

# The tmRNA System for Translational Surveillance and Ribosome Rescue

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Annu. Rev. Biochem. 2007. 76:101–24

First published online as a Review in Advance on January 2, 2007

The *Annual Review of Biochemistry* is online at [biochem.annualreviews.org](http://biochem.annualreviews.org)

This article's doi:  
10.1146/annurev.biochem.75.103004.142733

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0066-4154/07/0707-0101\$20.00

## Key Words

A-site cleavage, mRNA swapping, degradation of ssrA-tagged proteins, ribosome pausing and stalling

## Abstract

The tmRNA system performs translational surveillance and ribosome rescue in all eubacteria and some eukaryotic organelles. This system intervenes when ribosomes read to the 3' end of an mRNA or pause at internal codons with subsequent mRNA cleavage. A complex of alanyl-tmRNA (which functions as a tRNA and mRNA), SmpB protein, and EF-Tu·GTP binds stalled ribosomes, the nascent polypeptide is transferred to the alanine on tmRNA, and translation switches from the original message to a short tmRNA open reading frame (ORF) that encodes a degradation tag. Translation of the ORF and normal termination releases the tagged polypeptide for degradation and permits disassembly and recycling of ribosomal subunits for new rounds of protein synthesis. Structural and biochemical studies suggest mechanisms that keep tmRNA from interrupting normal translation and target ribosomes stalled with very short 3' mRNA extensions. Additional biological roles of tmRNA include stress management and the regulation of transcriptional circuits.

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## HISTORY

The tmRNA story started inconspicuously in 1978 with the observation of a band on an RNA gel (1). Although this molecule was present in *Escherichia coli* at roughly 10% of ribosomal RNA levels, its biological function remained a mystery for almost two decades (2). During this time, the sequence of the tmRNA gene (designated *ssrA*) was determined, the details of tmRNA transcript processing to a mature length of approximately 350 nucleotides were discovered, and some similarities between tmRNA and tRNA were established (3–6). For example, the 5' and 3' ends of tmRNA appeared to form a structure similar to the acceptor arm and T arm

of a tRNA, and tmRNA could be charged with alanine by alanyl-tRNA synthetase (4). The next functional clue emerged from studies showing that expression of a foreign protein in *E. coli* resulted in nested sets of truncated polypeptide fragments, each with the sequence AANDENYALAA at its C terminus (7). Remarkably, the last 10 residues of this “ssrA” tag sequence were encoded by a short open reading frame (ORF) in tmRNA. How were these unusual hybrid protein fragments produced? Splicing was considered as an obvious way to explain their genesis, but Northern blots revealed no spliced mRNAs (7).

The solution to this puzzle began with the observation that the C-terminal residues of

the *ssrA* tag were similar to sequences known to target *E. coli* proteins for degradation (2, 8). This finding suggested that tmRNA was part of a quality-control system, which in turn led to the idea that tmRNA might recognize ribosomes that were unable to finish protein synthesis and then act as a tRNA and an mRNA to provide an alternative mechanism for completing translation and marking the nascent polypeptide for degradation (2). For example, a stalled ribosome with an unoccupied A site could recruit alanine-charged tmRNA, which would initially function as a tRNA to transfer Ala to the nascent polypeptide. If translation then switched from the original message to the tmRNA ORF, the remaining portion of the *ssrA* tag would be added, and translation could terminate normally at a stop codon at the end of this ORF. The net results of these reactions would be to liberate the stalled ribosome and to target the tagged polypeptide for proteolytic destruction (**Figure 1**). This model was tested by engineering mRNAs without stop codons because ribosomes stall when they reach the 3' end of these nonstop messages. As predicted, the proteins expressed from nonstop mRNAs were untagged and stable in cells lacking tmRNA but terminated with AAN-DENYALAA and were rapidly degraded in cells containing tmRNA (2).

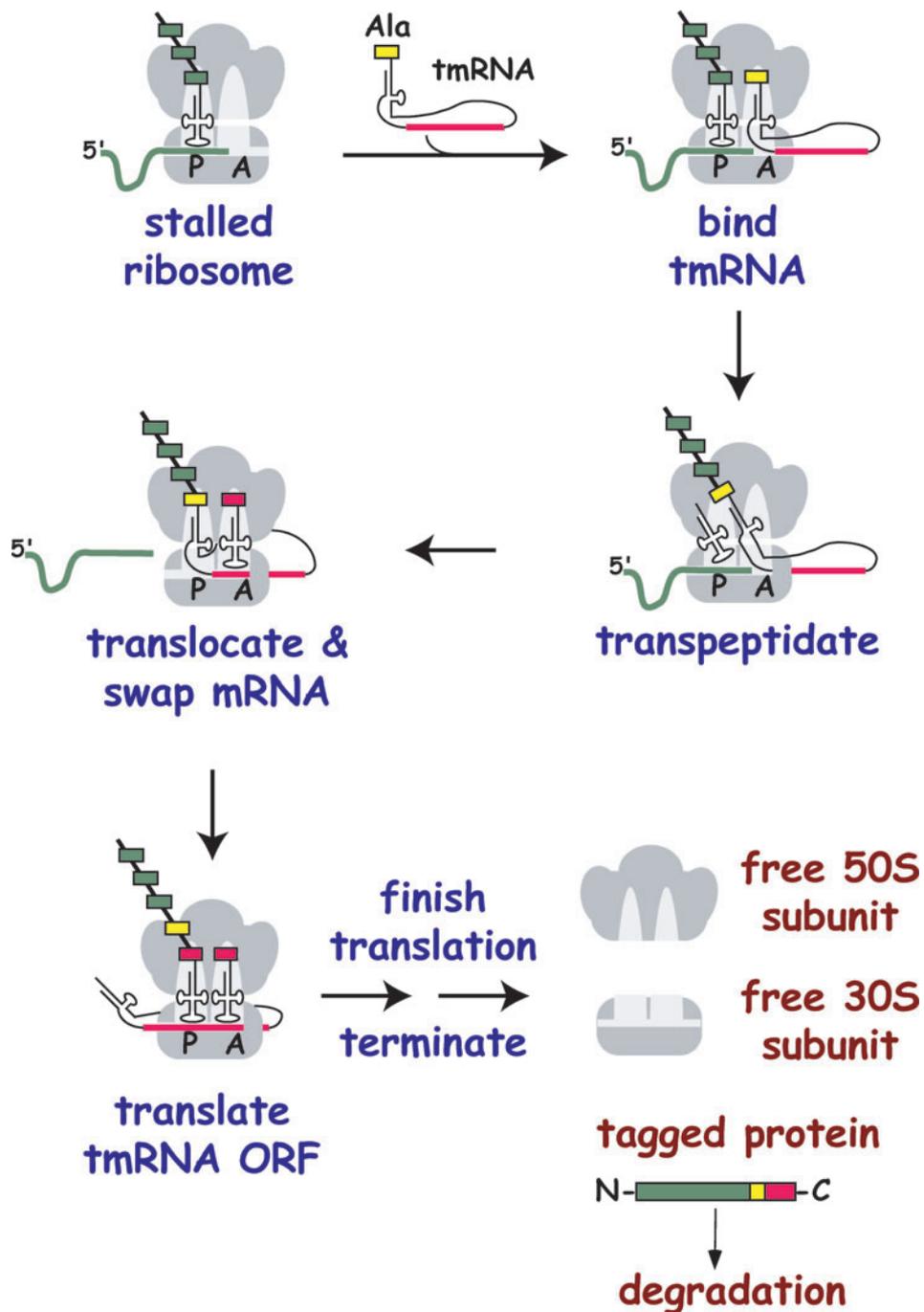
The general features of the original tmRNA model for translational quality control have now been supported by many additional studies. More importantly, this work has deepened our understanding of the underlying biochemical mechanisms and expanded our appreciation of the biological roles of tmRNA. For example, it is now known that tmRNA works in concert with SmpB (9), a dedicated protein partner, and cryo-EM (electron microscopy) studies have visualized a complex of SmpB, tmRNA, and EF-Tu entering the A site of a ribosome (10). Both the discovery of pausing-dependent mRNA cleavage and studies in vitro show that similar mechanisms mediate tmRNA recognition of all stalled ribosomes (11–13). Roles for tmRNA-

mediated protein degradation in the regulation of transcriptional circuits and cell-cycle timing have been elucidated (14–16). Moreover, “ribosome rescue” rather than targeted protein degradation has been shown to be the key function of tmRNA in many instances (17–20). Normal translation termination is an active process that requires a stop codon to recruit a release factor to the A site, where it catalyzes hydrolysis of the bond linking the P-site tRNA to the nascent polypeptide (21, 22). This event leads to disassembly and recycling of the 30S and 50S ribosomal subunits for new rounds of translation. When ribosomes stall, and neither continuation of translation nor normal termination are possible, tmRNA-mediated rescue allows otherwise trapped ribosomal subunits and the P-site tRNA to be returned to the active translation pool.

The last major tmRNA review was written in 2002 (23), and many advances have occurred in the interim. As a result, this is an excellent time to revisit this field. In the sections that follow, we discuss our current level of understanding of the structure and function of tmRNA and SmpB, potential mechanisms of translational switching and recognition of stalled ribosomes, and the multitude of ways in which tmRNA-mediated protein degradation and/or ribosome rescue are employed in biological systems.

## SYNTHESIS, PROCESSING, AND STABILITY

The tmRNA molecule begins life as a precursor, which needs to be processed before it becomes functional. In *E. coli*, for example, the primary transcript of 457 nucleotides is ultimately cleaved to a length of 363 bases by cellular ribonucleases (3, 4). Endonucleolytic and/or exonucleolytic removal of 5' and 3' nucleotides is necessary to create a tmRNA acceptor arm that can be charged with alanine. The site of tmRNA charging (as in tRNA) is the 3'-hydroxyl group of the terminal 5'-CCA-3' trinucleotide sequence. In certain



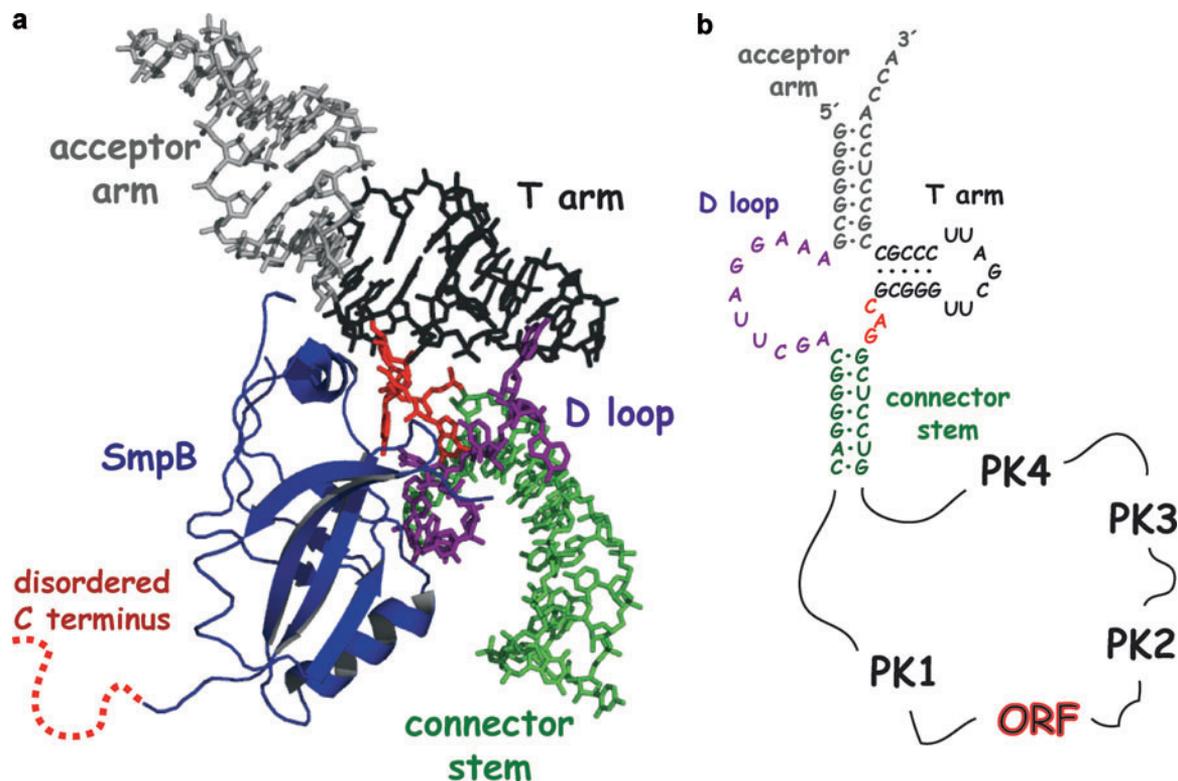
**Figure 1**

Model for tmRNA-mediated tagging and ribosome rescue (2). Alanyl-tmRNA recognizes ribosomes stalled at the end of an mRNA fragment and adds the alanine (yellow rectangle) to the C terminus of the nascent polypeptide chain. Following mRNA swapping, the tmRNA ORF (red) is translated, and RF1/RF2-mediated termination releases the tagged protein for degradation by cellular proteases and liberates the 30S and 50S subunits from the previously stalled ribosome for new rounds of protein synthesis.

bacteria (e.g., *Bacillus subtilis*), this CCA is added to the processed transcript by tRNA nucleotidyltransferase (5). Some tmRNA genes are circularly permuted, and an additional excision event during processing results in a two-piece tmRNA (24–26). In both one-piece and two-piece tmRNAs, however, the mature molecule consists of a tRNA-mimic domain, an ORF encoding the *ssrA* tag, and three or four pseudoknots (27–29). In *E. coli* tmRNA and many other tmRNAs, for example, the 5' portion of the tRNA-mimic domain is followed by pseudoknot 1, the

peptide-tag ORF, pseudoknots 2–4, and then the 3' portion of the tRNA-mimic domain (Figure 2).

Under typical intracellular conditions in *E. coli*, there are approximately 700 tmRNA molecules per cell, corresponding to one tmRNA for every 10–20 ribosomes (1, 30). A mature tmRNA is quite resistant to intracellular ribonuclease degradation, with a half-life that normally exceeds the doubling time of the bacterium (31). However, tmRNA stability depends on the presence of sufficient SmpB. When this tmRNA-binding protein is absent



**Figure 2**

tmRNA structure and SmpB binding. (a) A complex of *Aquifex aeolicus* SmpB (blue) and the tRNA-mimic domain of tmRNA (multicolored) is shown (37). SmpB contacts part of the D loop (purple) and a loop (red) linking the connector stem and T arm. The conformation of the acceptor arm (gray) is not observed in the cocrystal structure and was modeled from the structure of yeast tRNA<sup>Phe</sup> (37). (b) The linkage of the nucleotide sequence of the tRNA-mimic domain to the pseudoknots (PKs) and open reading frame (ORF) for a typical one-piece tmRNA is displayed. In both panels, only part of the complete “connector” region is shown.

or present at substoichiometric levels in *E. coli*, tmRNA is degraded faster, and its steady-state levels can drop fourfold or more (30–32). In *Caulobacter crescentus*, cell cycle-dependent changes in the rates of tmRNA transcription and degradation result in intracellular levels that peak at about 2000 tmRNA molecules per cell just before the onset of DNA replication and then decline rapidly (33). Intriguingly, a decrease in intracellular SmpB from roughly 2000 proteins per cell to undetectable levels is responsible for the increased rate of tmRNA degradation (34; K.C. Keiler, personal communication). In *B. subtilis*, tmRNA levels can increase as much as 10-fold in response to heat shock and other environmental insults because enhanced tmRNA transcription is a programmed response to cellular stress (19).

## tmRNA STRUCTURE AND FUNCTION

In the sections below, we discuss our current understanding of the tmRNA molecule and the structural and functional roles of its domains.

### tRNA-Mimic Domain

In one-piece tmRNAs, folding of the 5' and 3' ends creates the tRNA-mimic domain (4, 27, 28). This structure includes an acceptor arm, a T arm, and a D loop (**Figure 2**). A “connector” with several helical stems and intervening loops replaces the normal anticodon stem-loop of a conventional tRNA and links the tRNA-like domain to the rest of tmRNA. The tRNA-mimic domain is essential for tmRNA function. For example, mutations in the acceptor stem that prevent charging with alanine abrogate known tmRNA biological activities (4, 17–20, 35). The tRNA-mimic domain also contains the recognition sites for alanyl-tRNA synthetase, SmpB, and EF-Tu (4, 10, 32, 36, 37). A crystal structure of a complex with SmpB provides the highest resolution view of the tRNA-mimic domain, although

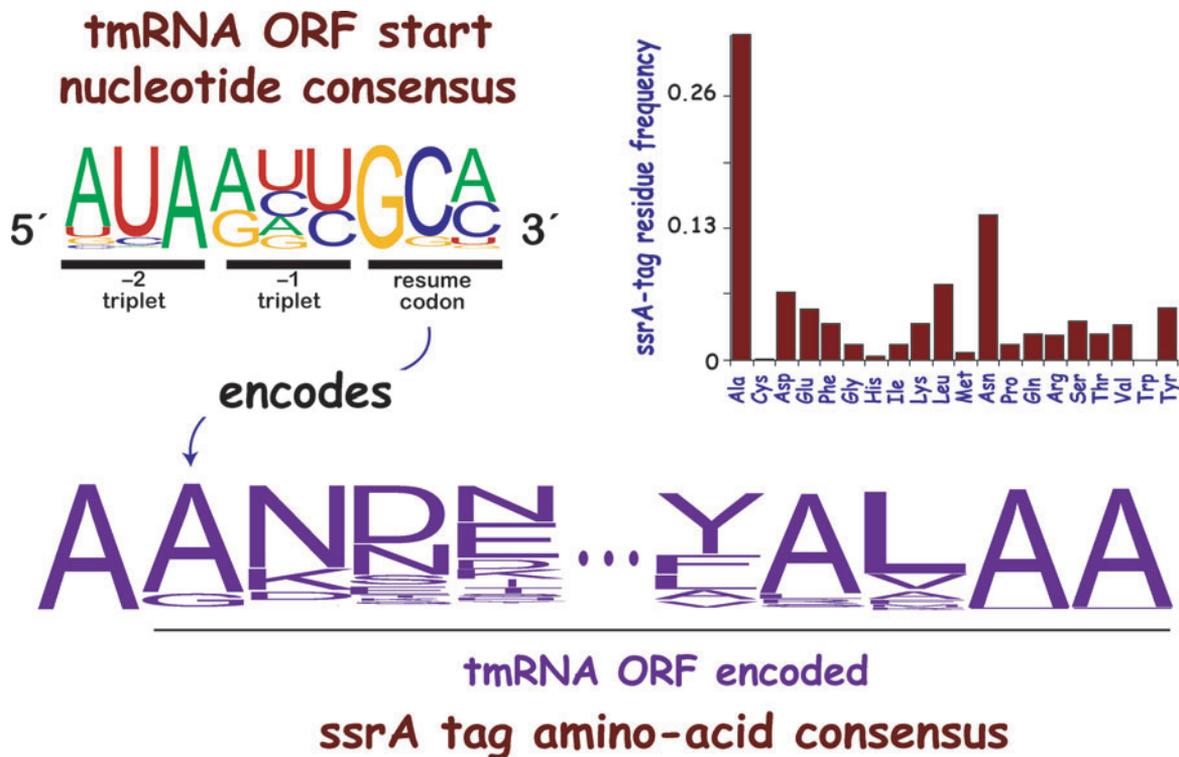
electron density for the acceptor arm and the 5' portion of the D loop is absent or poor (37). If the acceptor stem is modeled as a helical extension of the T stem, then the domain has an overall shape like a slightly bent arm with the D loop and T loop forming the elbow (**Figure 2**).

### Pseudoknots

The role of pseudoknots in tmRNA function is currently controversial. On the one hand, the fact that all tmRNAs seem to contain these structures supports the idea that they play a role in biological fitness. Indeed, mutations in pseudoknot 1, whose structure is known, can make tmRNA nonfunctional, and mutations in pseudoknots 2, 3, and 4 can reduce tagging activity in the cell (38–42). On the other hand, tagging or transpeptidation activity *in vitro* is observed for *E. coli* tmRNA variants that lack pseudoknots 1, 2, 3, and/or 4 (32, 40, 43). Moreover, complete randomization followed by an elegant genetic selection demonstrated that a simple hairpin could replace *E. coli* pseudoknot 1 and still support near wild-type function *in vivo* (44). Similarly, replacing pseudoknot 3 in *E. coli* tmRNA with an aptamer supports biological activity (45). These results suggest that replacing a tmRNA pseudoknot with single-stranded RNA or disrupting its structure is significantly more deleterious than replacing it with a folded element. Suggested roles for pseudoknots include aiding in overall tmRNA folding, slowing tmRNA degradation, maintaining the correct geometry for efficient translation switching to the tmRNA ORF, and serving as binding sites for proteins that facilitate tmRNA function.

### The Open Reading Frame of tmRNA

The mRNA-like ORF plays a critical role in tmRNA function. The sequence encoded by this ORF dictates whether tagged proteins are targeted for degradation by specific proteases (2, 46–49) (see below).



**Figure 3**

The histogram on the upper right shows the distribution of amino acids in the portion of the *ssrA* tag after the first uncoded residue. The panel on the upper left shows nucleotide conservation preceding the resume codon in tmRNA open reading frames (the height of each base is proportional to its occurrence in known tmRNAs). The bottom panel shows the most common amino acids at the first five positions and last five positions of known *ssrA* tags (the height of each residue symbol is proportional to its frequency of occurrence). Sequences were taken from the tmRNA website <http://www.indiana.edu/~tmrna/> (29). Sequence logos were prepared using WebLogo version 2.8.2 at <http://weblogo.berkeley.edu/> (138).

Moreover, translation of this ORF is required to allow normal termination of translation during ribosome rescue. Currently, 610 tag sequences are listed on the tmRNA website <http://www.indiana.edu/~tmrna/> (29). If the uncoded alanine is not counted, the most common length of these peptide segments is 10 residues (range 8 to 35 residues). The amino-acid composition of the encoded peptides is highly skewed. In a total of roughly 7500 tag residues, Ala (2472 residues) and Asn (1098 residues) are significantly over-represented, whereas Trp (1 residue) and Cys (12 residues) are very rare (Figure 3). As discussed below, at least part of this striking com-

positional bias is related to the role of the peptide tag in promoting degradation.

Tag sequences are probably also constrained by a need for ORF translation to be highly efficient, especially under conditions in which ribosomes stall frequently on other mRNAs. For example, because Cys and Trp are highly susceptible to oxidation (50), charging of tRNA<sup>Cys</sup> and tRNA<sup>Trp</sup> could be compromised during oxidative stress, explaining the scarcity of these amino acids in most tmRNA tags (Figure 3). Indeed, two of the other least common residues in the *ssrA* tag, His and Met, are also sensitive to oxidation and other modifications. Rare codons,

sense codons prone to misreading as nonsense codons, and codons prone to frame shifting are also likely to be used infrequently (51). Finally, the 3' codons of the tmRNA ORF form part of a stem-loop structure (27–29). Although mutations that disrupt this structure do not prevent tagging or ribosome rescue, recent studies suggest that the 3' bases of the ORF play a role in tmRNA-mediated degradation of rescued mRNAs (52) (see below).

## PROTEIN PARTNERS

All molecules needed for protein synthesis, for tmRNA synthesis and processing, and for degradation of *ssrA*-tagged proteins could be considered as a part of the tmRNA system. However, three proteins—alanyl-tRNA synthetase, EF-Tu, and SmpB—play direct and highly specific roles in tmRNA function. For example, because tmRNA variants that cannot be charged are biologically inactive, the synthetase-catalyzed charging reaction is functionally indispensable (4, 17–19, 35). Indeed, tmRNA-mediated tagging in vitro requires alanyl-tRNA synthetase or precharged alanyl-tmRNA (13, 53–55). Although tRNA<sup>Ala</sup> and tmRNA differ significantly in overall size and structure, alanyl-tRNA synthetase charges both molecules because simple determinants in their acceptor stems are sufficient for recognition (4, 56, 57). tmRNA can be charged by alanyl-tRNA synthetase alone, but an increase in aminoacylation is observed if SmpB is also present (32, 58, 59).

### EF-Tu, the G Protein Chaperone

The function of elongation factor Tu (EF-Tu) in normal translation (60, 61) provides a basis for appreciating both the similarities and differences in its role in supporting tmRNA activity. In its GTP-bound form, EF-Tu binds an aminoacylated tRNA (aa-tRNA) and dramatically slows hydrolysis of the ester linking the charged amino acid to the tRNA. This aa-tRNA·EF-Tu·GTP complex binds

in the A site of the ribosome. If cognate codon-anticodon pairing occurs, then GTP hydrolysis is stimulated causing a conformational change, EF-Tu·GDP dissociation, and movement of the acceptor end of the tRNA into the peptidyl-transfer center of the ribosome. These post-hydrolysis events result in a rearrangement and accommodation of the aa-tRNA in the A site. If the tRNA anticodon does not match the mRNA codon, however, then GTP hydrolysis is very slow, and aa-tRNA·EF-Tu·GTP almost always dissociates before a protein synthesis error is made. Hence, EF-Tu plays a major role in ensuring that a cognate aa-tRNA occupies the A site before protein synthesis proceeds.

EF-Tu·GTP binds to the acceptor arm and T arm of alanyl-tmRNA and protects the ester from hydrolysis, as it does with aa-tRNA (10, 62, 63). Because tmRNA does not have an anticodon, however, GTP hydrolysis and subsequent A-site accommodation must be controlled by a different mechanism than the one used by conventional tRNAs (see below). tmRNA-mediated addition of alanine to a stalled peptide or protein can occur in the absence of EF-Tu in vitro, but the rate is very slow (31, 43). Hence, EF-Tu is almost certainly required for normal tmRNA-mediated tagging in vivo. Interestingly, EF-Tu·GDP binds regions of tmRNA outside the tRNA-mimic domain (64). Whether such complexes, which are less stable than the canonical complex, play a role in tmRNA function is unknown.

### SmpB, an Essential Partner in Tagging and Rescue

Genetic clues initially led to the discovery that SmpB binds tmRNA and is required for ribosome rescue and tagging in *E. coli* (9). SmpB has been shown subsequently to be necessary for tmRNA activity in other bacteria (15, 65) and for tmRNA-mediated tagging in vitro (13, 31, 32, 54, 55). One function of SmpB is mediating tmRNA binding to ribosomes. The tmRNA molecule cosediments with 70S

ribosomes in wild-type cell lysates but not in lysates from SmpB-deficient cells unless purified SmpB is added (4, 9, 32, 66, 67). Several SmpB molecules can bind to a single tmRNA molecule, but binding to one high-affinity site in the tRNA-mimic domain appears to be the functionally important interaction (9, 10, 32, 36, 37, 58, 59, 68).

Three-dimensional structures of SmpB in solution (69, 70) and bound to the tRNA-mimic domain have been determined (37). The main body of the SmpB protein ( $\approx 135$  amino acids) consists of an oligonucleotide-binding fold with a central  $\beta$ -barrel and three flanking  $\alpha$ -helices; a C-terminal tail of roughly 20 residues is disordered (69). Phylogenetically conserved residues define two discrete surface patches on opposite sides of the folded portion of SmpB (69). One of these patches coincides with the SmpB surface that binds to the 3' end of the D loop and also to a short loop that links the T arm to the connector stem in the cocrystal structure (**Figure 2**) (37). Mutations confirm the importance of these contacts (32, 68, 71). The function of the conserved tmRNA-distal patch on SmpB has not been established but almost certainly involves contacts with the ribosome.

The disordered C-terminal tail of SmpB plays a critical role in tmRNA tagging and function (72, 73). Variants in which this tail is deleted or mutated bind tmRNA normally and allow tmRNA binding to 70S ribosomes (72). However, they fail to support biological activity, to add the *ssrA* tag, or even to allow attachment of the charged alanine to nascent polypeptides on stalled ribosomes. Thus, the C-terminal tail of SmpB must be needed for a tmRNA activity that occurs after ribosome binding but before transpeptidation is completed (72).

### Structure of an SmpB·tmRNA·EF-Tu Entry Complex

A cryo-EM structure of a complex of tmRNA, SmpB, and EF-Tu·GDP in the A site of a 70S ribosome has been determined (10). To obtain

this structure, the antibiotic kirromycin was used to block EF-Tu conformational changes and dissociation, which normally occur after GTP hydrolysis. In this structure, EF-Tu interacts with the ribosome and the acceptor arm of tmRNA in the same fashion observed for complexes with tRNA. SmpB binds to the elbow region of tmRNA and also interacts with several helices of 23S RNA in the 50S subunit (10).

A different model for A-site binding is obtained by superimposing the T arm of tmRNA from the cocrystal structure onto the T arm of tRNA bound in the A site of the ribosome (37). This procedure directs the tmRNA-distal face of SmpB and its C-terminal tail away from the 50S subunit and toward the decoding center of the 30S subunit. The cryo-EM structure is likely to represent an initial mode of binding of SmpB·tmRNA·EF-Tu to the A site. The cocrystal model represents a plausible structural rearrangement that could occur in the accommodation step after GTP hydrolysis and EF-Tu dissociation (10, 37). Interactions mediated by the C-terminal tail of SmpB are probably important in stimulating GTP hydrolysis and EF-Tu release and/or in allowing transpeptidation following accommodation (72). Indeed, SmpB stimulates GTP hydrolysis by EF-Tu in a ribosome-dependent and tmRNA-dependent manner (43). Thus, tmRNA-bound SmpB seems to mediate interactions with the ribosome that would normally be made by the D arm or anticodon regions of a conventional tRNA.

### Does SmpB Recognize Stalled Ribosomes?

SmpB can bind tightly to 70S ribosomes or to isolated 50S and 30S subunits in the absence of tmRNA (31, 74, 75). In the 50S subunit, footprinting shows that the site of SmpB interaction with 23S RNA is close to the one predicted from the cryo-EM structure (10, 74). In the 30S subunit, SmpB interacts with 16S RNA bases that are normally close to the anticodon regions of P-site or

E-site tRNAs (74). Hence, following transpeptidation and elongation, SmpB might stabilize tmRNA binding in the P site and subsequently in the E site of the ribosome. Two SmpB molecules can bind to a single 70S ribosome, leading to the proposal that tagging and ribosome rescue require two SmpBs for each tmRNA (31). Experiments *in vitro* do show increasing activity when the SmpB concentration exceeds the tmRNA concentration (31), but this result is consistent with many possible models. Moreover, analysis of affinity-purified tmRNA·ribosome complexes suggests that only one SmpB molecule is bound at a late stage in ribosome rescue (45). Whether tmRNA function requires two SmpB molecules per ribosome or a single SmpB that transits through the ribosome with tmRNA is an open question.

Does SmpB bind to ribosomes first and subsequently recruit tmRNA·EF-Tu or does a ternary complex of SmpB·tmRNA·EF-Tu recognize stalled ribosomes? Order-of-addition experiments suggest that tmRNA tagging *in vitro* proceeds at comparable rates whether SmpB is prebound to stalled 70S ribosomes or is added along with tmRNA and EF-Tu (31). In the cell, however, normal ribosomes are present in significant excess over SmpB and stalled ribosomes. Moreover, there is no current evidence that SmpB binds preferentially to stalled ribosomes (31, 74, 75). In fact, aa-tRNA can bind to the A site of a ribosome and carry out transpeptidation even when SmpB is present (74). In our view, it makes little biological sense for SmpB to be prebound to ribosomes, as most SmpB would then be sequestered and unavailable to support tmRNA function. By contrast, if ternary complexes of alanyl-tmRNA, SmpB, and EF-Tu were responsible for translational surveillance, then the system would be poised to respond quickly with all required components when any ribosome stalled. We also note that intracellular levels of both tmRNA and SmpB are reduced when the other component is absent or present at reduced levels (30–34),

making it likely that these molecules are normally present as a complex resistant to cellular nucleases and proteases.

## Other Molecular Partners

Additional proteins bind tmRNA. For example, RNase R, ribosomal-protein S1, phosphoribosyl-pyrophosphate synthase, and a protein with homology to met-tRNA formyl transferase copurify with His<sub>6</sub>-tagged SmpB and tmRNA in *E. coli* (76). RNase R degrades tmRNA in an SmpB-regulated fashion in *C. crescentus* (34) and participates in tmRNA-mediated degradation of nonstop mRNAs in *E. coli* (76a), supporting a biological role for the observed binding interaction. Ribosomal-protein S1 helps translation initiation on many mRNAs and binds specific regions of tmRNA (77), but S1 mutants that affect mRNA translation have little if any effect on tmRNA tagging (78). Moreover, S1 orthologues are absent in some bacteria with tmRNA systems, and S1 does not appear to play a role in tmRNA-mediated tagging using purified components *in vitro* (13, 31, 32, 54, 55). Because S1 contains six RNA-binding domains that bind to a broad spectrum of pseudoknots and single-stranded RNAs (79), the interactions observed with tmRNA may not be functionally relevant.

The finding that mutations in *E. coli* phosphoribosyl-pyrophosphate synthase and tmRNA are synthetically lethal (80) was originally suggested as evidence for a functional physical interaction (76). However, tmRNA also suppresses the conditional phenotypes of several unrelated mutants, apparently by increasing the steady-state expression levels of these mutant proteins (81). Lon protease binds specifically to tmRNA *in vitro* (K.E. McGinness, personal communication), and *lon* mutations were isolated in a screen for tmRNA function in *E. coli* (J. Choy, L.L. Aung, A.W. Karzai, submitted). Thus, there are intriguing physical and genetic connections between tmRNA and a handful of cellular proteins. However, the biological and/or

functional significance of many of these interactions remains to be firmly established.

## MESSAGE SWAPPING AND TAG TRANSLATION

For tmRNA-mediated tagging to occur, the translational machinery must disengage from the original “stalled” mRNA and engage or switch to the tmRNA ORF. As long as the P-site tRNA remains attached to the nascent chain, stalled mRNAs remain stably bound to ribosomes in biochemical experiments (83). Upon addition of SmpB, alanyl-tmRNA, and EF-Tu-GTP, transpeptidation occurs, and stalled mRNAs can dissociate at a moderate rate (83). EF-G catalyzed translocation of peptidyl-tmRNA into the P site subsequently increases the rate of mRNA dissociation by an additional factor of roughly 10-fold. Thus, swapping of the tmRNA ORF for the original mRNA begins following the initial tmRNA-mediated transpeptidation reaction and is probably finished once the first elongation step has been completed (83).

### Resuming Translation on the tmRNA ORF

One of the most interesting aspects of the translational swap is the mechanism that allows selection of the start or “resume” codon on the tmRNA ORF (84). This process depends neither upon a specific initiator tRNA nor on a Shine-Dalgarno-like pairing of tmRNA bases with 16S ribosomal RNA and is very different from start-codon selection during normal translation. After the initial transpeptidation step and movement of peptidyl-tmRNA into the P site, the resume codon must somehow be positioned in the A site to allow tRNA-mediated addition of the first encoded tag residue (**Figure 1**). The six bases immediately upstream of the resume codon, which are reasonably well conserved in different tmRNAs (**Figure 3**), appear to be important determinants of the resume mechanism (53, 84). For example, an A→U muta-

tion four bases upstream of the resume codon in *E. coli* tmRNA prevents addition of a peptide tag in vivo (84). The adenine at this position is highly conserved phylogenetically (**Figure 3**) and in randomization/selection experiments. There is no absolute requirement for a specific “resume” tRNA or residue. Natural tmRNAs and/or mutants with insertions/deletions in and around the resume codon can use a variety of amino acids as the first encoded residue of the tag, although the vast majority of tmRNAs from different organisms use alanine as the resume residue (29, 53, 84) (**Figure 3**).

If the three tmRNA bases immediately preceding the resume codon (the -1 triplet) interacted with the ribosome decoding center in the 30S subunit, then the reading frame of the peptide tag would be correctly set once peptidyl tmRNA moved from the A site into the P site during the first elongation cycle (85). In the -1 triplets of different tmRNAs, however, the first base is generally a purine, and the third base is usually pyrimidine, but there is almost no conservation of the central base (**Figure 3**). Thus, strict sequence specific recognition seems unlikely. One interesting proposal is that the propensity of the sugar-phosphate backbone to assume an A-form conformation dictates which -1 triplets interact favorably with the decoding center (85). This speculative model rightly predicts that certain triplets are not found at the -1 position in natural tmRNAs and also provides an explanation for the results of mutagenesis experiments. Moreover, adjacent bases in the tmRNA -2 triplet could interact with the ribosome near the decoding center, helping to explain the importance of these nucleotides in the resume mechanism. In the cryo-EM structure, a conserved loop in the tmRNA connector is next to the decoding center, and the -1 triplet of tmRNA is far away (10). However, the -1 triplet could move into the decoding center during accommodation or following transpeptidation but prior to EF-G-mediated elongation. Interactions between the C-terminal tail of SmpB and the -1

and -2 triplets of tmRNA conceivably could also play some role in establishing the correct reading frame on tmRNA.

## RECOGNITION OF STALLED RIBOSOMES

What features of a stalled ribosome allow it to be identified and distinguished from an actively translating ribosome? As noted previously, tmRNA and SmpB molecules are present at intracellular levels that are only 5–10% of the total ribosome population. This fact makes it unlikely that tmRNA and/or SmpB preassociate with ribosomes and simply wait for stalling to occur. Moreover, tmRNA-mediated tagging activity increases dramatically when cells are treated with translational inhibitors, when mRNA fragments accumulate because degradation by exonucleases is disrupted, or when a nonstop mRNA is overexpressed (20, 30, 86), suggesting that the tmRNA system surveys the translation pool for structural changes that distinguish stalled ribosomes from active ribosomes.

### Pausing and mRNA Cleavage

Tagging and rescue by tmRNA occurs when ribosomes reach the 3' end of an mRNA, pause at sense codons or termination codons for long periods because the cognate aa-tRNA or release factor is scarce, or stop translation for other reasons (2, 47, 48, 87–92). Although these events initially seemed quite different, we now know that mRNA cleavage can convert paused complexes, which are able to resume normal translation, into complexes stalled at or near the 3' end of a message fragment (11, 12, 47, 90, 91). Two types of pausing-dependent mRNA cleavage are possible. One type of cleavage occurs within or very near the A-site codon. The second type of cleavage occurs 10–20 bases downstream of the A-site codon in the 3' direction, near the position where the mRNA emerges from a ribosome tunnel. Hence, this “edge cleavage”

occurs at external mRNA positions that are not protected by the paused ribosome.

Pausing-dependent cleavage in the A-site codon of mRNA was first demonstrated at an inefficient stop codon and depends both on translation and on the duration of the pause (11). The last result suggests a kinetic competition between a nuclease that can access the A site and release factors or aa-tRNAs that mediate resumption of translation. The site of cleavage is not precise, and the resulting 3'-stalled ribosome can have an A site that is empty or contains a partial or complete codon (11, 12). Edge cleavage is observed during pausing at stop codons, rare codons, and other types of translational blocks (11, 47, 90, 91). It is not known why pausing leads to A-site cleavage in certain cases and edge cleavage in other instances. Nevertheless, as discussed below, pausing-dependent mRNA truncation together with poor occupancy of the A site appear to govern recognition by SmpB·tmRNA·EF-Tu.

### Discrimination Based on Steric Conflicts

Steric overlaps would prevent tmRNA complexes from binding the A site at the same time as an aa-tRNA or release factor, explaining why an unoccupied A site is one determinant of tmRNA recognition. It is less clear, a priori, how truncated mRNA affects tmRNA recognition and/or activity. Biochemical experiments have clarified this issue (13, 55). Using ribosomes programmed to stall at a specific codon on defined mRNAs, high levels of tmRNA tagging in vitro are observed if no more than six mRNA nucleotides follow the P-site codon (13). Thus, the precise position at which a stalled mRNA is cleaved in or near the A site does not appear to be a critical determinant of tmRNA recognition. As the length of the 3' extension increases past six nucleotides, however, tagging activity falls off until little or no activity is observed at lengths exceeding 15 bases. These biochemical results in combination with the observation

of pausing-dependent cleavage suggest that ribosomes paused at internal codons are not recognized directly but require mRNA truncation before tmRNA recognition can occur.

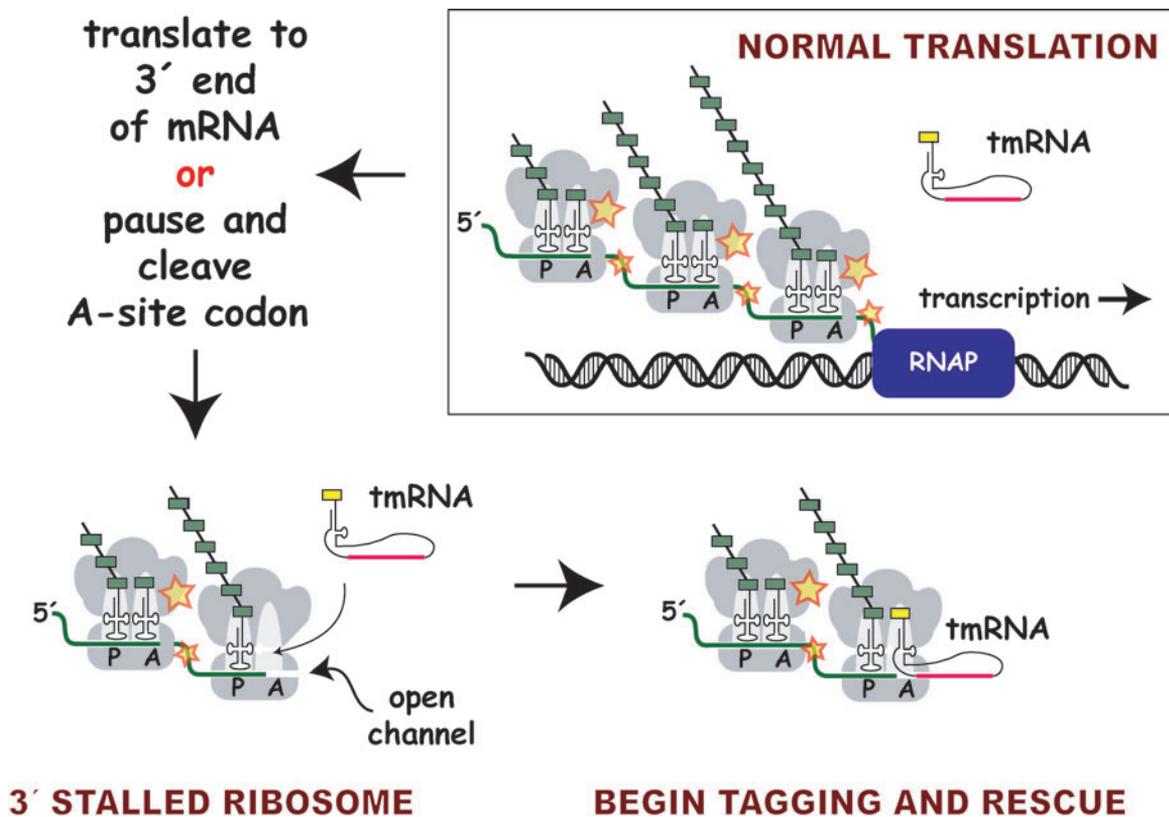
How does mRNA truncation allow tmRNA recognition? In structures of the ribosome, approximately 10 bases of mRNA following the A-site codon pass through a tunnel formed by the head and shoulder regions of the 30S subunit (93, 94). The site on the ribosome surface where mRNA begins to enter this tunnel overlaps the site occupied by the ORF in the cryo-EM structure of the tmRNA entry complex (10). Thus, in a full-length message undergoing translation, the mRNA sequence entering this tunnel would clash sterically with SmpB·alanyl-tmRNA·EF-Tu complexes trying to enter the A site. Clashes of this type would explain why paused ribosomes in which model mRNAs protrude from this entrance tunnel by more than a few bases are very poor substrates for tmRNA *in vitro* (13, 55). Transcription and translation are coupled in bacteria, and ribosomes usually queue on an mRNA (see Reference 95). As a consequence, 3' mRNA sequences that had not yet entered the tunnel would be bound to another ribosome or RNA polymerase, which would create additional steric clashes and make tmRNA binding even more difficult (Figure 4). Thus, structural and biochemical observations suggest that steric conflicts prevent or severely weaken tmRNA binding to actively translating ribosomes. By contrast, entry-mode binding of SmpB·alanyl-tmRNA·EF-Tu to ribosomes with an empty A site and little or no 3' protruding mRNA would be allowed (Figure 4).

### Positive Identification of Stalled Ribosomes

A second mechanism of discrimination between active ribosomes and ribosomes with short 3' mRNA extensions is suggested by structural and biochemical experiments. In the mRNA entrance tunnel, basic residues

from 30S ribosomal proteins neutralize the phosphate backbone of part of the mRNA chain (93), helping to keep the tunnel closed during active translation. If the tunnel were empty or incompletely filled with 3' mRNA (following a ribosome reading to the 3' end, for example), then electrostatic repulsion could drive conformational changes that open the tunnel and form the surface channel seen in some 30S subunit structures (see Reference 96). Tunnel opening would allow the tmRNA ORF, which is positioned nearby in the entry complex, to gain admittance to the mRNA channel as a prelude to positioning the resume codon in the A site. Indeed, because the tmRNA ORF is topologically restricted in one-piece tmRNAs and flanked by structured pseudoknots in all tmRNAs (Figure 2), it would not be possible to thread the ORF into the closed tunnel.

Hence, we anticipate that ribosomes stalled at or very near the 3' end of an mRNA would have an open-channel conformation, providing a positive mechanism of tmRNA identification (Figure 4). Alternatively, entry-mode tmRNA binding might help open the tunnel. Either model explains why ribosomes stalled on model mRNAs with very short 3' extensions are better substrates than those with 9 or 12 base extensions (13). In the latter cases, partial filling of the tunnel with mRNA would result in a dynamic equilibrium in which only a fraction of the ribosomes in a population had or could access the open-channel conformation. We note that tunnel opening would facilitate recognition of stalled ribosomes that read to the 3' end of mRNA or that were produced by A-site cleavage. However, extensive tunnel opening would not be expected following edge cleavage. It has also been proposed that destabilization of contacts between short 3' mRNA extensions and the ribosome would also allow easier displacement of any mRNA bases in the A site and tunnel and thus facilitate engagement of the tmRNA ORF during message swapping (13, 55).



**Figure 4**

Ribosomes engaged in active translation (*upper panel*) are not substrates for tmRNA because their A sites are usually occupied and because 3' mRNA and associated ribosomes or RNA polymerase (RNAP) would clash with tmRNA attempting to enter the A site (clash positions depicted by *stars*). Close packing of ribosomes along the mRNA also affords protection from edge cleavage. Ribosomes stalled at the 3' end of an mRNA (*lower panel*) have an unoccupied A site and may have an “open” mRNA entrance channel that allows the tmRNA ORF to be engaged. Although protein partners of tmRNA are not shown in this cartoon, a complex of alanyl-tmRNA, SmpB, and EF-Tu-GTP probably recognizes the stalled ribosome (see text).

### A-Site Competition on Truncated mRNA

tmRNA-mediated tagging does not increase markedly when tmRNA and SmpB are over-expressed 20-fold in *E. coli* (30). This fact suggests that tmRNA does not compete for transiently unoccupied A sites during normal translation, as expected if reading to the 3' end of an mRNA or pausing-dependent cleavage is needed to convert a translating ribosome into a substrate for tmRNA (**Figure 4**). What if pausing-dependent mRNA cleavage

leaves a complete codon in the A site? On the basis of experiments *in vitro*, tmRNA would now compete for the A site with a release factor or aa-tRNA (13, 55). If the release factor wins this competition with tmRNA, however, then translation would terminate normally, and the ribosome and nascent polypeptide would be released without need for tmRNA. If aa-tRNA wins the competition, then the 3' extension would become shorter by three bases, and tmRNA-mediated tagging and termination would simply be delayed.

## The Elusive A-Site Nuclease

At present, pausing-dependent A-site cleavage has only been documented in *E. coli*, and the identity of the nuclease has not been determined. The RelE toxin, which is normally inhibited by the RelB antitoxin, can cleave the A-site mRNA codon in paused ribosomes (97). Moreover, RelE cleavage activates tmRNA recognition of translational complexes that would otherwise be very poor substrates in vitro (13), and the deleterious effects of RelE cleavage in vivo are ameliorated by tmRNA-mediated ribosome rescue (98). However, A-site mRNA cleavage and subsequent tmRNA tagging are observed in strains lacking RelE and other known toxin-antitoxin systems (11). Moreover, A-site cleavage does not require tmRNA, SmpB, RNase R, RNase E, RNase G, RNase III, or (p)ppGpp (11, 12; C. Hayes, personal communication). It is possible that the ribosome itself cleaves mRNA in the A site after a long translational pause, but such cleavage has not been observed in vitro (13, 55).

The RelE example makes it most likely that a protein enters the empty A site to carry out cleavage or to stimulate the ribosome to cleave the paused mRNA, but this mechanism has not been verified. It is also probable that edge cleavage and A-site cleavage are mediated by different mechanisms. For example, pausing of one ribosome in a queue would allow adjacent leading ribosomes to move away, leaving flanking 3' mRNA sequences susceptible to cleavage by an endonuclease and subsequent trimming to the ribosome boundary by 3' to 5' exonucleases. Indeed, changes in the length of the 3' extensions are observed when specific exonucleases are absent (98a).

## Ribosomes that Pause with Occupied A Sites

Translation can also be interrupted when the A site is occupied. For example, a SecM peptide sequence causes ribosomes to stop with peptidyl-tRNA in the P site and prolyl-

tRNA<sup>Pro</sup> in the A site (98a, 99). In this instance, interaction of the SecM nascent chain with the exit channel causes structural changes that propagate throughout the ribosome and inhibit continuation of translation (100, 101). Active export of SecM normally creates a pulling force that counteracts this programmed stall (102). When the SecM stall sequence is transplanted into other mRNAs, cleavage and tmRNA-mediated tagging are observed (91, 92). However, as might be expected if the A site is usually occupied, pausing-dependent mRNA cleavage events at positions other than the A site are dominant (11, 91, 98a). It is possible that these mRNA processing events help relieve the pause, freeing the A site and allowing subsequent tagging and rescue.

## PHYLOGENY

The tmRNA system is amazingly widespread in eubacteria (24, 103, 104). For example, at the time of this review, each of the more than 240 completed eubacterial genomes contained genes for tmRNA and SmpB. Even *Mycoplasma genitalium*, a parasitic bacterium with a tiny genome of fewer than  $6 \cdot 10^5$  base pairs, encodes tmRNA and SmpB. The apparent universality of tmRNA translational surveillance and ribosome rescue in eubacteria implies a critical role for this system.

The tmRNA system also appears to function in the eubacterial-like organelles of some eukaryotes. For instance, tmRNA molecules are encoded in the genomes of photosynthetic or storage organelles of algae (e.g., *Cyanophora paradoxa*) and diatoms (e.g., *Thalassiosira pseudonana*), whereas SmpB is encoded with a signal sequence for organelle import in the nuclear genomes of these simple eukaryotes (73, 104, 105). Interestingly, tmRNA-like genes, which lack a peptide ORF, have been identified in the mitochondrial genomes of some protozoa, but it is not known whether these molecules have a partner SmpB or can function in ribosome rescue (24, 106). Although some organelle genomes encode

components of the tmRNA/SmpB system, it may be more significant that the majority do not. Moreover, genes for tmRNA have not been identified in any nuclear genome, and genes for SmpB are rarely present. Thus, the tmRNA system does not seem to be used in the nucleus/cytoplasm of eukaryotes and appears to be an exception rather than the rule in eukaryotic organelles.

The tmRNA system is also absent in archaea; neither genes for tmRNA nor SmpB have been identified in any of the 35 archaeal genomes whose sequences have been completed. Like eubacteria, archaea and eukaryotes must also face the problem of ribosome stalling. Indeed, recent studies show that when translation cannot continue in yeast, the stalled mRNA is cleaved in a process that depends on a protein homologous to the eRF1 eukaryotic translation termination factor (107). In this regard, it is interesting that the translation termination factors of archaea are more similar to those of eukaryotes than eubacteria. For example, protein orthologues of RF1 and RF2, which are required for translation termination in eubacteria, are not found in archaea. Eukaryotic/archaeal termination factors or related proteins may serve functions similar to tmRNA/SmpB in mediating translation termination that does not depend upon recognition of a normal stop codon. Whether mechanisms exist in archaea or eukaryotes to target incomplete proteins produced by premature termination for degradation is not known.

## DEGRADATION OF *ssrA*-TAGGED PROTEINS

Incomplete protein fragments have the potential to harm cells by misfolding, aggregating, or expressing unregulated activities. Ribosomes can also stall during translation of proteins destined for the cytoplasm, inner or outer membranes, periplasm, or extracellular space. Thus, it is not surprising that bacteria contain many proteases that degrade *ssrA*-tagged proteins. There do not appear to be

extracellular proteases that recognize and degrade *ssrA*-tagged proteins specifically (108), but partial translation products would pose little threat after secretion. *SsrA*-tagged proteins in the periplasm of *E. coli* are degraded by Tsp protease (2). Following binding to the *ssrA* tag of substrates (109), Tsp relies upon spontaneous unfolding of native substrates to expose sites for degradation (110). The fact that *ssrA*-tagged molecules are typically incomplete proteins, which are likely to be unfolded or metastable, probably assists Tsp degradation of these substrates.

## Energy-Dependent Cytoplasmic Proteases

Truncated protein fragments in the bacterial cytoplasm could interfere with metabolic and central-dogma processes and consequently be very toxic. Three proteases specifically degrade *ssrA*-tagged substrates in the cytoplasm of *E. coli*. ClpXP and ClpAP are soluble enzymes consisting of the ClpP<sub>14</sub> peptidase and hexamers of ClpX or ClpA (see Reference 111). The proteolytic active sites of ClpP are located in an internal chamber, accessible through pores that exclude native proteins and unstructured polypeptides larger than 30 residues. ClpX and ClpA are ATP-fueled machines that recognize *ssrA*-tagged substrates, unfold them if necessary, and translocate the denatured polypeptide into ClpP for degradation (46, 112, 113). Thus, ClpXP and ClpAP can degrade stable full-length cytoplasmic proteins that acquire *ssrA* tags. FtsH is hexameric protease anchored to the inner side of the cytoplasmic membrane (114). FtsH also uses the energy of ATP hydrolysis to feed target proteins into a proteolytic chamber, but this enzyme is unable to unfold and degrade highly stable *ssrA*-tagged proteins (115). FtsH degrades misassembled integral membrane proteins (114) and probably also plays a role in degradation of some *ssrA*-tagged proteins in the inner membrane.

The proteases described above recognize the *ssrA* tags of substrates. Because many

ssrA-tagged proteins in the cell are probably unfolded, however, they may also be degraded by proteases that recognize peptide signals that become accessible in the unfolded state. This type of ssrA-tag-independent proteolysis may explain why higher levels of ssrA-tagged proteins are observed in *lon*-deficient *E. coli* (30; J. Choy, L.L. Aung, A.W. Karzai, submitted; K.E. McGinness, personal communication), even though Lon protease does not recognize the ssrA tag (J. Yakamavich & E. Gur, personal communication).

### Adaptors and ssrA-Tagged Proteins

Adaptor proteins consort with intracellular proteases to control substrate degradation (116). Several adaptors that modulate degradation of ssrA-tagged substrates in *E. coli* have been discovered. SspB, which is present in  $\alpha$ -,  $\beta$ -, and  $\gamma$ -proteobacteria, binds to a portion of the ssrA tag and tethers substrates to ClpX, thereby enhancing degradation of ssrA-tagged substrates by ClpXP (49, 117, 118, 118a). At the same time, SspB binding to the ssrA tag blocks ClpAP recognition and degradation of these substrates (49). The ClpS adaptor is found in most strains that contain ClpA, and one of its activities is inhibition of ClpAP degradation of ssrA-tagged substrates (119). Thus, although ClpXP and ClpAP degrade ssrA-tagged substrates equally well in vitro (46), degradation of these substrates is usually directed away from ClpAP and to ClpXP by adaptor proteins in the cell.

### Conserved Tag Sequences Are Important for Degradation

The sequence of the ssrA tag shows substantial phylogenetic conservation. For example, the consensus sequence for the C-terminal ssrA-tag residues is YALAA (Figure 3). In *E. coli*, the first residue of this sequence is part of the recognition motif for SspB, whereas the last four residues are important for ClpX and/or ClpA recognition of the tag (49). The consensus for the N-terminal residues of the ssrA tag

is AANDN (Figure 3). In *E. coli*, the first four residues are recognized by SspB, and the first two are also important for ClpA recognition (49, 120). Thus, much, if not all, of the sequence conservation in the tmRNA-encoded tag is important for recognition by cellular proteases and/or adaptor proteins. This broad phylogenetic conservation suggests constant selective pressure for degradation of proteins rescued by the tmRNA system.

ClpXP accounts for most cytoplasmic degradation of ssrA-tagged substrates in *E. coli* and *B. subtilis* (46, 65, 121, 122) and presumably in the vast majority of eubacteria, as ClpX, ClpP, and the ssrA tag are all highly conserved. However, ClpXP orthologues are absent in a few bacteria, and the tag sequences in these organisms can differ substantially from the consensus. For example, *Ureaplasma parvum* has no ClpXP, and its ssrA tag ends in FAY instead of the canonical LAA. Which proteases degrade rescued proteins in such cases is not known.

## BIOLOGY

The tmRNA system may be present in all eubacteria, but it serves an essential genetic function in only a subset of this domain. For example, disruption or deletion of the genes encoding tmRNA and/or SmpB is lethal in some bacteria (18, 123) but not in others (9, 16, 19, 33, 65, 124–127). In *E. coli*, disrupting the gene encoding tmRNA or deleting the SmpB gene causes only a small decrease in growth rate (9, 124). By contrast, *Neisseria gonorrhoeae* mutants are not viable if tmRNA charging is prevented or its gene is disrupted (18).

Could tmRNA be dispensable in *E. coli* and certain other bacteria because translational fidelity is high and ribosome stalling is not a significant problem? The answer is no. In *E. coli*, for example, tmRNA tagging and ribosome rescue terminates approximately 1 of every 250 translation events (30). At this frequency of translational stalling, all of the ribosomes in the cell would be removed from the active

translation pool in less than one generation if there were not some way to release the translational blocks because each ribosome synthesizes approximately 100 proteins per generation and because multiple ribosomes would be queued and trapped on each stalled mRNA.

### Alternative Rescue

The facts discussed above suggest that an “alternative” mechanism for ribosome release exists in *E. coli* and other eubacteria in which tmRNA is nonessential. Indeed, proteins synthesized from nonstop mRNAs accumulate in a free active form in *E. coli* lacking tmRNA (2, 84), suggesting that another mechanism releases these proteins and concurrently liberates the stalled ribosome. When nonstop mRNAs are overexpressed in wild-type *E. coli*, most if not all of the encoded protein is *ssrA* tagged, indicating that tmRNA-mediated rescue occurs at a faster rate than alternative rescue (2). However, the use of tmRNA variants that encode different tag sequences frequently results in a mixture of tagged and untagged proteins (30, 47, 48), suggesting that these tmRNA mutants are less efficient than the wild-type molecule, which allows alternative rescue to become kinetically competitive.

Because stalled ribosomal complexes are very stable *in vitro* (83), it is unlikely that alternative rescue occurs passively. Energy is presumably required to break contacts between the P-site tRNA, the ribosome, and the mRNA and to pull the nascent polypeptide out of the exit channel. One speculative possibility is that translation by lagging ribosomes provides the force to push the stalled ribosome off of the 3' end of the mRNA, with subsequent disassembly of this ribosome and hydrolysis of the linkage between tRNA and the nascent chain by peptidyl-tRNA hydrolase. At present, it is not known if alternative rescue and peptidyl-tRNA drop-off represent distinct mechanisms or the same process (see Reference 128). Interestingly, however, tmRNA-mediated ribosome rescue in

*E. coli* suppresses the detrimental effects of mutations in peptidyl-tRNA hydrolase (128).

### Translational Stress Relief

Many of the documented roles of tmRNA can be understood in terms of preventing translational collapse when the frequency of ribosome stalling is very high. For example, tmRNA increases the resistance of bacteria to antibiotics that cause ribosomes to read through termination codons or stop at internal mRNA codons (16, 20, 129). Similarly, tmRNA lessens the deleterious effects of suppressor tRNAs that result in ribosomes reading through termination codons (130). In *B. subtilis* lacking the 3'-5' exoribonuclease polyribonucleotide nucleotidyltransferase, the accumulation of mRNA fragments creates an increased demand for ribosome rescue, and higher levels of tmRNA are required for growth (86).

The need for tmRNA increases as environmental stress in *B. subtilis* increases (19). Moreover, tmRNA allows *E. coli* to recover more rapidly from the stress of carbon starvation (124). The tmRNA system helps *Salmonella* and *Yersinia pseudotuberculosis* survive in macrophages, where host mechanisms attack these bacterial pathogens (16, 125, 127). Stress presumably leads to increased ribosome stalling, which is normally ameliorated by the tmRNA system. Conversely, tmRNA action can prevent stress. In *E. coli* lacking tmRNA, for instance, the heat shock response is constitutively induced, suggesting a continual state of stress (131).

### Use in Control Circuits

The tmRNA system appears to function as a component of regulatory circuits in many bacteria. For example, tmRNA and SmpB levels fluctuate during the *C. crescentus* cell cycle, and tmRNA-defective mutants show delayed DNA replication (15, 33, 34). The reduced virulence of *Y. pseudotuberculosis* lacking tmRNA or SmpB is correlated with reduced

levels of type III secretion components needed for efficient host infection and colonization (16). A related phenotype may prevent colonization of the root nodules of soybeans by tmRNA-defective *Bradyrhizobium japonicum* (132). Both *E. coli* and *Y. pseudotuberculosis* have reduced motility when tmRNA is absent (4, 16). In the latter case, flagella fail to assemble (16). Because tmRNA activity is known to affect the cellular levels and/or activities of some transcription factors (see below), it seems likely that many of the phenotypes discussed here involve changes in gene expression.

Phage may use control circuits that involve tmRNA to evaluate the translational capacity and fitness of a host bacterium before making irreversible decisions that involve a commitment to lytic or lysogenic growth. For example, tmRNA-mediated function is required for growth of hybrid  $\lambda$ -P22 phage in *E. coli* (17). This phenotype depends on the C1 transcriptional activator, as C1-defective phage plate efficiently on strains lacking tmRNA (133, 134). Efficient induction of phage Mu lysogens in *E. coli* requires tmRNA-mediated relief of stalling during translation of the Mu repressor (35). Both  $\lambda$ -P22 growth and Mu induction can be supported by tmRNA variants that fail to target tagged proteins for degradation. In these cases, rescue of a single stalled ribosome has been proposed to allow the ribosomes queued behind it to complete normal translation without further need for tmRNA (17, 35). However, this model requires tmRNA rescue at an internal mRNA site without mRNA cleavage, as pausing-dependent production of a nonstop fragment would require tmRNA rescue or alternative rescue when each ribosome reached the 3' end. Although the exact mechanism may be in question, it is clear that tmRNA-mediated relief of stalling without degradation of the rescued protein is sufficient for biological function in numerous cases (18–21, 86). By contrast, the defects that cause cell-cycle delay in *C. crescentus* and nonmotility in *Yersinia* when tmRNA is absent are not complemented by variants that

rescue ribosomes but do not lead to degradation of rescued proteins (15, 16).

In *E. coli*, the levels of Lac repressor appear to be fine tuned via the actions of tmRNA (14). The Lac repressor tetramer binds to an operator site situated in the C-terminal coding region of its own gene. RNA polymerase cannot transcribe through the bound repressor, and transcription terminates prematurely creating a nonstop message (14). In cells lacking tmRNA, the Lac repressor fragments are released in an untagged form and contribute to increased activity because they bind operator DNA (14). In wild-type cells, however, the truncated repressor translated from the nonstop fragment is tagged by the tmRNA system and subsequently degraded. The truncated mRNA is presumably also degraded more rapidly than the complete mRNA (see below), again keeping Lac repressor levels low. Thus, when Lac repressor levels are high enough to bind the operator in its gene, tmRNA activity precludes the synthesis of even higher levels and enforces an upper limit on the steady-state repressor concentration. Keeping Lac repressor levels low is important for inducer-mediated derepression of the lac operon (14). This example highlights a simple mechanism that utilizes tmRNA to create feedback circuits that help control steady-state levels of regulatory proteins. In this regard, it is intriguing that many sites of ribosome stalling in *C. crescentus* are associated with a nucleotide sequence motif that might represent a protein-binding site (S.J. Hong, K.C. Keiler, personal communication).

### Quality Control for mRNA

Translation begins soon after transcription of the 5' portion of a bacterial mRNA, and rates of transcriptional and translational elongation are similar (95). Hence, closely packed ribosomes protect the newly transcribed mRNA from nuclease cleavage (**Figure 4**). Ribosomes remain closely spaced on mRNAs even after transcription is complete. Mature

mRNAs typically have 3' stem-loop structures that protect this end of the message from digestion, and endonucleases bind to the 5' end of mRNAs to initiate degradation in *E. coli* and *B. subtilis* (95, 135, 136). A general 5' to 3' direction of mRNA degradation allows ongoing translation to finish, prevents new initiation, and consequently minimizes partial translation and tmRNA rescue. Because exoribonucleases degrade RNA in a 3' to 5' direction, however, overall mRNA turnover requires a combination of endonucleolytic and exonucleolytic digestion. Problematic nonstop mRNAs or fragments can arise when transcription terminates prematurely, when endonucleases cleave mature mRNAs internally when transcription and translation become uncoupled, or when ribosomes pause for long periods during translation.

The tmRNA system plays a role in degradation of nonstop mRNAs (137). For example, a normal mRNA and closely related nonstop variant had similar half-lives (2–3 min) in *E. coli* lacking tmRNA. The half-life of the normal mRNA was unchanged when tmRNA was present, but the half-life of the nonstop mRNA decreased to 0.6 min (137). It appears likely that tmRNA contributes to degradation of nonstop mRNAs by recruiting ribonucleases, such as RNase R, during tagging and ribosome rescue (76, 76a). Consistent with a recruitment model, the levels of tmRNA-mediated protein tagging increase in *E. coli* strains lacking RNase R, as expected if this enzyme participates in degradation of mRNAs released during tmRNA-mediated rescue (76, 76a). However, a role for RNase R in degrading nonstop mRNAs has been observed in some studies (76a) but not in others (137). The rate at which the swapped mRNA dissociates from the ribosome may influence the outcome of such studies. Experiments *in vitro* show that some mRNAs are released more slowly than others from rescued ribosomes (83). A swapped mRNA that remained bound longer would be more susceptible to ribonucleases recruited by

tmRNA than a comparable mRNA that dissociated rapidly.

### Regulatory Opportunities or Housekeeping Failures?

As we have noted, ribosomes paused at rare codons and inefficient stop codons are substrates for mRNA cleavage and subsequent tmRNA-mediated tagging and rescue (11, 12, 48, 87–90). Tagging caused by pausing at rare codons generally requires more than one such codon in a short stretch of mRNA, rare codons in combination with an inefficient stop codon, or depletion of the rare aa-tRNA. Tagging caused by pausing at termination codons depends on the stop codon, the following base, the identity of the penultimate and antepenultimate amino acids and/or codons, and the concentration of the cognate release factor. For example, roughly 40% of the protein molecules expressed from a coding region ending in Pro-Pro with a UAA stop codon were found to be tagged by the tmRNA system in *E. coli* (87).

It is possible that pausing-dependent cleavage and subsequent tmRNA activity simply provide a fail-safe housekeeping mechanism, allowing translation reactions that cannot be completed in a timely fashion to be abandoned. Alternatively, ribosome pausing at internal mRNA codons could also be genetically programmed to allow post-transcriptional regulation of protein and/or mRNA levels through the actions of pausing-dependent cleavage and the tmRNA system. The latter model is more appealing because it provides an evolutionary rationale for the use of rare sense codons or inefficient stop codons.

### CLOSING COMMENTS

A role for tmRNA in translational surveillance and ribosome rescue was first proposed in 1996 (2). Since then, substantial progress has been made in understanding the structure and function of tmRNA and its binding

partner SmpB, in elucidating the biochemical mechanisms of tmRNA-mediated tagging and rescue, in deciphering how tmRNA avoids actively translating ribosomes and recognizes stalled ribosomes, and in uncovering myriad biological roles for this fascinating

molecule. This period has coincided with a renaissance in structural and biochemical studies of the ribosome and translation. The field is now poised to advance understanding of the biochemistry and biology of tmRNA to far deeper mechanistic levels.

## ACKNOWLEDGMENTS

We thank Chris Hayes, Natalia Ivanova, Wali Karzai, Ken Keiler, Kathleen McGinness, and Tom RajBhandary for helpful discussions and communication of unpublished results. Our work is supported by NIH grants AI-16892 and AI-15706.

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## Errata

An online log of corrections to *Annual Review of Biochemistry* chapters (if any, 1997 to the present) may be found at <http://biochem.annualreviews.org/errata.shtml>