Resolving Nonstop Translation Complexes Is a Matter of Life or Death

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Problems during gene expression can result in a ribosome that has translated to the 3' end of an mRNA without terminating at a stop codon, forming a nonstop translation complex. The nonstop translation complex contains a ribosome with the mRNA and peptidyl-tRNA engaged, but because there is no codon in the A site, the ribosome cannot elongate or terminate the nascent chain. Recent work has illuminated the importance of resolving these nonstop complexes in bacteria. Transfer-messenger RNA (tmRNA)-SmpB specifically recognizes and resolves nonstop translation complexes in a reaction known as trans-translation. trans-Translation releases the ribosome and promotes degradation of the incomplete nascent polypeptide and problematic mRNA. tmRNA and SmpB have been found in all bacteria and are essential in some species. However, other bacteria can live without trans-translation because they have one of the alternative release factors, ArfA or ArfB. ArfA recruits RF2 to nonstop translation complexes to promote hydrolysis of the peptidyl-tRNAs. ArfB recognizes nonstop translation complexes in a manner similar to tmRNA-SmpB recognition and directly hydrolyzes the peptidyl-tRNAs to release the stalled ribosomes. Genetic studies indicate that most or all species require at least one mechanism to resolve nonstop translation complexes. Consistent with such a requirement, small molecules that inhibit resolution of nonstop translation complexes have broad-spectrum antibacterial activity. These results suggest that resolving nonstop translation complexes is a matter of life or death for bacteria.

Bacteria perform transcription and translation in the same cellular compartment because they do not have nuclei. One advantage to this arrangement is that bacteria can rapidly respond to environmental challenges by producing new proteins. The time between transcription of a gene and the availability of the corresponding protein is minimized because the mRNA does not have to be processed or exported, and translation of an mRNA can initiate before transcription is complete. However, using a single compartment for transcription and translation has serious consequences for protein quality control because there are limited opportunities for mRNA proofreading. Mechanisms used by eukaryotes to ensure that the mRNA is intact are generally absent in bacteria. For example, in eukaryotes, 3' polyadenylation is used as a signal that the mRNA transcript is complete. This signal is read at several steps, including nuclear export and translation initiation, which requires interaction between poly(A)-binding proteins and translation initiation factors (1, 2). In contrast, the bacterial ribosome does not require any information from the 3' end of the mRNA to initiate translation, so there is no assurance that the mRNA is complete or intact (3). mRNAs can be truncated by many events, including premature termination of transcription, nuclease activity, and physical damage. As a consequence, bacterial ribosomes frequently translate mRNAs that do not have a stop codon ("nonstop" mRNAs). When a ribosome reaches the 3' end of a nonstop mRNA, it is trapped in a nonstop translation complex. In this complex, the mRNA and peptidyl-tRNA in the P site prevent dissociation of the ribosome, but the complex cannot elongate or terminate because there is no codon in the A site. A nonstop complex can also be formed when a ribosome stalls during translation and the mRNA is cleaved in the A site (4–6). Estimates from Escherichia coli suggest that 2% to 4% of translation reactions end in a nonstop translation complex (7). At that rate, an average ribosome is involved in ~5 nonstop translation complexes per cell division cycle. Clearly, the protein synthesis capacity of the cell would be severely compromised if these complexes could not be quickly resolved. To cope with the prevalence of nonstop translation complexes, bacteria have a remarkable mechanism known as trans-translation, which can release the ribosome and target the nonstop mRNA and nascent polypeptide for rapid degradation.

RESOLUTION OF NONSTOP TRANSLATION COMPLEXES BY tmRNA-SmpB

trans-Translation is performed by a ribonucleoprotein complex consisting of transfer-messenger RNA (tmRNA), a specialized RNA molecule, and SmpB, a small protein. tmRNA has elements of both a tRNA and an mRNA. The 5' and 3' ends of tmRNA form a structure resembling the acceptor arm and TΨC arm of alanyl-tRNA (8, 9). The remainder of tmRNA includes several pseudoknots and a specialized reading frame that is decoded during trans-translation (8, 10–12). SmpB binds tightly with tmRNA and completes the tRNA-like structure by mimicking the anticodon stem (13–15). The acceptor arm of tmRNA is charged with alanine by alanyl-tRNA synthetase and bound by EF-Tu in the same manner as tRNAAla (8, 16, 17). During trans-translation, tmRNA-SmpB specifically recognizes a nonstop translation complex and is accommodated in the ribosomal A site (Fig. 1) (18–21). The nascent polypeptide is transferred to the alanine charged to tmRNA, and SmpB-tmRNA is translocated to the P site. During translocation, a large swivel of the 30S head of the ribosome allows the reading frame of tmRNA to enter the mRNA channel (22). The first codon of the tmRNA reading frame is aligned in the A site, and translation resumes using the tmRNA reading frame as a mes-

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Correct alignment of tmRNA in the mRNA channel requires sequence-specific contacts between tmRNA and SmpB (23). Translation of the tmRNA reading frame terminates at a stop codon, releasing the ribosome and a protein that includes the tmRNA-encoded peptide tag at the C terminus (24). The peptide tag is recognized by multiple proteases in the cell, ensuring that the protein is rapidly degraded (24–28). The nonstop mRNA is also targeted for degradation during trans-translation (29–31). Thus, the overall effect of the reaction is to remove the problematic mRNA and the incomplete protein and to release the ribosome (Fig. 1).

A crystal structure from Neubauer et al. captures an early step of trans-translation and shows how tmRNA-SmpB recognizes nonstop translation complexes (32) (Fig. 2). In the structure, the...
tRNA-like domain of tmRNA, bound with SmpB and EF-Tu, is trapped in the A site of a nonstop translation complex during accommodation using the drug kirromycin. Overall, the structure resembles an elongation complex with tmRNA-SmpB in place of the acylated tRNA. The acceptor arm of tmRNA is in the same orientation as the acceptor arm of the acylated tRNA, and SmpB takes the place of the anticodon stem. However, SmpB also makes contacts in the decoding center and empty mRNA channel that appear to mimic the missing mRNA. The 16S rRNA residues A1492, A1493, and G530, which interact with the mRNA in an elongation complex, directly contact SmpB in the nonstop complex. In addition, the C terminus of SmpB forms a helix that extends into the empty mRNA channel between the decoding center and the leading edge of the ribosome. Chemical footprinting and mutational studies support the hypothesis of the presence of these interactions during trans-translation (33, 34). This crystal structure suggests that tmRNA-SmpB could not be accommodated in elongating ribosomes because the mRNA would obstruct SmpB interactions with the 16S rRNA (Fig. 2). Consistent with this model, competition experiments show that tmRNA-SmpB does not interfere with translation elongation or termination in vivo (35).

Whereas the crystal structure suggests that the mRNA channel downstream of the A site must be empty for tmRNA-SmpB to bind, kinetic data indicate that the mRNA channel does not always have to be empty for trans-translation to occur. The rate of trans-translation in vitro was measured using ribosomes stalled on mRNAs of different lengths (36). When the ribosomes were stalled with the mRNA channel completely occupied (with >15 nucleotides downstream of the P site), the reaction was extremely slow, consistent with the mRNA blocking tmRNA-SmpB. However, the reaction was rapid when the ribosomes were stalled with 0 to 6 nucleotides of mRNA downstream of the P site and was inhibited only partially with 9 to 12 nucleotides downstream of the P site. These results imply that mRNA in the A site, and even several codons downstream of the A site, does not interfere with trans-translation. The substrates used for the kinetic measurements were generated by omitting a tRNA from the reaction, so they probably do not occur frequently in vivo. However, the issue of whether tmRNA-SmpB can act on ribosomes with mRNA extending past the A site has important implications for the mechanism of trans-translation. It is possible that the interactions between SmpB and 16S rRNA observed in the crystal structure represent the lowest energy conformation, but these interactions are not required for tmRNA-SmpB to initiate trans-translation. Alternatively, when a ribosome stalls on an mRNA that does not completely fill the mRNA channel, it might undergo a structural change that allows SmpB access to the 16S rRNA. For example, the 3′ end of the mRNA might loop out of the A site, or the ribosome could slide to the 3′ end of the mRNA, leaving the A site empty. Such rearrangements could be facilitated by communication between the mRNA channel and the decoding center of the ribosome. Further biochemical experiments are required to determine whether trans-translation always requires an empty mRNA channel.

**SUBSTRATES FOR trans-TRANSLATION**

Some of the known substrates for trans-translation are consistent with nonstop translation complexes generated by mRNA damage, but others suggest nonrandom or intentional mRNA cleavage to target translation reactions to trans-translation. Truncation of mRNA by premature termination of transcription, damage to the mRNA, or 3′-5′ exonucleolytic mRNA turnover would be expected to be largely random and should produce nonstop translation complexes at a variety of positions along many mRNAs. Two proteomic-analysis-scale studies identified proteins tagged by trans-translation in Caulobacter crescentus and Francisella tularensis. Both studies found that many proteins are tagged and that tagging occurs at locations throughout the protein sequence, as would be expected for activity on damaged mRNAs (37, 38).

On the other hand, investigation of *E. coli* proteins that are tagged with high frequency indicates that there are some sequences prone to generation of nonstop translation complexes (39). For example, in some substrates, tagging occurs with high frequency after runs of rare codons or highly inefficient translation termination sequences (40-42). The mRNA is initially complete in these cases, but ribosome stalling during translation elongation or termination exposes the downstream mRNA to exonucleases, which chew back the mRNA to the leading edge of the ribosome to generate substrates for trans-translation (4, 43-45). Exonuclease activity by RNase II can promote cleavage of the mRNA in the A site through an unknown mechanism, but RNase II and the corresponding A-site cleavage are not essential for trans-translation on known substrates (46, 47). Redundant nuclease activities may ensure that translation complexes stalled for an extended time are targeted for resolution by trans-translation.

In addition to ribosome stalling, errors during translation can lead to trans-translation. Suppressor tRNAs and drugs that promote miscoding increase the number of proteins tagged by trans-translation, demonstrating that readthrough of the stop codon and frameshifting can result in nonstop translation complexes when there is not an in-frame stop codon downstream (48, 49). The examples described above all result in nonproductive translation complexes, which could sequester ribosomes and limit new protein synthesis. The main purpose of trans-translation on these substrates is likely to be release of the ribosomes to maintain protein synthesis capacity.

There is also evidence that trans-translation is used to ensure the quality of the protein pool. Trans-Translation increases on large proteins when dnaK is deleted, suggesting that misfolding of the nascent polypeptide might trigger mRNA cleavage to target the nascent polypeptide for proteolysis (50). It is now clear that interactions of the nascent chain in the peptide exit tunnel and communication between ribosome-associated chaperones and the catalytic center of the ribosome can affect the rate of translation (51, 52). Terminally misfolded nascent proteins might be targeted to trans-translation to ensure that they are rapidly degraded. It is not yet known whether there is a dedicated pathway for generating nonstop complexes that is triggered by misfolding or whether misfolding slows elongation enough to expose the mRNA to nonspecific exonuclease activity.

Finally, trans-translation is used intentionally as part of several regulatory circuits. RNase toxin components of toxin-antitoxin systems such as RelE and MazF cut most mRNAs in the cell, generating a large number of nonstop mRNAs and nonstop translation complexes (53, 54). Toxin activity is used to induce stasis, allowing the cell to conserve resources during severe stress (53, 54). Toxins are also activated in a small percentage of cells under optimal growth conditions to generate persister cells that can survive sudden stresses (55). *E. coli* mutants lacking trans-translation...
activity are defective in recovery from toxin-induced stasis, indicating that resolution of the nonstop translation complexes resulting from toxin activity is important for resuming growth after severe nutritional stress or persistence (56, 57). Individual proteins are also targeted for trans-translation through truncation of the cognate mRNAs. Nuclease cleavage sites or transcriptional terminators 5′ of the stop codon have been found in some arfA and kinA genes (58–60). Translation of these genes results in proteins that are rapidly degraded unless trans-translation is impaired, making the encoded protein activity dependent on the state of trans-translation. The arfA example is described in more detail below. trans-Translation is used by LacI in E. coli to prevent excess protein accumulation (61). At high concentrations, LacI binds within the 3′ end of its own gene. LacI binding to this site blocks transcription elongation and generates a nonstop mRNA, thereby targeting all newly expressed LacI for proteolysis. The use of trans-translation in regulatory circuits may be important for individual species or behaviors, but the evolutionary conservation of trans-translation is almost certainly due to the ability to maintain the protein synthesis capacity of the cell.

PHYSIOLOGY OF AND ALTERNATIVES TO trans-TRANSLATION

Genes encoding tmRNA (ssrA) and SmpB (smpB) have been identified in all sequenced bacterial species, including those with severely reduced genomes (62). This conservation suggests that trans-translation confers a selective advantage in all environments that support bacterial life. In fact, tmRNA and SmpB have been shown to be essential in several species, including Neisseria gonorrhoeae, Shigella flexneri, Helicobacter pylori, and Mycobacterium tuberculosis (63–66). Saturating genome-wide mutagenesis experiments suggest that tmRNA and SmpB are also required for viability in Haemophilus influenzae, Mycoplasma genitalium, and Staphylococcus aureus (67–69). In other bacteria, tmRNA can be deleted with widely varying consequences. In some species, phenotypes of mutants lacking trans-translation activity are severe, including defects in virulence (Salmonella enterica, Yersinia pestis, Francisella tularensis, and Streptococcus pneumoniae), symbiosis (Bradyrhizobium japonicum), and cell cycle control (C. crescentus) (38, 70–75). However, E. coli and Bacillus subtilis mutants that lack trans-translation have relatively mild phenotypes, such as increased antibiotic susceptibility and stress response defects (48, 76–78). Recent discoveries have shown that most or all species that do not require trans-translation have backup systems that resolve nonstop translation complexes when trans-translation activity is not available.

ArfA

On the basis of the evolutionary conservation of trans-translation and the differences in phenotypes between E. coli and species in which tmRNA is essential, Chadani and coworkers performed a screen for genes that are essential in strains deleted for ssrA (79). They identified a single gene, arfA, and showed that the ArfA protein can promote hydrolysis of peptidyl-tRNA on nonstop translation complexes in an in vitro translation reaction. Release of the ribosomes by ArfA requires RF2, suggesting that ArfA recognizes the empty mRNA channel and recruits RF2 to hydrolyze the peptidyl-tRNA (Fig. 1) (80). However, it is not yet clear how ArfA recognizes nonstop translation complexes.

ArfA is a true backup system for trans-translation in that it is active only when trans-translation activity is not available. The arfA mRNA in E. coli includes a cleavage site for RNase III before the stop codon and is efficiently cut by RNase III to produce a nonstop mRNA (58). Translation of arfA when trans-translation is active results in a tagged ArfA protein that is rapidly degraded. When ssrA is deleted, stable and active ArfA protein is produced. Presumably, regulation by trans-translation allows ArfA to release nonstop complexes only under physiological conditions where trans-translation is inactive or saturated. Most arfA genes from other species encode the RNase III cleavage site, but some use a transcriptional terminator before the stop codon to produce a nonstop mRNA (60). Thus, regulation of ArfA by trans-translation is conserved even though the mechanism for producing the nonstop mRNA is not.

Genetic experiments with arfA suggest that release of ribosomes from nonstop translation complexes is essential in E. coli and related species. In E. coli, deletion of arfA and ssrA is synthetically lethal (79). In contrast, ssrA is essential in S. flexneri, which does not have arfA, but ssrA can be deleted in S. flexneri cells that are engineered to express E. coli arfA (64). arfA genes have been identified in only a subset of beta- and gammaproteobacteria and a few other species (60). However, the small size of arfA makes bioinformatic identification in distantly related bacteria difficult. The presence of arfA does not ensure that trans-translation is dispensable. N. gonorrhoeae has an arfA gene, and yet trans-translation is essential. The N. gonorrhoeae arfA is active when expressed in E. coli (60), so either arfA is not expressed in N. gonorrhoeae or its activity is not sufficient to support viability in the absence of trans-translation.

ArfB

A second alternative system, ArfB, was discovered in a multicopy suppressor screen for genes that allowed E. coli to survive without tmRNA or ArfA (Fig. 1) (81). Peptidyl-tRNA hydrolase (Pth) activity had been predicted for ArfB on the basis of the presence of a GGQ motif common to release factors and peptidyl-tRNA hydrolases (82). In fact, purified ArfB specifically hydrolyzes peptidyl-tRNA in nonstop translation complexes in vitro (81, 83). Structural studies show that ArfB recognizes nonstop complexes in a manner similar to that of SmpB-tmRNA: a C-terminal helix of ArfB extends into the empty mRNA channel, and residues in this helix make contacts with 16S rRNA that are important for activity (Fig. 2) (84, 85). The physiological role of ArfB in E. coli is not clear. The chromosomal copy of arfB will not support growth of E. coli in the absence of tmRNA and ArfA, and ssrA is essential in S. flexneri even though arfB is present (64, 79). Either ArfB is reserved for special conditions in these species or the availability of ArfA has made ArfB redundant and control of its expression has been lost. In contrast, ArfB in Caulobacter crescentus is functional in its chromosomal context and allows cells to survive without trans-translation. The C. crescentus arfB gene was identified in transposon sequencing (Tn-Seq) experiments as a gene that is essential in cells lacking ssrA but not in wild-type cells (H. A. Feaga and K. C. Keiler, unpublished data). ArfB homologs are widely distributed throughout bacterial species. No regulation of ArfB by trans-translation has been identified, so, unlike ArfA, ArfB may provide a constitutive, low level of resolution activity that becomes significant only when trans-translation is saturated or inactivated.

Mitochondria also have an ArfB homolog, which is named
ICT1 (85, 87). ICT1 hydrolyzes peptidyl-tRNA on the ribosome, and this activity is essential for human cells (87). ArfB and ICT1 both contain an N-terminal GGQ motif and a C-terminal R(X3)K(X6)K(X2)R motif that are required for peptidyl-tRNA hydrolyase activity (85). As in bacteria, transcription and translation are performed in a single compartment in mitochondria, so ICT1 may serve to release nonstop complexes and maintain protein synthesis capacity in these organelles. tmRNA has been identified in organelles of some primitive eukaryotes but is not retained in metazoans (62, 88). It appears that most eukaryotic mitochondria kept ArfB and dispensed with trans-translation, whereas all bacteria retained trans-translation.

The discoveries of ArfA and ArfB have important implications for understanding the role of trans-translation and the consequences of nonstop translation complexes. With the exception of B. subtilis and F. tularensis, all species in which ssrA or smpB has been deleted encode either ArfA or ArfB (Fig. 3). Moreover, in all cases that have been tested, the ArfA or ArfB backup system becomes essential when ssrA is deleted. Therefore, at least one mechanism to resolve nonstop complexes may be required for viability in most or all bacteria. Investigation of unknown alternative resolution mechanisms in B. subtilis and F. tularensis would test how universal this requirement is. Some nonstop translation complexes may be resolved by “drop-off,” dissociation of the peptidyl-tRNA from the ribosome followed by hydrolysis of the free peptidyl-tRNA by peptidyl-tRNA hydrolase (Pth). Drop-off occurs with some nascent chains of two to five amino acids, but longer chains have not been shown to dissociate without prior peptidyl-tRNA hydrolysis within the ribosome (89, 90). Interactions between the nascent polypeptide and the exit channel may prevent drop-off in most cases. The discoveries of ArfA and ArfB make it clear that drop-off alone cannot support viability for most species in the absence of trans-translation.

Why is it that all bacteria use trans-translation to resolve non-stop complexes, and some use only trans-translation, but none use only ArfA or ArfB? ArfA and ArfB do not completely mimic trans-translation, because they do not directly target the nascent polypeptide for proteolysis. Presumably, incomplete proteins released by ArfA or ArfB activity must be recognized and degraded by other proteolytic pathways in the cell. The fate of the mRNA during ArfA and ArfB activity is not yet known. It is likely that trans-translation is the preferred pathway because it promotes degradation of the incomplete proteins and damaged mRNAs from non-stop complexes in addition to releasing the stalled ribosomes.

**TARGETING trans-TRANSLATION FOR ANTIBIOTICS**

The trans-translation pathway is an attractive target for development of new antibiotics because it is required for viability or vir-
High-throughput screening assay to identify trans-translation inhibitors. The reporter contains a gene encoding luciferase with a strong translation terminator inserted before the stop codon, such that transcription results in a nonstop mRNA. E. coli cells containing the reporter were screened in high-throughput format to identify compounds that inhibit trans-translation. When no inhibitor is present, translation of the nonstop mRNA results in trans-translation followed by proteolysis of luciferase, and cells produce no luminescence. Conversely, active luciferase is produced when a trans-translation inhibitor is present, resulting in luminescence.

ullence in many pathogenic strains and is not found in metazoans. Therefore, compounds that specifically inhibit trans-translation and not translation are likely to be effective for treating infections and yet have low toxicity for host cells. Compounds that inhibit trans-translation should kill trans-translation and yet have low toxicity for host cells. Compounds that inhibit and not translation are likely to be effective for treating infections trans-translation followed by proteolysis of luciferase, and cells produce no luminescence. Conversely, active luciferase is produced when a trans-translation inhibitor is present, resulting in luminescence.

FIG 4 High-throughput screening assay to identify trans-translation inhibitors. The reporter contains a gene encoding luciferase with a strong translation terminator inserted before the stop codon, such that transcription results in a nonstop mRNA. E. coli cells containing the reporter were screened in high-throughput format to identify compounds that inhibit trans-translation. When no inhibitor is present, translation of the nonstop mRNA results in trans-translation followed by proteolysis of luciferase, and cells produce no luminescence. Conversely, active luciferase is produced when a trans-translation inhibitor is present, resulting in luminescence.

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