [121]. However, mRNA cleavage on stalled ribosomes occurs \textit{in vivo} and precedes tmRNA-mediated tagging also in the absence of RelE or other known toxins and nucleases [122] [123, 124]. This means that specific, so far unidentified endonucleases target mRNAs on stalled ribosomes. We suggest that the action of
one or other of these unknown endonucleases could be the com-
mon element in different eubacterial stress responses.

Our data on the trans-transfer efficiency and the comparison
with the termination efficiency show that mRNA truncation is a
major determinant for tmRNA targeting of stalled ribosomes.
Those findings also explain how negative interference between the
action of ternary complexes and peptide release factors, on the
one hand, and the action of tmRNA, on the other, is avoided.

mRNA release in trans-translation

The next question about trans-translation was when the prob-
lematic mRNA leaves the stalled ribosome.

To address that question we have used the ribosomal com-
plexes described above, containing mRNAs with weak and strong
SD sequences. This time the mRNAs were 5' labeled with \( \gamma^{32} \)P ATP
and the time course of their release was monitored upon addition
of Ala-tmRNA, SmpB and EF-TuGTP with and without EF-G pre-
sent. No significant mRNA release was detected in the absence of
tmRNA.

Without EF-G the release of mRNA occurred on a timescale of
minutes (Table I, paper III) and was much slower than the rate of
the trans-peptidation reaction, which could be completed in milli-
seconds (paper II). The release rate depended on the strength of
the SD:antiSD interaction and was accelerated tremendously by
EF-G catalyzed translocation.

Slow mRNA release can occur prior to translocation

In the case of weak-SD mRNAs with downstream extensions
ranging from zero to twelve bases, the rate constants for mRNA
release after trans-peptidation were all about 0.45 min\(^{-1}\) and
somewhat slower for the longer extensions. For the mRNAs with
the strong SD sequence the rate of tmRNA-induced mRNA release
was 2-3 times slower, and showed small variation with the length
of the mRNA extension. The cause of the slow release in this case
was, we propose, that the SD:antiSD contact was maintained in
the stalled ribosomal complexes.

In order to explain an apparent length-independence of mRNA
release after trans-peptidation it should be mentioned that in con-
trast to canonical peptidyl-tRNA, peptidyl-tmRNA does not interact
with mRNA in the A site via codon:anticodon base pairing. This
suggests that after trans-peptidation the mRNA is retained on the

45
ribosome mostly through its interaction with the deacylated tRNA in the P site. Hence, the rate-limiting step in mRNA release from the ribosome would be the dissociation of this deacylated P-site tRNA, which is expected to proceed independently on the mRNA extension length. The destabilizing effect of trans-peptidation on the P-site tRNA could also be enhanced by its direct contact with the tmRNA.

**EF-G induced translocation accelerates the mRNA release**

We demonstrated that EF-G was required for fast translocation of peptidyl-tmRNA into the P site (Fig. 3). This translocation accelerated the mRNA release from all ribosomal complexes but especially from complexes stalled on mRNAs with a weak SD sequence (Table 1, paper III). This, we propose, reflects rapid translocation of the P-site tRNA to the E site followed by its rapid dissociation from the ribosome [125]. In addition, the tmRNA translocation could have further destabilized the mRNA by pushing it in the E-site direction.

Translocation of tmRNA accelerated the dissociation of mRNAs with a strong SD as well but to a much lesser extent (Table 1) which, again, could be explained by the persistence of the SD:antiSD contact even after translocation.

In conclusion, our results suggest the following mechanism for the replacement of the original truncated mRNA with the ORF of tmRNA during trans-translation (Fig. 9). First, tmRNA enters the ribosome aided by its protein co-factors, SmpB and EF-Tu•GTP. This event is followed by rapid trans-peptidation, destabilizing the P-site bound tRNA. However, the presence of EF-G in the cell results in rapid translocation of tmRNA from the A to the P site and of the deacylated tRNA from the P to the E site, from which it rapidly dissociates. Afterwards, the mRNA devoid of all tRNA-mediated contacts with the ribosome dissociates rapidly, provided that the mRNA was truncated sufficiently close to the A site in its original position.

Fast release of truncated mRNAs with a weak SD element near the truncation site occurs after the peptidyl-tmRNA translocation to the P site. This observation suggests that trans-translation would normally proceed without interference from the original mRNA.
Structure probing of tmRNA in distinct stages of trans-translation and its interaction with SmpB

In this study we have used a lead (II) footprinting technique to study the interactions of tmRNA with SmpB and with other components of the translation machinery along the tmRNA passage through several steps of the trans-translation cycle.
Mapping tmRNA•SmpB interactions in vivo and in vitro

We observed distinct protection at positions A334-G336 and U16-U17 in the TLD of the tmRNA due to binding to the SmpB protein (Fig. 10 and 11). The footprint persisted for all studied in vitro steps of trans-translation. The protection was seen as well on tmRNA from a wild-type Escherichia coli strain, treated with lead(II) in vivo, and in vitro transcribed tmRNA in complex with SmpB. The persistence of this footprint implies that tmRNA is present in the cell mostly in complex with SmpB and that SmpB doesn't dissociate from the trans-translation complex during the whole process.

Footprinting the tmRNA interactions in different stages of trans-translation by lead(II) cleavage analysis

In order to follow tmRNA through the distinct steps of trans-translation ribosomal complexes were assembled in vitro, stalled at discrete stages, and purified (Fig. 10).

The ribosomal complexes contained: RC 0 - mRNA encoding MFTI, MFTI-tRNA\textsubscript{Ile} in P site and empty A site; RC I - MFTI-tRNA\textsubscript{Ile} in P site, Ala-tmRNA•SmpB•EF-Tu•GDP and kirromycin in A site; RC II - deacylated tRNA\textsubscript{Ile} in P site and MFTIA-tmRNA•SmpB in A site; RC III - deacylated tRNA\textsubscript{Ile} in E site, MFTIA-tmRNA in P site and the resume codon of tmRNAs internal ORF in A site; RC IV - MFTIAA-tRNA\textsubscript{Ala} in P site and empty A site with the 3\textsuperscript{rd} codon of tmRNAs internal ORF; RC V - MFTIAAN-DENYALAA-tRNA\textsubscript{Ala} in P site and UAA-stop codon of tmRNAs internal ORF in A site.

We analyzed the partial lead(II) cleavage patterns on all tmRNA-containing complexes (I-V), and compared it to results obtained for tmRNA both in vivo and in vitro (Fig. 11 and Table I in paper IV).

Upon lead(II) addition, specific cleavages were obtained, some of which were characteristic for tmRNA in discrete stages. Distinct nucleotides situated in single-stranded RNA stretches in PK2-4 were specifically protected or exposed to cleavage in some ribosomal complexes but not in others. Some footprints were also present in all complexes as compared to naked tmRNA. In addition to the SmpB footprint, five more nucleotides were protected in all stages of trans-translation (A197, A185, C211 and A32, A34) (Fig. 11 and Table I in paper IV). These protections may be due to the interaction with S1 ribosomal protein, as previously observed for some of these positions [97-99] or result from other interactions with the ribosome.
The observed similarities in the cleavage patterns for the different ribosomal complexes indicate that no major structural changes were induced during the different trans-translations steps and the overall conformation of tmRNA seems to be intact. Our data are in agreement with the model by Ivanov et al. suggesting that PK2-PK4 remain outside the ribosome during the whole course of trans-translation [80]. For the moment we can’t speculate about the specific roles of all mapped nucleotides and assign them interaction partners. However, footprinting studies of the ribosomal RNA in the same complexes are in progress as well as cryo-electron microscopy studies. The results from these studies will allow us to picture the exact ribosomal location and interactions in which the footprinted nucleotides participate during tmRNA movement through the ribosome in trans-translation.

**Figure 10.** The trans-translation steps, adopted from paper IV.
**Figure 11.** tmRNA secondary structure model with indicated protected/exposed positions after lead(II) acetate cleavage, adopted from paper IV.
Mapping the interaction of SmpB with ribosomes by footprinting of ribosomal RNA

In this work we have searched for possible interaction sites between SmpB and ribosomal RNA by chemical modification and primer extension analysis of SmpB-containing ribosomal complexes. We have also studied the binding of SmpB to ribosomes and ribosomal subunits by gel-filtration chromatography.

Stoichiometry of SmpB binding to ribosomes and ribosomal subunits estimated by gel-filtration chromatography

The ribosomal complexes containing 35S labeled SmpB were formed by incubating ribosomes or ribosomal subunits with 2.5-fold or 5-fold excess of SmpB over ribosomes/subunits and applied to a gel-filtration column. The elution profiles clearly show that SmpB forms a 2:1 complex with 70S ribosomes in agreement with earlier data (paper 1) whereas 30S and 50S subunits bind 0.8 and 0.6 molar equivalents of SmpB, respectively.

Probing the SmpB interaction with ribosomal RNA

Interactions between ribosomal RNA and SmpB were probed by DMS and kethoxal modifications or by hydroxyl radicals-induced cleavage of rRNA.

The results show that SmpB interacts with both 30S and 50S subunits and footprints both 16S and 23S rRNA. The positions of nucleotides that had altered accessibility to the probe upon addition of SmpB were mapped on the crystal structure of Ecoli 70S ribosome [126](see Fig. 12 and paper V).

Interaction of SmpB with 16S rRNA in 30S subunits and 70S ribosomes

Nucleotides in the central and 3´major domains were protected from chemical modification by the interaction of SmpB with the 30S subunit. No additional footprints were seen in 16S rRNA in 70S ribosomes. Instead the reactivity of several nucleotides disappeared, or was diminished, as a result of the interaction between the ribosomal subunits.

The SmpB-induced footprints in the 690-loop, at G690 and G691 and at positions G926 and G927 in helix H27 as well as the reactivity of C1400 were similar in 70S ribosomes and 30S subunits. However, we didn't see detectable SmpB interaction with the decoding region.
of 30S as it was proposed by Gutmann et al [77]. The observed SmpB localization on the ribosome may be due to the absence of tmRNA.

**Effect of SmpB on translational accuracy**

A modest effect of SmpB on the accuracy of translation was observed. The effective association rate constant $k_{cat}/K_m$ of tRNA$\text{Leu}$(GAG) to the ribosomes programmed with near-cognate UUU codon in the A site measured by dipeptide formation assay was 97 M$^{-1}$s$^{-1}$ in the presence and 140 M$^{-1}$s$^{-1}$ in the absence of SmpB. The $k_{cat}/K_m$ value in the cognate case was unaffected by the presence of SmpB.

**Interaction of SmpB with 23S rRNA in 50S subunits and 70S ribosomes**

No base-specific footprints in 23S rRNA in 50S subunits could be detected even at a 5-fold excess of SmpB over ribosomes. Instead, three regions were protected from hydroxyl radical cleavage within the SmpB 50S complexes.

The sugar-phosphate backbone in the 1100-region in helix H43 and in helix H44 of domain II was cleaved to similar extents within SmpB 50S and SmpB 70S-complexes. The accessibility of the 2475-loop on top of helix H89 in domain V was reduced in 70S ribosomes compared with 50S subunits but the SmpB-induced footprint was similar. In contrast, the SmpB-induced footprint in the $\alpha$-sarcin/ricin-loop, present within the SmpB 50S complexes, was abolished by subunit association.

The footprints on top of helix H89 in the 1100 region are consistent with the SmpB-positioning in the cryo CEM structure of the complex between Ala-tmRNA and 70S ribosomes in the presence of EF-Tu$\cdot$GTP$\cdot$Kirromycin [78].

Mapping of the interacting nucleotides in the crystal structure of the 70S ribosome [126] shows that the binding of at least two SmpB molecules is required to account for both the footprints on the 30S subunit and for footprints below the L7/L12-stalk on the 50S subunit in the assembled 70S ribosome. Thus, the probing results are in agreement with the gel-filtration data showing that 70S ribosomes bind two molecules of SmpB.
**Figure 12.** A summary of 23S rRNA and 16S rRNA positions protected by SmpB in the 70 ribosome. In solid colours to the left from the yellow A-site tRNA are the protected 16S rRNA nucleotides. Position to the right of the A-site tRNA are those protected by SmpB in the 23S rRNA. Coordinates of the Ecoli 70S ribosome were from PDB files 1PNS (30S) and 1PNU (50S). Adopted from paper V.
CONCLUSIONS

I. SmpB binds 70S ribosomes independently of the presence of tmRNA and this interaction is a prerequisite for the tmRNA binding to the ribosomes.

II. Two SmpB molecules bind per ribosome. SmpB prebound to ribosomes can recruit Ala-tmRNA and trigger trans-translation.

III. SmpB has a higher affinity for the 30S than for the 50S subunit and interacts with nucleotides below the L7/L12-stalk on the 50S subunit and near the subunit interface on the 30S.

IV. SmpB is the only factor sufficient and indispensable for the trans-peptidation to Ala-tmRNA and EF-Tu•GTP stimulates greatly the reaction rate.

V. SmpB remains bound to the ribosome rescue complex throughout the whole trans-translation cycle.

VI. The overall structure of tmRNA remains generally intact during the trans-translation process.

VII. mRNA truncation close to the P-site codon on the stalled ribosome should occur prior to trans-peptidation to Ala-tmRNA and is the major determinant for the efficiency of this process. The cleavage is not induced by tmRNA or the ribosome itself.

VIII. tmRNA-mediated trans-translation is efficient in comparison to elongation or termination only on mRNAs truncated close to the P-site codon.

IX. The original mRNA leaves the stalled ribosome after the EF-G mediated trans-translocation has occurred.
Hitta de okända faktörerna för trans-translation


Ala-tmRNA i komplext med eloneringsfaktorn EF-Tu och SmpB binder till ribosomens bindningsställe A för transportRNA (A site). Sedan flyttas peptidkedjan från transportRNAt i ribosomes bindningsställe P (P site) till Ala-tmRNA genom trans-peptidering (trans-transfer), och peptidkedjan märks sedan för nedbrytning genom avkodning av den öppna läsramen i tmRNA. När den märkta peptiden lämnat ribosomen efter termineringssteget, kan ribosomen återcyklas med hjälp av återcyklingsfaktorn RRF och eloneringsfaktorn EF-G så att den sedan kan syntetisera ett nytt protein.

I detta arbete har vi använt ett in vitro system för proteinsyntes med rena komponenter från E. coli för att i detalj studera hur tmRNA och dess hjälpprotein SmpB kan rädda ribosomer som fastnat på budbärarRNA.

Vi har undersökt i cellen såväl som i provröret var SmpB är lokalisert och hur trans-översättning initieras. Vi har funnit att SmpB binder till cellens ribosomer vare sig där finns tmRNA eller ej och vidare att SmpB måste vara närvarande för att tmRNA skall kunna binda till ribosomen och trans-peptidering äga rum. Vi har visat att två SmpBmolekyler binder till ribosomen och att förbindning av SmpB antingen till ribosomen eller till tmRNA resulterar i
snabb association av tmRNA till ribosomen och till snabb trans-peptidering. Vi har, dessutom, använt “fotavtrycksmetoden” (footprinting) och andra tekniker för att demonstera att SmpB förblir associerad med ribosomen under hela trans-översättningscykeln.

Vi har studerat hur hastigheten i trans-peptideringen beror på hur långt budbärarRNAs baskedja sträcker sig nedom ribosomens A site. Vi har visat att effektiviteten i trans-peptideringen skarpt avtar med ökande budbärarRNA-längd och blir nära noll när budbärarRNA-sträcker sig mer än femton baser nedom P site. Vi har demonstrerat hur initiiering av trans-översätting kraftigt stimuleras när budbärarRNA klyvs i A site av bakterietoxinet RelE. Vår slutsats är att tmRNA kan verka i cellen bara när budbärarRNA-trunkerats, och vi föreslår att sådana trunkeringar av mRNA utfördas av RelE eller andra, hittills oidentifierade, enzymer i cellen används av bakterierna för att hantera stressiga situationer, som t.ex. när det plötsligt blir brist på en aminosyra.

Vi har dessutom kunnat visa att dissociering av trunkerade budbärarRNA från ribosomen stimuleras av tmRNA-bindning till ribosomen och trans-peptidering. Snabb dissociation av mRNA kräver emellertid att tmRNA bundet till peptidkedjan flyttas (translokeras) till ribosomens P site i en reaktion katalyserad av elongeringsfaktorn EF-G. Dissociation av budbärarRNA blir långsammare när det kan binda till en komplementär sekvens i 16S ribosomalt RNA (Shine-Dalgarno-anti-Shine-Dalgarno bindning) eller när budbärarRNA-sträcker sig långt nedom ribosomens P site.

Med hjälp av fotavtrycksmetoden har vi undersökt SmpBs bindning till tmRNA, ribosomen och dess subenheter (30S och 50S). Vi har funnit RNAfotavtryck nära ribosomens P site i 30S subenheten och nedanför L7/L2 stjälken i 50S subenheten.

Med gelfiltrering och fotavtryck har vi kunnat bekräfta att två SmpBmolekyler binder till ribosomen, och visat att SmpB har högre affinitet till lilla (30S) än till stora (50S) subenheten.

Vi har studerat in vitro hur fotavtryck på tmRNA varierar när tmRNA genomlöper trans-översättnings olika steg och jämfört med fotavtrycken på tmRNA inne i cellen i närvaro såväl som i frånvaro av SmpB.

Vår slutsats är att den största delen av tmRNA inne i cellen normalt är bundet till SmpB och att SmpB förblir bunden till tmRNA genom hela trans-översättningscykeln. Vidare, att tmRNA-strukturens huvuddrag förblir oförändrade under trans-översättningen, men att det finns ett fåtal, unika fotavtryck på tmRNA för olika steg i trans-översättningscykeln.
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Nobody is forgotten! Nothing is forgotten!
REFERENCES


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112. Sunohara, T., et al., The C-terminal amino acid sequence of nascent peptide is a major determinant of SsrA tagging at all three stop codons, in RNA. 2002. p. 1416-27.
124. Sunohara, T., et al., Nascent-peptide-mediated ribosome stalling at a stop codon induces mRNA cleavage resulting in nonstop


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