Scaffolding as an Organizing Principle in Trans-translation

THE ROLES OF SMALL PROTEIN B AND RIBOSOMAL PROTEIN S1

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A eubacterial ribosome stalled on a defective mRNA can be released through a quality control mechanism referred to as trans-translation, which depends on the coordinating binding actions of transfer-messenger RNA, small protein B, and ribosome protein S1. By means of cryo-electron microscopy, we obtained a map of the complex composed of a stalled ribosome and small protein B, which appears near the decoding center. This result suggests that, when lacking a codon, the A-site on the small subunit is a target for small protein B. To investigate the role of S1 played in trans-translation, we obtained a cryo-electron microscopic map, including a stalled ribosome, transfer-messenger RNA, and small protein B but in the absence of S1. In this complex, several connections between the 30 S subunit and transfer-messenger RNA that appear in the +S1 complex are no longer found. We propose the unifying concept of scaffolding for the roles of small protein B and S1 in binding of transfer-messenger RNA to the ribosome during trans-translation, and we infer a pathway of sequential binding events in the initial phase of trans-translation.

Trans-translation is a bacterial quality control mechanism that allows the rescue of stalled ribosomes by transfer-messenger RNA (tmRNA),4,5 a unique chimeric molecule acting as both tRNA and mRNA. tmRNA is ~230 – 400 nucleotides long, depending on the bacterial species, and its consensus secondary structure encompasses a tRNA-like domain (TLD), a small internal open reading frame (ORF, sometimes referred to as mRNA-like domain), and a string of pseudoknots (1). Although the secondary structure of the TLD is very similar to that of a canonical tRNA, it lacks an anticodon loop, possesses a reduced D-domain, and the x-ray structure shows an increased inter-domain angle at the elbow level (120° versus 90°) (2, 3). SmpB (small protein B) is a basic protein interacting with tmRNA during trans-translation (4). Its gene is conserved among prokaryotes, and its deletion causes phenotypes similar to those observed when tmRNA is inactivated.

The process of trans-translation has been described as follows (5, 6). The TLD in tmRNA is aminoacylated with alanine, and then the complex of tmRNA, SmpB, and EF-Tu binds to the stalled ribosome, characterized by the absence of a stop codon in the A-site because of a defective mRNA. Subsequent insertion of the ORF into the ribosome and its complete translation, which requires the presence of protein S1 in solution, leads to the release of both the tagged polypeptide to be degraded and the stalled ribosome to be recycled.

An earlier cryo-EM study (7) identified the position of the pseudoknots and the TLD in tmRNA, as well as a single copy of SmpB. Some portions of the density map were less well resolved, leaving ambiguities in the path of ORF and the number of SmpBs. Both ambiguities were recently resolved by an improved cryo-EM map (8), whose interpretation could take advantage of new x-ray data on a complex between SmpB and TLD (3). Accordingly, two SmpB molecules were identified, one (SmpB-2) bound in the decoding site and the other (SmpB-1) perched at the GTPase-associated center. This result is in line with the earlier suggestion by Wower et al. (9) that several SmpB molecules might interact with tmRNA, both on its CCA stem and its pseudo-anticodon stem. Indeed, a recent biochemical study demonstrates that during trans-translation in vivo, two SmpB's interact with the stalled ribosome, one with the small and one with the large subunit of a stalled ribosome (10).

According to the biochemical data (11, 12), SmpB primarily has three functions as follows: (i) facilitation of the binding of tmRNA to the ribosomes; (ii) enhancement of tmRNA alanylation by the alanyl-tRNA synthetase; and (iii) protection of tmRNA from degradation. Essentially, no trans-translation can be triggered in the absence of SmpB. The finding of two copies
of SmpB bound to the stalled ribosome raised questions on functional identity of the two SmpBs and the sequence in which the binding occurs in the initiation of trans-translation. This study takes a cue from the *in vivo* study by Hallier *et al.* (13) who found that SmpB can bind to the stalled ribosome independently from the presence of tmRNA, suggesting that the stalled ribosome is first targeted by SmpB and that this might be the first step of trans-translation. However, the questions that remained to be addressed are as follows. 1) When SmpB binds to the stalled ribosome independently, then where precisely is its binding position? 2) Given the binding position of the SmpB on the stalled ribosome, what is the likely mechanism of the targeting of the ribosome by tmRNA? 3) Which of the two molecules of SmpB, which are associated with the ribosome-bound tmRNA according to Kaur *et al.* (8), is used to protect free tmRNA from degradation?

In an effort to address these questions, we used cryo-EM to study two complexes, one obtained by incubating SmpB with stalled ribosomes and the other (the control) with nonstalled ribosomes that had a sense codon in the A-site. Comparison of these maps revealed the presence of SmpB at the decoding site of the stalled ribosome, roughly in the position of SmpB-2 as defined by Kaur *et al.* (8). This study demonstrates that the presence of a codon in the A-site prevents the binding of SmpB. This result gives a rationale as to why SmpB does not interfere with canonical translation. Furthermore, to elucidate the role played by the ribosomal protein S1 in solution during this early step of trans-translation, we also obtained a cryo-EM reconstruction (at a resolution of 13 Å) of a stalled ribosome bound with tmRNA, EF-Tu-GDP, and SmpB in a pre-accommodated state but in the absence of S1. In this map, the ORF in the tmRNA is well structured and continuous outside the decoding center, unlike the discontinuous, disrupted structure seen in the presence of S1.

Following the study by Kaur *et al.* (8), this study was aimed at determining the roles of both SmpB and S1 in the assembly and function of the trans-translation complex. Our findings can be summarized by one unifying concept, the concept of scaffolding. Trans-translation, specifically the formation of a productive tmRNA structure, ORF insertion into the mRNA channel, and TLD insertion into the A-site all appear to be mediated and controlled by these structural proteins.

**EXPERIMENTAL PROCEDURES**

**Preparation of Components and Formation of SmpB-Ribosome Complexes—Thermus thermophilus** ribosomes were prepared as described previously (14). C-terminal His-tagged SmpB was overexpressed in *Escherichia coli* using the T7 expression system and isolated by Ni²⁺-charged His-Trap chelating column, according to the manufacturer’s procedures (Amersham Biosciences, Orsay, France). Ribosomal P-site tRNA complexes were obtained by incubating 70 S ribosomes (1 μM) with 2 μM of mRNA consisting of the sequence GGCAGGGUAAAAUUG for the stalled one and UCGAGAAGGAGCUUAAAGUGGAGUACCCUUCUUCUCC for the nonstalled, and *E. coli* fMet-tRNAfMet (2 μM) for 30 min at 37 °C in a 48-μl medium containing 5 mM Hepes-KOH, pH 7.5, 50 mM KCl, 10 mM NH₄Cl, 10 mM MgOAc, 6 mM β-mercaptoethanol. An initiation complex, including an SD sequence, an AUG on the mRNA, and an initiator tRNA, was used to simulate a rescue complex because it gives a stable complex that mimics a stalling situation in the most simple case. Complexes were then obtained by adding SmpB (4 μM) for 15 min at 37 °C. No S1 was used in the complexes. Pre-accommodated complexes in the absence of the ribosomal protein S1 were prepared as described earlier (7).

**Equilibrium Binding Measurement by Filter Binding—Ribosome-SmpB complexes (100 μl) were applied to a Superdex 200 HR 10/30 column (GE Healthcare, Orsay, France) equilibrated with 5 mM Hapes-KOH, pH 7.5, 10 mM NH₄Cl, 10 mM MgOAc, 50 mM KCl, at 4 °C. The fractions containing the purified 70 S ribosome were pooled and concentrated. *In vitro* transcribed tmRNA was end-labeled with [γ-32P]ATP and phage T4 polynucleotide kinase after dephosphorylation with alkaline phosphatase. Following purification (MegaClear, Ambion, Austin, TX) and ethanol precipitation, tmRNA was denatured for 2 min at 80 °C in a folding buffer (5 mM MgCl₂, 20 mM NH₄Cl, 10 mM Hapes-KOH, pH 7.5) and slowly cooled down to room temperature for 30 min before being applied to the SmpB-ribosome complexes described above. 1 μl of labeled tmRNA was added to the ribosomal complexes for either 2 or 5 min (similar results) at 20 °C, in a total volume of 15 μl in a buffer containing (5 mM Hapes-KOH, pH 7.5, 50 mM KCl, 10 mM NH₄Cl, 10 mM MgOAc, 6 mM β-mercaptoethanol). 13 μl were then filtered under vacuum suction over nitrocellulose filters (Schleicher & Schuell), followed by rapid washing with 3 × 1 ml of the same cold buffer. Membranes were dried, and the holding radioactivity was measured by scintillation counting (Wallac 1409).

**Sucrose Density Gradient Centrifugation and Analysis—**Crude ribosomes from *E. coli* were obtained as described previously (13). 70 S ribosomes were dissociated into 50 S and 30 S subunits by diluting the crude ribosome with a 10-fold volume of Mg²⁺-free lysis buffer. Ribosome subunits were fractionated on a 10–30% sucrose gradient in 25 mM Tris-HCl, pH 7.5, 100 mM ammonium acetate, 1 mM magnesium acetate for 15 h at 27,000 rpm and at 4 °C. RNAs were isolated from one-half of each fraction by phenol extraction followed by ethanol precipitation. For Northern hybridization, RNAs were separated by electrophoresis on a 1% (w/v) agarose gel, and transferred to polyvinylidene difluoride membranes. SmpB was then detected by using a rabbit polyclonal antibody as described previously (13).

**Cryo-electron Microscopy and Image Processing—**Ribosomal samples were diluted to a final concentration of 32 nm and used directly for cryo-EM grid preparation following standard procedures. Micrographs at ×50,000 (±2%) magnification were taken on a Philips FEI (Eindhoven, The Netherlands) Tecnai F20 with field emission gun operated at 200 kV and with a low electron dose (~15 e⁻ Å⁻²). The micrographs were scanned
with a pixel size corresponding to 2.82 Å on the object scale with a Zeiss Imaging scanner (Z/I Imaging Corp., Huntsville, AL). The image processing was carried out with the SPIDER package (15), including a reference-guided projection classification and alignment, contrast transfer function correction of segregated defocus groups, and correction of the high frequency amplitudes using low angle x-ray scattering data (16). The numbers of particles used in the three-dimensional reconstructions were 48,138, 52,829, and 31,731 for the 70 S tRNA with a sense codon in the A-site, the 70 S tRNA-SmpB lacking a codon in the A-site, and the 70 S tRNA-tmRNA-56EF-Tu-SmpB-kir lacking a sense codon in the A-site and in the absence of S1 for a pre-accommodated complex, respectively. The map resolutions were 10, 11.8, and 13.1 Å, respectively, using a 0.5 cutoff in the Fourier shell correlation. The docking of atomic model of tmRNA into the cryo-EM maps was done using O, and the visualization was performed using IRIS Explorer (Numerical Algorithm Group Ltd., Downers Grove, IL), Ribbons (17), and INSIGHT II (Accelrys Inc., San Diego).

**RESULTS**

**Binding of SmpB to the Stalled Ribosome**—Our study was motivated by the finding, using biochemical methods, that SmpB binds the stalled 70 S ribosome in the absence of tmRNA (13), suggesting that this binding may be an initial step of trans-translation. We set out to visualize this binding complex by means of cryo-EM and single particle reconstruction (15). Two cryo-EM samples were made by incubating SmpB protein with the 70 S ribosome, tRNA, and two different mRNAs, one bearing a stop codon at the A-site and the other lacking any codon at the A-site (see “Experimental Procedures”). Both SmpB and the ribosome were from *T. thermophilus*. By using these two samples, two cryo-EM maps were produced, at resolutions of 10 and 11.8 Å, respectively (Fig. 1, A and B). These two maps promised to provide a comparison for binding of SmpB under the two conditions.

The difference between the two maps proved to be an extra piece of density located at the decoding center of the ribosome (Fig. 1). This density indicates high (close to 100%) occupancy of a protein ligand. To ascertain SmpB as the origin, its atomic conformation, which is atomic structure (PDB 1P6V) was fitted into the cryo-EM map by visual criteria, leading to a good match with the map in both size and shape (Fig. 2). We regard this result as a positive identification of SmpB, which was confirmed by Western blot analyses using anti-SmpB antibodies (not shown). The x-ray structure shows long random coils in both the N and C termini. In addition, residues 73–76 are missing in the x-ray structure, which leave the two disconnected ends less structured. In addition to the overall match between the density of the current cryo-EM map and the x-ray structure, the best candidate orientation of this SmpB can be inferred by comparing it with the SmpB bound to the A-site (i.e. SmpB-2) in the complex with tmRNA (8). Placing the SmpB at the position and orientation of SmpB-2 in the tmRNA-SmpB model leads to a fairly good match with the current cryo-EM map. Thus, our cryo-EM results indicate that SmpB is recognized by the stalled ribosome but not by the ribosome encountering a stop codon in the A-site in the process of normal translation.

In the fitting position, SmpB fills the space near the decoding center. Based on this fitting position, the potential binding sites are on different sides of the molecule and include the position for normal A-site codon, helix 44 of the 30 S subunit, which are on different sides of the molecule, exposing more than one potential intermolecular binding site. This suggestion thus fits our current observation. The orientation of the SmpB seems plausible in facilitating the entry of tmRNA into the ribosome (see “Discussion”).

To check this hypothesis and to investigate further the contribution by stalled ribosomes to SmpB recognition, measurements of the binding of tmRNA to the ribosomal complexes described above were carried out by filter-binding assays (20). The complexes were loaded on a gel filtration Superdex 200 column (GE Healthcare) to eliminate the free fraction of SmpB that could interfere with tmRNA. Equilibrium dissociation experiments were then carried out between radiolabeled tmRNA and each of the purified complexes (see “Experimental Procedures”). According to the data, tmRNA binds to stalled ribosomes once the empty A-site has been recognized and occupied by SmpB (Fig. 3; compare curves with squares to diamonds).

**Experiments Related to the Assembly Pathway**—To ascertain specificity of SmpB for an empty A-site, a control experiment was carried out by preparing a sample in which all conditions are kept the same, except for placement of a sense codon at the A-site (see “Experimental Procedures” and Fig. 1C). Despite the presence of SmpB in the initial mix, tmRNA is apparently unable to target the stalled ribosome (Fig. 3, open squares). Altogether, these results confirm the cryo-EM data (Fig. 1) showing that SmpB binds exclusively to stalled ribosomes bearing an empty A-site, thereby facilitating their subsequent interaction with tmRNA.

Our cryo-EM study and the finding by Hallier et al. (13) agree with the result that in the absence of tmRNA one SmpB molecule binds stably to the stalled 70 S ribosome and that this molecule is found on the 30 S subunit. We designed biochemical experiments to investigate whether or not this 30 S tmRNA-SmpB complex has any relevance in vivo and whether under these conditions SmpB molecules strongly bind with tmRNA. Translating 70 S ribosomes were purified in vivo and dissociated into their large and small subunits on a sucrose density gradient at a low salt concentration (Fig. 4A). After gel separation, the 23 S and 16 S ribosomal RNA were detected by ethidium bromide staining, allowing a correct matching of the two subunits extracts on the gel (Fig. 4B). tmRNA and SmpB distribution were monitored in each fraction by Northern and Western blots, respectively. Dissociation of the two subunits releases tmRNA from the ribosomes to the soluble fraction (Fig. 4C). tmRNA was not detected in the 50 S or in the 30 S subunits. Using an antibody raised against SmpB, immunoblots of these fractions indicate that, in the same way, the protein mostly co-sediments with tmRNA outside the separated ribosomal subunits. The SmpB protein coming from a mixture containing ribosomes in all stages of trans-translation is released
with tmRNA, whereas a small portion of SmpB bound alone to the stalled ribosomes is found associated with the 30 S fraction (Fig. 4D).

These results confirm that in vivo one SmpB molecule binds to a fraction of the stalled 70 S ribosomes on the small subunit and, furthermore, that once the trans-translation complex is formed, tmRNA and two SmpB molecules form a strong association that even persists after forced subunit dissociation. Given the fact that two copies of SmpB are found in the stalled trans-translation 70 S ribosome (8, 10), the assembly of the trans-translation complex brings two SmpBs in close contact so that they form a structural scaffold.

SmpB Forms a Binding Framework for tmRNA—The in vitro experiment demonstrates that when tmRNA is present, two molecules of SmpB on the 70 S ribosome are required for trans-translation (13). One is tightly bound to the 30 S subunit, whereas the other contacts the large subunit transiently (10). Accordingly, a recent cryo-EM reconstruction at higher resolution has identified density for two molecules of SmpB in a pre-accommodated state as follows: the one depicted above (SmpB-2) and the other directed toward the large subunit (SmpB-1) (8).

On the side view depicted in Fig. 5, interacting regions of the two SmpB proteins with the TLD portion of tmRNA are emphasized. Both SmpB proteins interact with tmRNA such that the two molecules come in close contact (Fig. 5). In addition, SmpB-1 contacts SmpB-2 through the unstructured C terminus of SmpB-1. In the fitting position (3), the bi-lobed shape of the density for SmpB-1 near the 50 S subunit was fitted quite well by placing $\beta_{\alpha\gamma}\beta_5$ and $\beta_{\gamma}o_5$ into the two lobes. However, this placement causes noticea-
ble overlap between the C terminus of SmpB-1 and the T stem-loop. In the model shown in Fig. 5, the x-ray position of the C terminus (residues 125–130) is highlighted to indicate that we are dealing with a highly flexible portion of the molecule, so that concerns about the overlap may be moot. In addition, we made a slight adjustment for the position of SmpB-1 to release some residual regional overlapping. Furthermore, we noticed that the bi-lobed shape of the density can also be fitted by a position of the x-ray structure related to the current position through rotation by 180° about an axis normal to the plane of the figure, but this would remove the potential dimeric contact via C-terminal binding. It is hoped that a future higher resolution map will provide a decisive answer for the position of SmpB-1.

Intriguingly, when monitoring the interactions of SmpB with a full-length thermophile tmRNA, not all the footprints identified on the TLD are clustered around the 5′ end of the D-loop (21), as expected from the x-ray crystal data of the complex between the tRNA-like domain of tmRNA and SmpB. Additional protections are in the T stem and loop, reflecting either indirect changes because of a rearrangement of the binding to the elbow or a direct interaction between the protein and the RNA, as suggested by the position of SmpB-1 in our model. In the same way, a combination of enzymatic probing and UV cross-linking experiments indicates that the acceptor and T arms constitute a major binding site for SmpB (9). Thus the two SmpB proteins surrounding the TLD create a stable scaffold that allows the RNA to be connected with two essential structural elements of the ribosome as follows: the decoding site on the small subunit and the GTPase-associated center on the large subunit. Both biochemical data and the structural evidence depicted here strengthen the idea of the SmpB molecules forming a protein scaffold that provides a stabilizing framework for the flexible tmRNA molecule on the ribosome, allowing its accurate positioning and tuning of its activity.

**Regulation of tmRNA Activity by Ribosomal Protein S1**—The crucial role played by SmpB in scaffolding tmRNA into an active conformation for aminoacylation and recognition by the ribosome raises the question about the involvement of the other protein partners during the process. Among these, the role of ribosomal protein S1 is still not completely understood despite numerous studies. Two recent studies have suggested that S1 might play a role at a stage prior to the recruitment of tmRNA to the ribosome (22, 23).

To investigate the way in which ribosomal protein S1 regulates the activity of tmRNA in trans-translation, a higher resolution 13-Å map (as compared with the earlier 18-Å map by Valle et al. (7)) of the pre-accommodated complex in the absence of S1 was obtained (Fig. 6A). Assignment of the atomic coordinates for each portion of the tmRNA complex was done as described in our previous cryo-EM study (8), which allowed us to refine the previously published model (7) of the tmRNA-SmpB complex. Although in agreement with the previous map in all essential features, the new map shows the complex with much higher definition. We see the 70 S ribosome and a density attributed to the complex of tmRNA, SmpB, and EF-Tu. This map must be compared with the map showing the complex in the presence of S1, to clarify whether or not this
complex is associated with the functionally related structural feature. Evidently, the absence of S1 produces a complex that differs from the pre-accommodated complex in the presence of S1 in four major features as follows (for the placement of structural elements in the tmRNA map, see Fig. 6). First, the formation of a closed ring by the tmRNA elements ORF, PK1, PK2, PK3, PK4, and helix 5 is observed. Presumably related to the stabilized structure of the ORF region, tmRNA in the absence of S1 forms a closed loop rather than an open one-turn spiral (7). The closed loop places the ORF outside the decoding center so that trans-translation cannot start, as this process relies on the recognition of codons included in the ORF. The presence of S1 influences the position and structure of the ORF, implying S1 as a decisive factor instrumental for sending the ORF into the decoding site. The density appearing between PK1 and helix 5 is strong enough to account for a single-stranded RNA in a slightly curved path, reflecting the fact that the ORF is highly structured in the absence of S1. These data are consistent with the prediction for the secondary structure of tmRNA, according to which the ORF contains a structured single-stranded domain (24). Second is the absence of a direct contact between the ORF and the beak of the 30 S subunit. Third is the appearance of an enlarged area of contact between helix 5 of tmRNA and the head of the 30 S subunit, as well as an extra contact between PK2 and the head of the 30 S subunit (Fig. 6B). Fourth is the weakening of the connection between the loop linking H2a and H2b of tmRNA and the head of 30 S subunit.

DISCUSSION

Trans-translation requires a complex assembly of several elements in the correct order and correct spatial arrangement. Our study, in conjunction with the study presented by Kaur et al. (8), indicates that this process can be understood as the result of the interaction of the main players (tmRNA and the 70 S ribosome) with two proteins, SmpB and S1. Both interactions can be subsumed under the single perspective of scaffolding. In the following, the separate roles of these proteins, in assembling an A/T-like pre-trans-translation complex and in guiding the ORF into the mRNA entrance channel, will be illuminated.

Scaffolding of tmRNA by S1—The new cryo-EM data show that the presence of S1 directly affects the conformation of tmRNA even though S1 does not appear at the canonical ribosomal binding site (16) (Fig. 6B, indicated by asterisk) or around tmRNA. Our previous observations indicated that S1 binds to the ribosome rather weakly (16). The fact that S1 interacts with tmRNA in solution has been supported by biochemical data (22, 23).

Going by its size (25), S1 could approximately match the inner diameter of the circle, about 80 Å, of the one-turn spiral formed by tmRNA, which is composed of the sequence of pseudoknots from PK1 to PK4. Biochemical studies revealed that the S1-binding sites along the tmRNA include PK2, PK3, and mRNA-like domain (26, 27). Our cryo-EM map shows that these tmRNA domains interact with the 30 S subunit after the tmRNA complex enters the ribosome. Our interpretation is that these binding elements could be actively involved in the binding with S1 in the pre-binding stage. In view of the normal role of S1 as a ribosomal protein, and because it is known to bind loosely,
it is interesting to ask if the absence of S1 in solution might have
an indirect effect, in depleting ribosome-bound S1 and thereby
affecting the conformation of the ribosome adversely, such that
the formation of the productive spiral conformation of tmRNA
is disfavored. One observation is relevant here, although it falls
short of answering this question. We see some differences in
the conformation of the 30 S subunit head between the
\[\text{S1}\] complexes in the region of the PK2 contact point
(arrow in Fig. 6).

By taking into account the available data, we can postulate the
following pathway. First, S1 binds with tmRNA in solution and
thereby activates tmRNA into a functional conformation. (We
cannot exclude at the present time that S1 might play an additional
role in binding to the 30 S subunit and effecting a change in its
conformation.) Then, upon the initial SmpB-mediated binding of
tmRNA to the stalled ribosome, S1 is released from the pre-bind-
ing complex. The 30 S subunit head replaces S1 in its stabilizing
function and positions tmRNA such that the ORF is placed in the
decoding site, as required for trans-translation. Accordingly, the
S1 protein is dispensable for the tRNA-like function of tmRNA but
is essential for its mRNA function in vitro and in vivo.6

The present cryo-EM map shows that although the absence
of S1 does not prevent tmRNA recruitment, and the ORF in
such a complex is highly stable and structured, the position of
this structured ORF is remote from the decoding site, so the
complex cannot be in a functional state. In fact, the effect of S1
in destabilizing the ORF region was already seen for the tmRNA
ribosome complex at lower resolution (7).

**Scaffolding of the tmRNA-Ribosome Complex by SmpB—**
Both biochemical results and cryo-EM studies have demonstrated that two molecules of SmpB bind to the complex of the
stalled 70 S ribosome with tmRNA when trans-translation is
initiated. The positions of these two SmpB molecules have been
identified in the cryo-EM maps as follows: SmpB-2 in the
decoding site on the small subunit and SmpB-1 toward the
GTPase-associated center on the large subunit (Fig. 5). Both
SmpB-1 and SmpB-2 interact with the TLD in tmRNA such
that a stable scaffold is created, allowing tmRNA to be correctly
positioned into the decoding site. Although not present in our
map in the absence of tmRNA, the SmpB protein directed
toward the large subunit (SmpB-1) probably plays a major role
in the early events of trans-translation because it makes close
interactions with helix 69 in the 23 S RNA, which is involved in
the ribosomal subunit association as well as in the GTPase acti-

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6 Saguy, M., Gillet, R., Skorski, P., Hermann-Le Denmat, S., and Felden, B. (2007)
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trans-translation empty, precisely corresponding to the situation triggering relevant, stalled state, with mRNA present but truncated, and the protein. In our study the ribosomes were in a functionally tRNA sites (A, P, and E) empty and available for interacting with the ribosomes used in their study were vacant, leaving all three but in the vicinity of the P-site. It should be noted here that subunit, close to the GAC, and the second toward the small one for SmpB were detected per ribosome, one toward the large one in placing the TLD correctly, in line with the central theme of modulated state of tmRNA. The fact that the two copies of SmpB position at the decoding center is retained in the intact messages. As another cryo-EM study shows (8), this cryo-EM data directly show the binding of SmpB to the stalled pre-loading of SmpB into the stalled ribosome. The present until recently (13) that trans-translation might be triggered by Sauer and co-workers in the late 90s (4, 30), it has been known intriguing questions about trans-translation is how tmRNA... ever the presence of a codon in the decoding site of the small components associated with tmRNA entry into the ribosome. However, the presence of a codon in the decoding site of the small ribosomal subunit prevents this binding from occurring. Thus, the SmpB at the decoding site acts as a sentinel, able to discriminate stalled ribosomes from the active ones that are translating intact messages. As another cryo-EM study shows (8), this SmpB position at the decoding center is retained in the accommodated state of tmRNA. The fact that the two copies of SmpB tightly interact with each other in the pre-accommodated state suggests that the association of these molecules is instrumental in placing the TLD correctly, in line with the central theme of scaffolding in trans-translation.

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REFERENCES
1. Gueneau de Nova, P., and Williams, K. P. (2004) Nucleic Acids Res. 32, D104–D108
2. Stagg, S. M., Frazer-Abel, A. A., Hagerman, P. J., and Harvey, S. C. (2001) J. Mol. Biol. 309, 727–735
3. Gutmann, S., Haebel, P. W., Metzinger, L., Sutter, M., Felden, B., and Ban, N. (2003) Nature 424, 699–703
4. Karzai, A. W., Susskind, M. M., and Sauer, R. T. (1999) EMBO J. 18, 3793–3799
5. Karzai, A. W., Roche, E. D., and Sauer, R. T. (2000) Nat. Struct. Biol. 6, 449–455
6. Saguy, M., Gillet, R., Metzinger, L., and Felden, B. (2005) Biochimie (Paris) 87, 897–903
7. Valle, M., Gillet, R., Kaur, S., Henne, A., Ramakrishnan, V., and Frank, J. (2003) Science 300, 127–130
8. Kaur, S., Gillet, R., Li, W., Gursky, R., and Frank, J. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 16484–16489
9. Wower, J., Zwieb, C. W., Hoffman, D. W., and Wower, I. K. (2002) Biochemistry 41, 8826–8836
10. Hallier, M., Desreac, J., and Felden, B. (2006) Nucleic Acids Res. 34, 1955–1963
11. Hanawa-Suetsugu, K., Takagi, M., Inokuchi, H., Himeno, H., and Muto, A. (2002) Nucleic Acids Res. 30, 1620–1629
12. Shimizu, Y., and Ueda, T. (2002) FEBS Lett. 514, 74–77
13. Hallier, M., Ivanova, N., Rametti, A., Pavlov, M., Ehrenberg, M., and Felden, B. (2004) I. Biol. Chem. 279, 25978–25985
14. Clemons, W. M., Jr., Brodersen, D. E., McCutcheon, J. P., May, J. L., Carter, A. P., Morgan-Warren, R. J., Wimberly, B. T., and Ramakrishnan, V. (2001) J. Mol. Biol. 310, 827–843
15. Frank, J. (2006) Three-dimensional Electron Microscopy of Macromolecular Assemblies, Oxford University Press, New York
16. Gabashvili, I. S., Agrawal, R. K., Spahn, C. M., Grassucci, R. A., Svergun, D. I., Frank, J., and Penczek, P. (2000) Cell 100, 537–549
17. Carson, M. (1991) J. Appl. Crystallogr. 24, 103–106
18. Ogle, J. M., Murphy, F. V., Tarry, M. J., and Ramakrishnan, V. (2002) Cell 111, 712–732
19. Dong, G., Nowakowski, J., and Hoffman, D. W. (2002) EMBO J. 21, 1845–1854
20. Cochella, L., and Green, R. (2005) Science 308, 1178–1180
21. Metzinger, L., Hallier, M., and Felden, B. (2005) Nucleic Acids Res. 33, 2384–2394
22. McGinness, K. E., and Sauer, R. T. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 13454–13459
23. Okada, T., Wower, I. K., Wower, J., Zwieb, C. W., and Kimura, M. (2004) Biosci. Biotechnol. Biochem. 68, 2319–2325
24. Felden, B., Himeno, H., Muto, A., McCutcheon, J. P., Atkins, J. F., and Gesteland, R. F. (1997) RNA (Cold Spring Harbor) 3, 89–103
25. Sengupta, J., Agrawal, R. K., and Frank, J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 11991–11996
26. Wower, I. K., Zwieb, C. W., Guven, S. A., and Wower, J. (2000) EMBO J. 19, 6612–6621
27. Bordeau, V., and Felden, B. (2002) Biochimie (Paris) 84, 723–729
28. Maivali, U., and Remme, J. (2004) RNA (N. Y.) 10, 600–604
29. Ivanova, N., Pavlov, M. Y., Bouaziz, E., Ehrenberg, M., and Schiavone, L. H. (2005) Nucleic Acids Res. 33, 3529–3539
30. Keiler, K., Shapiro, L., and Williams, K. P. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7778–7783