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How a circularized tmRNA moves through the ribosome

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Abstract

During *trans*-translation, transfer-messenger RNA (tmRNA) and small protein B (SmpB), together rescue ribosomes stalled on a truncated mRNA, and tag the nascent polypeptide for degradation. We used electron cryo-microscopy to determine structures of three key states of the tmRNA-SmpB-ribosome complex during *trans*-translation at 3.7–4.4 Å resolutions. The results show how tmRNA and SmpB act specifically on stalled ribosomes and how the circularized complex moves through the ribosome enabling translation to switch from the old defective message to the reading frame on tmRNA.

Ribosomes that reach the 3' end of a messenger RNA (mRNA) without terminating at a stop codon stall in an unproductive state, forming “non-stop” translation complexes (1). In bacteria, these ribosomes are primarily rescued by *trans*-translation (2), without which the accumulation of non-stop translation complexes is inevitable and lethal (3–5).

Previous studies have described the roles of transfer-messenger RNA (tmRNA) and its binding partner, small protein B (SmpB), in *trans*-translation (6, 7). As its name implies, tmRNA contains both a tRNA-like and an mRNA-like domain (TLD and MLD, respectively) (Fig. S1). The TLD resembles the acceptor arm of tRNA^{Ala}, which together with SmpB mimics a tRNA (7–9). tmRNA is charged with alanine (8) and binds elongation factor Tu (EF-Tu) for delivery to the ribosome (10, 11). After accommodation into the A site, the nascent peptide is transferred to the alanine on tmRNA through a peptidyl transferase reaction. Elongation factor G (EF-G) then translocates tmRNA-SmpB through the ribosome similarly to canonical translation (12). The ribosome swaps messages, abandoning the original mRNA and restarting translation on a reading frame in the MLD of tmRNA. Translation on tmRNA adds a polypeptide tag to the nascent chain and guarantees that the ribosome will terminate at a stop codon and be released. Despite previous low-resolution electron cryo-microscopy (cryo-EM) structures showing the overall position of

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Author contributions: C.D.R. cloned, expressed and purified all *trans*-translation components, prepared samples and performed electron cryo-microscopy data collection, processing, model building and analysis. Y.G. purified ribosomes and tRNA^{Ala}. C.D.R. and V.R. wrote the manuscript.

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Data and materials availability: Cryo-EM density maps have been deposited with the Electron Microscopy Data Bank (accession numbers EMD-4475, EMD-4476, EMD-4477, EMD-4478), and coordinates have been deposited with the Protein Data Bank (entry codes 6Q95, 6Q97, 6Q98, 6Q9A).

tmRNA-SmpB in and between the A and P sites (12, 13), an atomic-level understanding of *trans*-translation is still lacking.

Here, we have determined structures of three key *trans*-translation intermediates by cryo-EM: tmRNA with the TLD-SmpB in the A site, P site, and in a location past the E site of the 70S ribosome at 3.9, 4.4 and 3.7 Å resolution, respectively (Fig. S2, Table S1). Non-stop ribosomes were affinity-purified from an *E. coli in vitro* translation system via 3xFLAG affinity tags on their nascent chains. *Trans*-translation was initiated by adding alanyl-tmRNA-SmpB-EF-Tu-GTP to the non-stop ribosomes to trap tmRNA-SmpB in the A site. The state after translocation, with tmRNA in the P site, was trapped by including EF-G. The addition of Ala-tRNA^{Ala} with EF-G caused a second round of translocation to move tmRNA past the E site.

Full-length tmRNA-SmpB in the A site (Fig. 1A) binds a non-stop ribosome in two coordinated ways: (i) SmpB binds in the decoding center and mRNA channel, taking the place of a codon-anticodon interaction and downstream mRNA and (ii) pseudoknot 2 (PK2) and helix 5 (H5) of tmRNA bind the solvent side of the ribosome and crowd the entrance of the mRNA channel. SmpB is 'decoded' by the ribosome in a manner analogous but not identical to the usual codon-anticodon interaction in the A site. As in the pre-accommodated structure of the TLD-SmpB with EF-Tu trapped during delivery to the ribosome by Neubauer et al. (14), His136 of SmpB maintains a stacking interaction with G530. Additionally, the conserved aromatic residue, His22 of SmpB, stacks with A1493 (Fig. 1B). This observation agrees with earlier biochemical probing experiments, which suggest that the binding of SmpB in the A site can protect decoding center nucleotides (15). Unlike canonical decoding of tRNA, however, A1492 does not participate in decoding SmpB and remains only partially flipped out as in Neubauer et al. 2012 (14). Nevertheless, the small subunit is in a closed conformation that resembles that of canonical decoding.

SmpB also occupies part of the mRNA channel (Fig. S3A). Highly conserved, charged residues (Fig. S3B) in the tail of SmpB interact with the mRNA channel to position SmpB in the A site. In the structure of *E. coli* tmRNA-SmpB bound in the A site, the helix formed by the tail of SmpB is longer and shifted compared to that of the *T. thermophilus* pre-accommodated crystal structure (14) (Fig. 1C). The corresponding structure determined using entirely *T. thermophilus* components showed that the tail of SmpB remains in a nearly identical position after accommodation (Fig. 1C), thus arguing that the shift is due to differences between species.

An interaction between a single-stranded loop of RNA from PK2 and ribosomal protein uS3 anchors tmRNA to the small subunit (Fig. 1D). This is consistent with the binding position of tmRNA in previous EM studies (12, 13). The loop of PK2 is sandwiched in a pocket of a helix-turn-helix motif of uS3. In this way, PK2 binding may explain why the tail of SmpB is dispensable for initial binding to the ribosome (16).

Additionally, H5 of tmRNA crowds the entrance of the mRNA channel in close proximity with the tail of SmpB (Fig. 1D). Arginine 132 and 143 of protein uS3 make electrostatic interactions with the phosphate backbone of H5. Protein uS3 can similarly stabilize mRNA

on the solvent side of the ribosome (17–19). Biochemical evidence shows that *trans*-translation activity is reduced on ribosomes that contain mRNAs with 9 nucleotide extensions downstream from the P site codon (20). Such an extension corresponds to the distance at which the mRNA would begin to clash with H5 at the entrance of the mRNA channel (Fig. S3C). Binding of PK2 and H5 may therefore play a role in initial recognition of a non-stop translation complex.

During accommodation, tmRNA-SmpB undergoes a conformational change in two regions: (i) The highly conserved Gly132 in the tail of SmpB flexes to permit the body of SmpB to rotate into the A site, and (ii) the elbow region of tmRNA acts as a hinge around which the acceptor arm of the TLD swings into the peptidyl transferase center (PTC) (Fig. 1E, Fig. S4). These movements are analogous to the distortions of the anticodon stem loop (ASL) and elbow of canonical tRNA during accommodation (21).

After accommodation, the 3' CCA of tmRNA is positioned in the PTC and the nascent peptide is transferred to the alanine on tmRNA during peptidyl transfer. Consistent with this notion, density for the nascent peptide is seen in all three active *E. coli* structures (Fig. S5). After peptidyl transfer, EF-G translocates tmRNA-SmpB into the P site and the ribosome switches messages from mRNA to tmRNA (12) (Fig. 2A). The C-terminal tail of SmpB remains alpha-helical but flips to vacate the A site, binding the mRNA channel in the E site and anchoring tmRNA-SmpB in the P site (Fig. 2B). The highly conserved Gly132 facilitates flipping by again acting as a flexible joint between the body and tail of SmpB. In addition, H5 moves to unblock the entrance of the mRNA channel and permit the MLD to pass through (Fig. 2C). The movement of SmpB and H5 of tmRNA away from their original positions in the A site allows the MLD of tmRNA to occupy the mRNA channel. The MLD must pass through the A-site latch (also called 30S latch) to load into the mRNA channel. The A-site latch is a physical barrier formed by the contact of the head (helix 34) and body (guanosine 530) of the 16S rRNA when the small subunit is in a closed conformation. Ramrath et al. (12) suggest that passing through the A-site latch occurs via an extra large head movement that opens the latch during translocation.

Apart from loading the MLD into the mRNA channel, the reading frame within the MLD must also be positioned correctly for the ribosome to terminate at an in-frame stop codon. We can trace otherwise unassigned density leading from PK1 to SmpB, across the A site and out of the mRNA channel, suggesting that the MLD interacts with the beginning of the tail of SmpB (Fig. 2D, Fig. S5). This is consistent with biochemical evidence which suggests that the five nucleotides upstream of the “resume” codon, the first codon on tmRNA to be decoded, are critical for positioning the reading frame (22, 23).

Ala-tRNA^{Ala} decodes the resume codon on the MLD in the A site, and EF-G then translocates peptidyl-tRNA^{Ala} into the P site, consequently forcing tmRNA-SmpB toward the E site. Unexpectedly, tmRNA-SmpB does not mimic a tRNA bound in the E site. Instead, tmRNA-SmpB moves past the E site to the solvent side of the ribosome (Fig. 3A). Although it is formally possible that an intermediate E-site tmRNA-SmpB complex was skipped in the *in vitro trans*-translation system, superimposing tmRNA-SmpB from our

structure onto a model of canonical tRNA in the E site induces clashes with the ribosome that make a stable E-site intermediate unlikely (Fig. S6).

To complete loading into the mRNA channel, the MLD must pass through another latch, this time located at the E site. The E-site latch joins the head (protein uS7) and body (protein uS11 and guanosine 693 of 16S rRNA) of the small subunit. We see the MLD loaded into the mRNA channel after the second translocation step, analogous to the first (Fig. 3B). PK1 and H5 flank the single-stranded MLD, and density is seen running through the mRNA channel (Fig. S5). The position of H5 is approximately the same as that of tmRNA-SmpB occupying the P site, thus continuing to provide room for the MLD to exit the channel.

During movement of tmRNA-SmpB between all three states, the single stranded loop of RNA from PK2 remains bound to uS3, anchoring tmRNA to the solvent side of the small subunit (Fig. S5). In this way, PK2 acts as a hinge about which tmRNA bends and pivots (Fig. 3C). Indeed, the anchoring interactions of PK2 coordinate the different positions of H5 seen during *trans*-translation and limit the position of tmRNA on the solvent side of the ribosome after the second translocation event. PK2 is highly conserved (2) and its interactions with uS3 may therefore represent a general function of tmRNA.

As tmRNA-SmpB moves through the ribosome, SmpB first binds in the space subsequently occupied by the MLD following translocation. Thus, SmpB identifies legitimate non-stop ribosomes by verifying that the mRNA channel is empty, and then vacates the space to make way for the MLD. Loading is necessarily mediated via a looping mechanism that passes through two latches, one during each translocation event (Fig. 4). Although the tmRNA here is a single, circularized molecule, this mechanism is likely applicable for two-piece tmRNA as well, since large secondary structures flank the MLD in many bacteria (24). This work shows how two translocation events move tmRNA-SmpB through the ribosome, resulting in complete loading of the MLD into the mRNA channel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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One Sentence Summary

Cryo-EM studies of key *trans*-translation intermediates reveal new mechanistic insight into the rescue of stalled ribosomes.

Fig. 1. tmRNA-SmpB accommodates into the A-site.

(A) Overview of the ribosomal complex with tmRNA-SmpB occupying the A site of a non-stop ribosome. (B) Aromatic residues of SmpB (teal) interact with decoding center nucleotides (gold). (C) Global superposition of ribosomes, showing the tail of SmpB from *E. coli* and *T. thermophilus* bound in the A site (blue vs. purple, respectively) and *T. thermophilus* SmpB bound in a pre-accommodated state [gray, PDB code 4V8Q (14)]. (D) A single stranded RNA loop from PK2 of tmRNA binds protein uS3 and arginine residues of protein uS3 interact with the phosphate backbone of H5 of tmRNA. (E) Global

superimposition of TLD of tmRNA bound to SmpB (left) in the A site (colored) or pre-accommodated (gray) compared to canonical tRNA (right) in the A site (purple) or pre-accommodated (gray).

Fig. 2. tmRNA-SmpB is translocated into the P site of the ribosome.

(A) Overview of the ribosomal complex with tmRNA-SmpB bound in the P site and the MLD occupying the A site. (B) Comparison of SmpB occupying the A site (gray) versus P site (blue). The tail of SmpB flips into the E site, anchoring tmRNA-SmpB in the P site. The conserved glycine residue at the junction between the body and the tail of SmpB is highlighted (red). (C) Helix 5 of tmRNA changes position from the A site complex (gray) to the P site complex (red), allowing the MLD to pass through the space previously occupied by the tail of SmpB. (D) The MLD contacts the junction of the body and tail of SmpB to set the tag reading frame correctly in the A site. The MLD has passed through the A-site latch (inset) when tmRNA-SmpB occupies the P site.

Fig. 3. tmRNA-SmpB is translocated from the P site past the E site.

(A) Overview of the ribosomal complex with tmRNA-SmpB bound on the solvent side of the E site after passing through the ribosome. Canonical tRNA (teal) bound to the resume codon occupies the P site. (B) Density for PK1, the MLD going through the mRNA channel, and H5 shows the MLD fully loaded into the mRNA channel. During the second translocation, the MLD passes through a second latch, this time in the E site (inset). (C) PK2 is anchored to protein uS3, acting as a flexible hinge point during the movement of tmRNA through the ribosome.

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reaction center (PTC) where it joins with the nascent peptide. PK2 interacts with protein uS3, binding tmRNA to the outside of the ribosome and coordinating the position of tmRNA as it moves through the ribosome. (4) Elongation factor G (EF-G) translocates tmRNA-SmpB from the A site into the P site and expels the original mRNA and tRNA. (5) During translocation, the MLD passes through the A-site latch and into the space in the mRNA channel previously occupied by the tail of SmpB. The tail of SmpB flips to the opposite side of the mRNA channel, binding in the E site. (6) Ala-tRNA^{Ala} decodes the first codon of the reading frame of tmRNA (the “resume” codon) and (7) a peptidyl transferase reaction transfers the peptide from tmRNA to tRNA^{Ala}. (8) EF-G translocates the peptidyl-tRNA^{Ala} into the P site and consequently shifts tmRNA-SmpB (9) past the E site to the solvent side of the ribosome. During this second translocation event, the MLD is again loaded into the mRNA channel through a latch, this time at the E site. The MLD is fully loaded into the mRNA channel and translation continues until terminating at a stop codon at the end of the reading frame. (10) The ribosome is released and the peptide is targeted for degradation by proteases that recognize the polypeptide tag.