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Ribosomal RNA Mutations That Inhibit the Activity of
Transfer-Messenger RNA on Stalled Ribosomes

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A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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ABSTRACT

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In eubacteria, stalled ribosomes are rescued by a conserved quality-control mechanism involving transfer-messenger RNA (tmRNA) and its protein partner SmpB. Mimicking a tRNA, tmRNA enters stalled ribosomes, adds Ala to the nascent polypeptide, and serves as a template to encode a short peptide that tags the nascent protein for destruction. To further characterize the tagging process, we developed two genetic selections that link tmRNA activity to cell death. These negative selections can be used to identify inhibitors of tagging or to identify mutations in key residues essential for ribosome rescue. Little is known about which ribosomal elements are specifically required for tmRNA activity. Using these selections, we isolated ribosomal RNA mutations that block the rescue of ribosomes stalled at rare Arg codons or at the inefficient termination signal Pro-opal. We find that deletion of A1150 in the 16S rRNA blocks tagging regardless of the stalling sequence, suggesting that it inhibits tmRNA activity directly. The C889U mutation in 23S rRNA, however, lowers tagging levels at Pro-opal and rare Arg codons but not at the 3'-end of an mRNA lacking a stop codon. We conclude that the C889U mutation does not inhibit tmRNA activity per se but interferes with an upstream step intermediate between stalling and tagging.

Keywords [rRNA, ribosome, helix 38, A-site finger, tmRNA, SmpB, trans-translation]

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LIST OF ABBREVIATIONS

A site	Aminoacyl or Acceptor site of the ribosome
aa-tRNA	Aminoacyl-tRNA
E site	Exit site
<i>E. coli</i>	<i>Escherichia coli</i>
EF-G	Elongation Factor G
EF-Tu	Elongation Factor Tu
fMet-tRNA	Formylated-methionine-initiator tRNA
GTP	Guanosine triphosphate
IF1	Initiation Factor 1
IF2	Initiation Factor 2
IF3	Initiation Factor 3
mRNA	Messenger RNA
P site	Peptidyl site
PTC	Peptidyl-transferase center
RF1	Release Factor 1
RF2	Release Factor 2
RF3	Release Factor 3
RRF	Ribosome Recycling Factor
rRNA	Ribosomal RNA
SmpB	Small Protein B
tmRNA	Transfer – messenger RNA
tRNA	Transfer RNA

CHAPTER 1 INTRODUCTION

Ribosomes synthesize proteins by polymerizing amino acids as directed by the information in messenger RNA (mRNA). Transfer RNAs (tRNAs) charged with amino acids bind mRNA codons within the ribosome. This binding of tRNAs positions the amino acids for the ribosome to create a peptide bond between them. The ribosome then shifts the mRNA and aminoacyl-tRNAs to allow the next appropriate aa-tRNA to enter and position its amino acid for peptide bond formation. In this way, the ribosome is capable of producing long chains of amino acids that are released into the cell and folded into active proteins.

Several ribosomes can be associated with a single mRNA at the same time. If the leading ribosome on a mRNA becomes stalled, then the ribosomes following it also stall and the ribosomal pool becomes depleted and can no longer support cellular life. To solve this problem, bacteria have evolved a rescue mechanism termed trans-translation that releases the stranded ribosomes and degrades the unproductive mRNA and unfinished proteins. The aim of our research is to better understand how trans-translation factors interact with stalled ribosomes. Our goal is to find ribosome mutants that inhibit trans-translational rescue of stalled ribosomes.

THE RIBOSOME

The *Escherichia coli* (*E. coli*) ribosome is comprised of two subunits. The large subunit (50S) contains thirty-six proteins and a 2,904 nucleotide structured ribosomal RNA (23S rRNA) and a 120 nucleotide structured ribosomal RNA (5S rRNA). The small subunit (30S) contains twenty-one proteins and a 1,542 nucleotide structured ribosomal RNA (16S rRNA). Together the large and small subunits form a functional 70S ribosome (Shown in Figure 1-1 is the *Thermus thermophilus* 70S ribosome¹ bound to mRNA and three tRNAs).

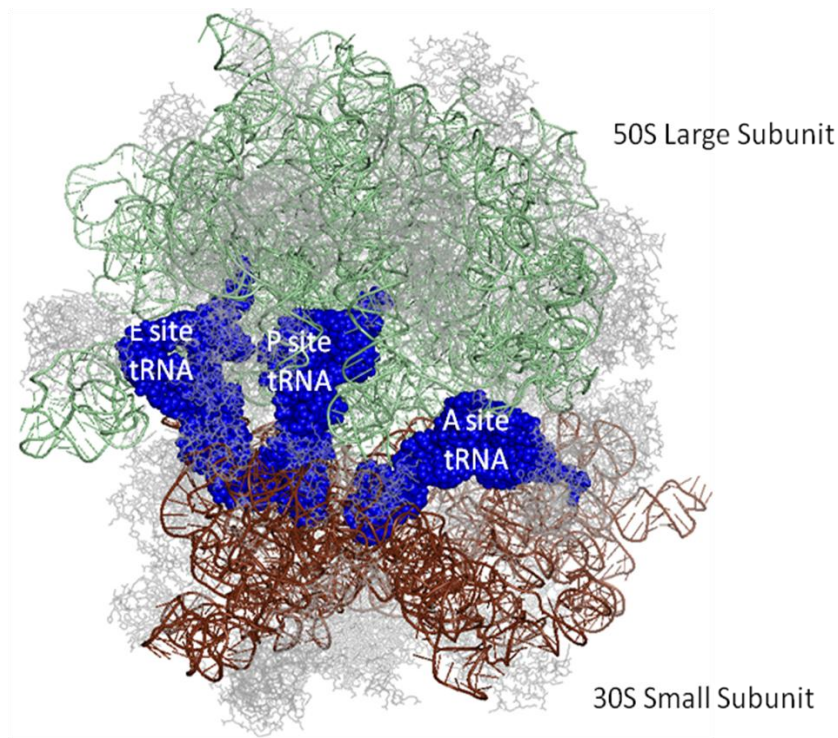


Figure 1-1 THE RIBOSOME

The 70S Ribosome of *Thermus thermophilus*¹. Identified are the 23S rRNA in green, the 16S rRNA in brown, tRNAs in blue and ribosomal proteins in grey. The 50S large subunit is made up of the 23S rRNA and the proteins within the green. The 30S small subunit is made up of the 16S rRNA and the proteins associated with the brown (PDB# 2WRN – 30s and 2WRO – 50s).

THE TRANSLATION CYCLE

Protein synthesis on the 70S ribosome is carried out in three phases known as initiation, elongation and termination. The ribosome cycles between these phases. Once a ribosome has terminated protein synthesis on a particular mRNA, it will re-initiate on a new mRNA, elongate the peptide, release it by termination, and re-initiate again (Figure 1-2).

Initiation begins when the 30S subunit associates with an mRNA template. This interaction is facilitated by the presence of the Shine-Delgarno sequence (consensus AGGAGG)

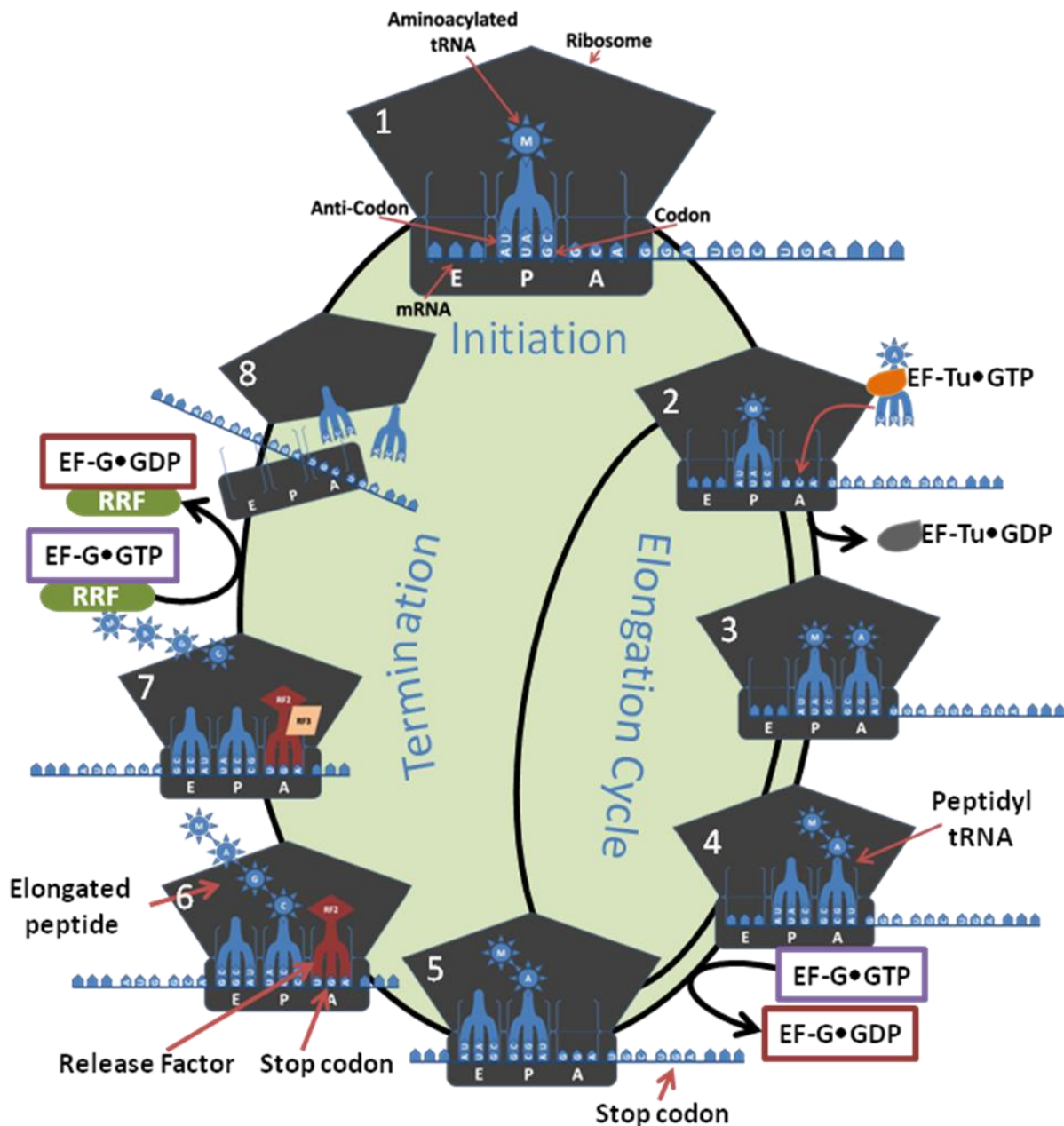


Figure 1-2. RIBOSOMAL CYCLES

(1) The 70S ribosome following initiation, with mRNA and fMet-tRNA in the P site. (2) EF-Tu (orange) delivers aminoacyl-tRNA to the A site. (3) and (4) Methionine is then transferred to the A-site aa-tRNA. (5) Translocation by EF-G (purple box) shifts the A-site peptidyl-tRNA into the P site and the elongation cycle starts over at 2 until the peptide is fully elongated and the stop codon is located in the A site. (6) With the stop codon in the A site, a release factor (red) enters and hydrolyzes the peptidyl-tRNA linkage, releasing the peptide. (7) RF3 is recruited to release the release factor. (8) RRF (green) and EF-G then dissociates the subunits and the cycle begins again with 1.

on the mRNA and a complementary sequence on the 16S rRNA. This binding interaction not only brings the mRNA to the small 30S subunit, but also helps select the reading frame. Three proteins known as initiation factors 1, 2 and 3 (IF1, IF2, IF3) bind the 30S-mRNA complex. IF2 bound by guanosine triphosphate (IF2-GTP) associates formyl-methionine-initiator tRNA (fMet-tRNA) with the AUG codon. Once this pairing occurs, IF3 is released. When IF3 is no longer bound, the 50S subunit binds, activating GTP hydrolysis by IF2, releasing IF1 and IF2. The binding of the 50S subunit signals completion of initiation and the beginning of elongation (Figure 1- 2).

Elongation proceeds with the help of two elongation factor proteins, elongation factor-Tu (EF-Tu) and elongation factor-G (EF-G). EF-Tu binds aminoacyl-tRNAs and escorts them into the ribosome (Figure 1-2, Step 2). Within the ribosome are three tRNA binding sites known as the A, P, and E sites. Incoming tRNAs first bind the A (aminoacyl-tRNA or acceptor) site, which selects for tRNAs that correctly pair with a given mRNA codon. During the first elongation step, fMet-tRNA is already positioned in the P (peptidyl) site. Once an aa-tRNA is delivered to the ribosomal A site by EF-Tu, a peptide bond forms between fMet and the A-site amino acid. The dipeptide is now attached to the A-site tRNA (Figure 1-2, Step 4). In a process known as translocation, EF-G shifts the mRNA, free tRNA and peptidyl tRNA three nucleotides (Figure 1-2, Steps 4-5). The deacylated tRNA moves to the E (exit) site where it is ejected. The peptidyl-tRNA occupies the P site. With an empty A site, the next aa-tRNA binds and the cycle of elongation continues until a stop codon is reached (Figure 1-2, Step 6).

Since tRNAs do not recognize the stop codons UAA, UAG or UGA, elongation is halted and either release factor 1 or 2 (RF1 or RF2) binds the stop codon and frees the peptide from the peptidyl-tRNA. Following the release of the peptide, release factor 3 (RF3) helps release the

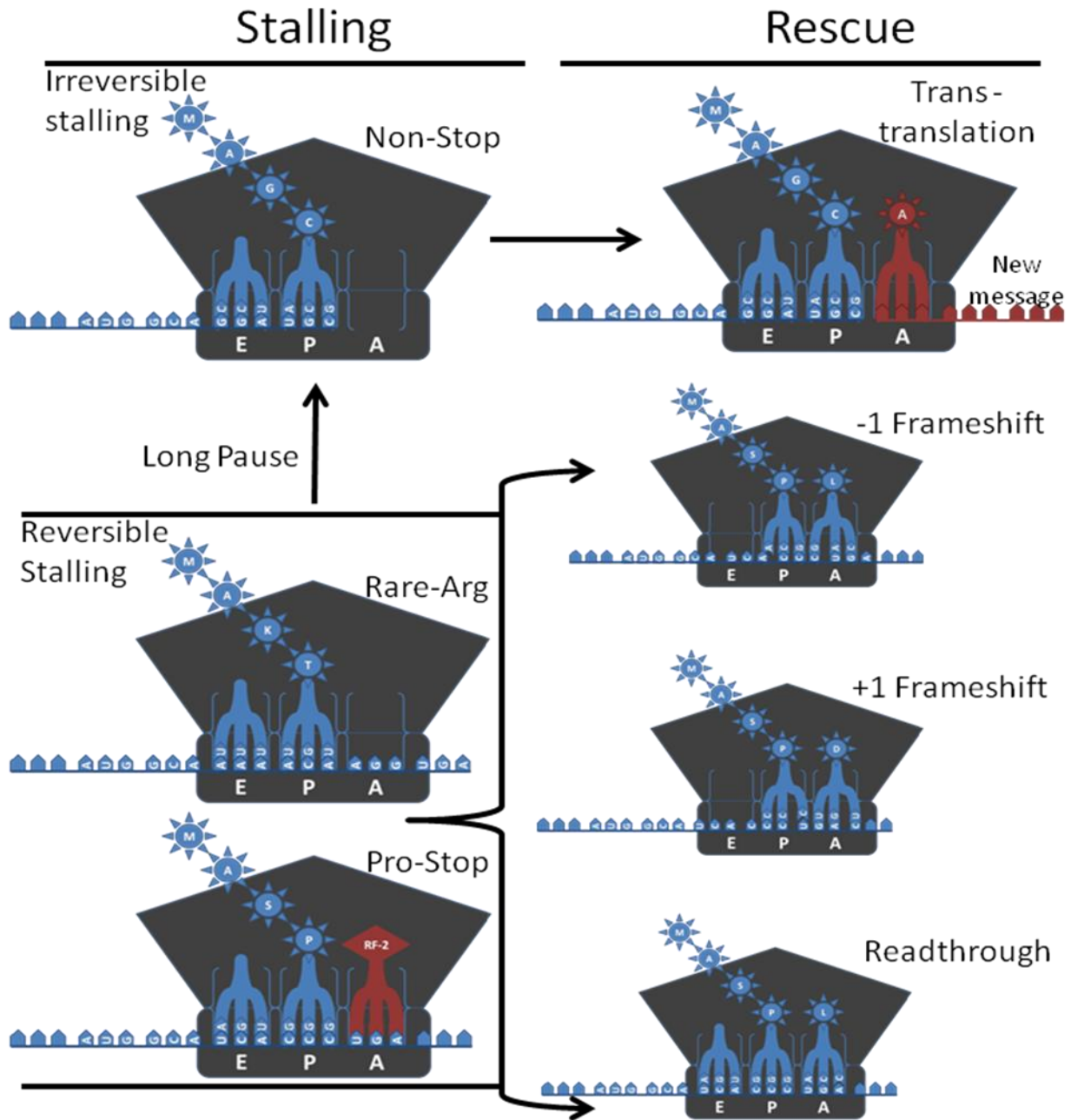


Figure 1-3. STALLING AND RESCUE

In the first column are shown three representative types of stalling: Non-stop mRNA, rare-arg, and pro-stop. Types of rescue are represented in the second column: Trans-translation, -1 frameshifting, +1 frameshifting and readthrough. Of these four rescue methods nonstop mRNAs can only be rescued by trans-translation. However, rare-arg and pro-stop stalling can be rescued by readthrough and frameshifting unless induced into a irreversible stalling non-stop mRNA. The frameshifting and readthrough situations are represented by rescue of pro-stop stalling.

bound release factors. The ribosomal subunits are then pried apart by ribosome recycling factor (RRF) and EF-G, releasing free subunits, the mRNA and tRNAs (Figure 1-2). Once the subunits dissociate they are free to initiate another round of translation.

STALLING AND RESCUE

Sometimes ribosomes encounter mRNAs that cause them to stall during protein synthesis. Ribosome stalling events can either be reversible (the ribosome pauses and then resumes translation) or irreversible (the ribosome stalls completely and cannot resume translation).

Irreversible stalling occurs when translation has reached the 3' end of the mRNA without having encountered a stop codon, and there is no message to be decoded in the A site. mRNA transcripts lacking a stop codon are referred to as non-stop mRNAs. In the bacterial cell, mRNAs are degraded from the 3' end relatively quickly; the average half-life of an mRNA in *E. coli* is ~6 minutes^{4,5}. This 3' degradation can create non-stop mRNAs if exonucleases that with the ribosome after removing the stop codon. The abundance of non-stop mRNAs, and the resulting irreversibly stalled ribosomes, poses a threat to cell viability. To rescue irreversibly stalled ribosomes, the cell uses trans-translation (Figure 1-3). Trans-translation keeps the cell healthy by recycling stalled ribosomes, adding a new message to the A site and continuing translation to a stop codon. Ribonucleases and proteases are then recruited to destroy the aberrant mRNA and peptide.

Reversible stalling occurs for many reasons, one of which is strings of rare codons translated by rare tRNAs. Rare codons are those codons that are rarely found in protein coding genes compared to other codons within the genome of a given species. Since the cognate tRNAs that recognize rare codons are also scarce, overexpressing the gene will deplete all the available tRNAs that decode the rare codon, leaving the ribosome with an empty A site (Figure 1-3). An example in *E. coli* is the rare arginine codon AGG. AGG represents only 0.2% of all the codons in protein coding genes in *E. coli*. In contrast the arginine codon CGC appears 2.2% of the time. When mRNAs containing AGG codons are overexpressed, the cell has insufficient tRNA to decode the A site codon and the ribosome becomes stalled. However, because there is mRNA message extending beyond the stalling site, translation can be rescued by the arrival of the proper tRNA, by misreading of another tRNA (readthrough), or by frameshifting⁶ (Figure 1-3).

Another example of reversible stalling is the inhibition of the termination reaction by proteins ending in proline. If peptidyl prolyl-tRNA is in the ribosomal P site and a stop codon is in the A site, as is the case at the sequence Pro-stop, the function of the release factor is inhibited (Figure 1-3). The strength of the stalling event is dependent upon the upstream (-2) amino acid. Sequences Glu, Asp, Ile, Val, or Pro-Pro-stop have the strongest stalling phenotypes⁷. The mechanism of stalling is not clear, but evidence suggests that the proline residue slows down its own release. This is supported by experimentation with RF bound at the A-site of the ribosome and peptidyl prolyl-tRNA in the P site. These experiments showed that catalysis of the nascent peptide from the prolyl-tRNA is inhibited to the point that it affects cell growth⁸. Further studies using the antibiotic puromycin, which reacts with and releases nascent peptides, demonstrated that peptide release of a peptidyl prolyl-tRNA was slower than release with any other C-terminal amino acid⁹. Furthermore, a recent cryo-EM structure which contained a peptidyl prolyl-tRNA

in the P site and a stop codon in the A site showed the proline amino acid making contacts with the peptidyl-transferase center (PTC) of the ribosome, resulting in an inactive conformation¹⁰.

Although trans-translation was first discovered because it rescues ribosomes on non-stop mRNAs, ribosomes stalled at rare codons or at termination can also be rescued by trans-translation. However, this is only possible if the transcript is converted into a non-stop mRNA by either endonucleolytic RNA digestion at the A site of the ribosome or by exonucleolytic RNA digestion of the 3' end of the mRNA¹¹. An example of this occurs during *E. coli* starvation. At starvation, *E. coli* elicit the stringent response to slow down protein production by generating non-stop mRNAs using the bacterial toxin RelE, a known endoribonuclease^{12, 13}. RelE cleaves mRNA at the A site of the ribosome, generating non-stop mRNAs and inducing irreversibly stalled ribosomes¹⁴. These ribosomes are then rescued by trans-translation.

TRANS-TRANSLATION

Trans-translation rescues irreversibly stalled ribosomes by adding new message to the A site of the ribosome so that translation can continue to a stop codon (Figure 1-3). Trans-translation has two main players: transfer-messenger RNA (tmRNA) and small protein B (SmpB). tmRNA acts both as a tRNA and an mRNA to rescue stalled ribosomes. This is first done by tmRNA-SmpB mimicking a tRNA and entering the empty A site of the ribosome (Figure 1-4). Since tmRNA is aminoacylated by alanyl-tRNA synthetase, it can add Ala to the growing polypeptide chain. The SmpB-tmRNA complex is then translocated to the P site, and the open reading frame of tmRNA is placed in the decoding center of the ribosome to be translated. Translation of the tmRNA open reading frame continues to a stop codon which releases the peptide and recycles the ribosome. The amino acid sequence encoded by tmRNA

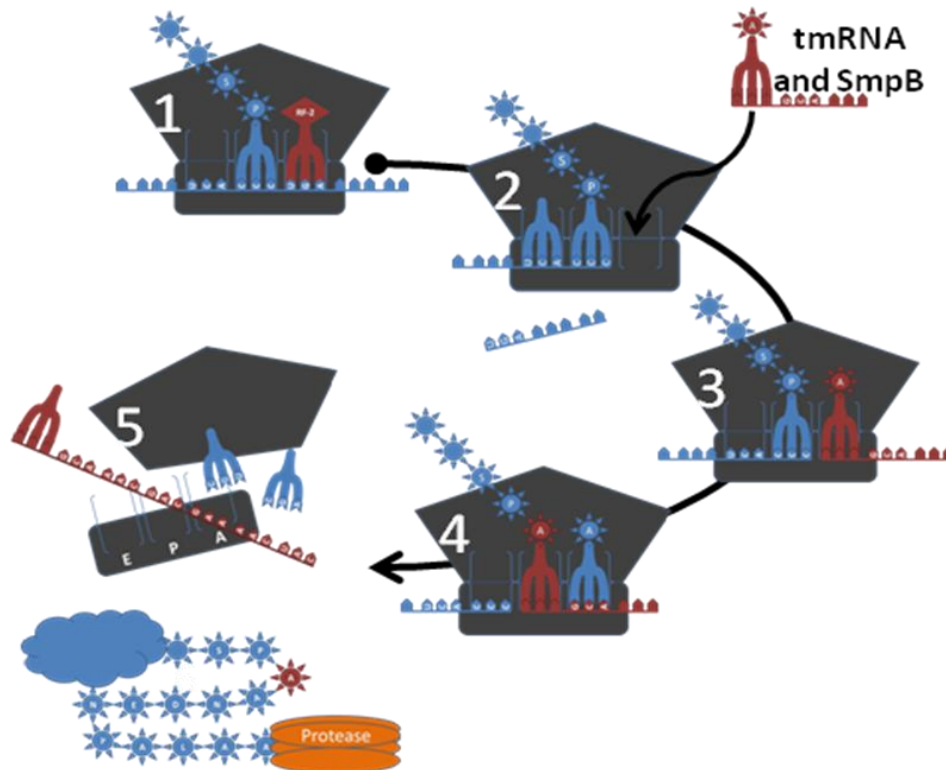


Figure 1-4 TRANS-TRANSLATION

When the ribosome stalls (1) the A-site mRNA is removed from the ribosome and the SmpB-tmRNA complex enters (2). The alanine of tmRNA is now positioned for peptidyl transfer (3). After peptidyl transfer and translocation, the open reading frame of tmRNA is positioned in the A site for elongation to resume (4). After termination at a stop codon on the tmRNA template, the ribosomal subunits are recycled and the tagged protein is degraded by proteases (5).

and added to the nascent peptide signals it to be destroyed by proteases. It has also been proposed that ribonucleases that associate with the tmRNA complex target and degrade the released mRNA to ensure it no longer can be translated¹⁵. It is because of this that tmRNA is conserved in all eubacterial genomes and has even been shown to be essential for a number of different bacterial species¹⁶⁻¹⁹.

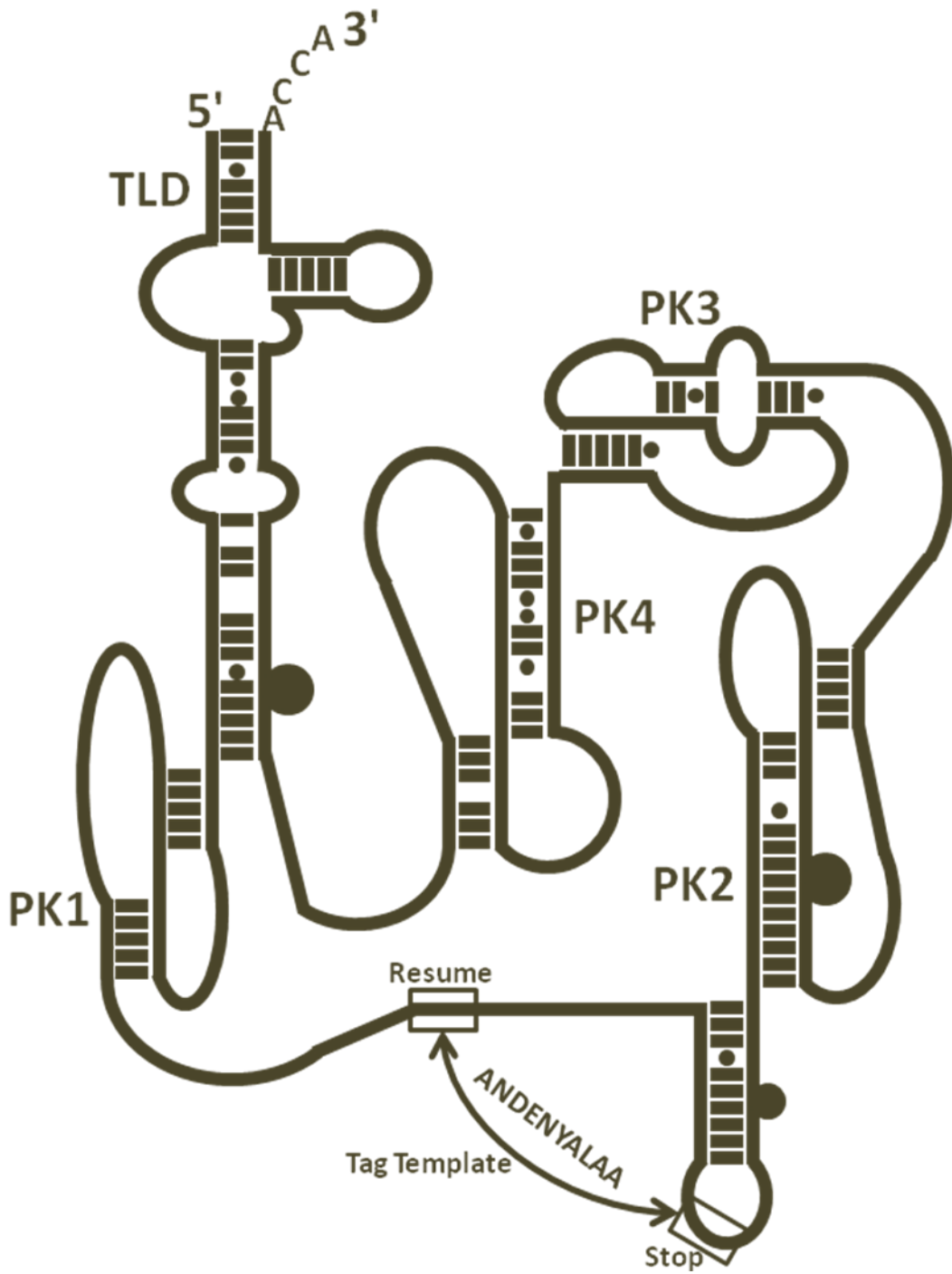


Figure 1-5. SECONDARY STRUCTURE OF tmRNA

The secondary structure of tmRNA with pseudoknots identified as PK1-PK4. The TLD is labeled at the 5' and 3' helix with the CCA at the very 3' end. Canonical base pairs are identified by dashes and non-canonical base pairs are identified by dots.

tmRNA AND SmpB STRUCTURE

tmRNA in *E. coli* is a 363 nucleotide RNA with four pseudoknots (PK) that form most of its structure (Figure 1-5). These pseudoknots convey structural stability. Although PK 2-4 can be deleted with little loss of function, PK1 needs a thermodynamically stable structure to maintain trans-translational activity²⁰. Connecting pseudoknots 1 and 2 is the open reading frame of tmRNA that encodes the degradation tag ANDENYALAA(Stop). The tRNA-like domain (TLD) is formed by the 5' and 3' ends through loops and helices that interact with SmpB (Figure 1-5 and Figure 1-6). SmpB is 160 amino acids long and folds into an antiparallel β -barrel and three helices. A 30 amino acid long C-terminal tail comes out of the protein and due to its lack of structure, cannot be seen by cryo-EM or crystal structures.



Figure 1-6. tmRNA-SmpB COMPLEX MOLECULAR MIMICRY

SmpB (light blue) and the TLD of tmRNA (dark blue) structurally mimic tRNAs. The structures shown are a yeast tRNA^{Phe} in red and a *T. Thermophilus* tRNA^{ser} in green³. Reprinted with permission by PNAS.

Functional activity of tmRNA is known to be linked to the cellular expression and association of SmpB¹⁸. Using its beta barrel core, SmpB binds tmRNA at its TLD with high affinity (Figure 1-6). This interaction between SmpB and tmRNA protect both molecules from

being degraded within the cell^{21,22}. The binding of tmRNA by SmpB creates a structural mimic of a cellular tRNA³ (Figure 1-6). This tRNA conformation is essential for tmRNA aminoacylation by alanyl-tRNA synthetase, acceptance into the A-site of the ribosome, and peptide transfer^{3, 18, 21-24}.

THE RIBOSOME AND TRANS-TRANSLATION

The goal of the research presented in this thesis is to find ribosomal RNA mutations that inhibit tmRNA function. Ideally, these mutations would specifically inhibit trans-translation and not canonical translation. Regions of the rRNA that are likely essential for tmRNA function are the peptidyl-transferase center, decoding center, or GTPase center of the ribosome. These regions play critical roles in canonical translation as well.

For example, the 16S bases A1492, A1493 and G530 in the decoding center (the 30S A site) undergo conformational changes upon correct pairing of the mRNA codon to the tRNA anticodon^{25,26}. These conformational changes are vital to tRNA selection and translational fidelity. It is also believed that the SmpB-tmRNA complex is accepted into the A site by SmpB interacting directly with these same bases at the decoding center. This is supported by cryo-EM data that orients SmpB to the decoding center of the ribosome and biochemical data that suggest that SmpB interacts directly with A1492, A1493 and G530^{27,28}. SmpB tail mutations and truncations abolish the ability of tmRNA to transfer Ala to the nascent peptide, though they have no effect on tmRNA binding to the ribosome²⁹. Therefore, mutations to the decoding center might decrease the translation of the tmRNA tag by rejecting the SmpB-tmRNA complex from being accepted into the A site. To the extent that tmRNA function relies on these same conserved

nucleotides, however, mutating them might also inhibit normal tRNA acceptance into the A site and overall protein production.

Another way that ribosomal mutations could inhibit trans-translation would be to reduce stalling. This could be accomplished at stalling sequences like Pro-stop by rRNA mutations that increased termination at C-terminal Pro residues. In the case of stalling at strings of rare Arg codons, mutations could increase the miscoding frequency. If readthrough or frameshifting rates increase in a given rRNA mutant, stalling could be reduced and the ribosomes would no longer need rescue by tmRNA.

Since the SmpB-tmRNA complex makes extensive contacts with the ribosomal rRNA and proteins^{27, 30} it is likely that ribosomal mutations could also decrease the binding affinity of tmRNA and or SmpB. This could prevent tmRNA recruitment to the A site. It could also prevent translocation of tmRNA to the P site. It has been shown that SmpB has an affinity for the A and P sites of the ribosome even without tmRNA³¹. As discussed above, SmpB plays an important role in the acceptance of the SmpB-tmRNA complex into the A site of the ribosome. Its highest affinity binding site, however, is the 30S P site, and so SmpB may also play a critical role for tmRNA translocation to the P site. This is most likely done by a different mechanism than translocation of tRNAs since they are attached to mRNA which is in contact with the P site. In the case of trans-translation SmpB contacts the P site. Translocation of tRNAs employs specific residues of the 16S rRNA³². Because these bases are most likely unimportant to translocation of the SmpB-tmRNA complex, then 16S mutations that did not include these bases could inhibit translocation of SmpB and tmRNA without affecting translocation of tRNA important to protein production.

A final way in which inhibition of trans-translation might be effected would be interfering with placement of the first codon of the tmRNA open reading frame in the A site. The role of tmRNA-ribosomal interactions in resume codon placement is minimal. Mapping of the interactions between tmRNA in the ribosomal P site showed few contacts at the TLD and PK-1³³. Translation resumes at the GCA Ala codon (underlined) in the sequence U₈₅AGUCGCA in tmRNA. For instance the U85A mutation causes the ribosome to resume in the -1 frame on the C₈₉GC codon rather than on the G₉₀CA resume codon³⁴. Likewise the A86C mutant resumes translation exclusively in the +1 frame placing C₉₁AA in the A site^{34,35}. SmpB is thought to be the element of the tmRNA-SmpB-ribosomal complex that interacts with the tmRNA upstream nucleotides. This is supported by the finding that several SmpB mutants restore translation in the proper frame on A86C tmRNA³⁶ and the finding that SmpB protects these nucleotides from reacting with chemical probes *in vitro*^{37,38}.

Mapping of SmpB with the ribosome showed several interactions with both the A and P sites of the 16S and 23S rRNA³¹. These tight interactions explain why SmpB has such a high affinity for the ribosomal P site *in vitro* even without tmRNA³⁹⁻⁴¹. Because SmpB is essential to the placement of the open reading frame of tmRNA it must associate with the ribosomal P site and have correct positioning for functional rescue to take place. The ribosomal P-site rRNA is therefore a potential target for ribosomal mutations that could inhibit translation of the tmRNA tag without affecting canonical translation.

The questions remain, what mutations of the ribosome could decrease stalling or inhibit the SmpB-tmRNA complex from interacting with the ribosome and carrying out efficient trans-translation and not effect translation? Since many mutations could likely affect both, we approached this question by randomization of the rRNA within the ribosomal subunits and a

selection against tmRNA tagging. This approach allows us to test many rRNA nucleotides for their ability to reduce trans-translation but not translation all at once.

CHAPTER 2 rRNA MUTATIONS THAT INHIBIT TRANSFER-MESSENGER RNA ACTIVITY ON STALLED RIBOSOMES

This chapter is modified from the published article: Crandall, J.; Rodriguez-Lopez, M.; Pfeiffer, M.; Mortensen, B.; Buskirk, A., rRNA mutations that inhibit transfer-messenger RNA activity on stalled ribosomes. *J Bacteriol* **2010**, 192, (2), 553-9.

INTRODUCTION

Defects in protein synthesis threaten cell viability in eubacteria. mRNA transcripts lacking stop codons (non-stop mRNAs) arise from premature termination of transcription and from mRNA decay by 3'-5' exonucleases. Since release factors cannot be recruited to non-stop mRNAs, ribosomes stall at the 3'-end and trap additional ribosomes upstream. In *Escherichia coli*, roughly 1 in every 250 translation reactions leads to ribosome stalling, so that if they were not rescued and recycled, all the ribosomes would be stalled in a single generation⁴².

A highly-conserved quality control system shields eubacterial cells from the negative effects of ribosome stalling (for a review, see¹⁹). All known eubacterial genomes contain genes encoding transfer-messenger RNA (tmRNA) and its protein partner SmpB⁴³. As implied by its name, tmRNA has two functions. First, tmRNA mimics a transfer RNA: it is aminoacylated with alanine, enters the A site of stalled ribosomes together with SmpB, and adds Ala to the nascent peptide chain. tmRNA then serves as an mRNA template, encoding a short peptide tag that is recognized by cellular proteases⁴⁴. After this tag is translated, the ribosome is released at a stop codon within tmRNA and the aborted protein is degraded. Because the ribosome translates a protein from two RNA templates, this tagging process is known as *trans*-translation.

In addition to rescuing ribosomes on non-stop mRNAs, tmRNA can also act on ribosomes stalled on intact transcripts. Stalling can occur during elongation at clusters of rare

codons, such as the Arg codons AGA, AGG, and CGA⁴⁵⁻⁴⁷. The ribosome pauses on rare codons because the cognate tRNAs are in low abundance and therefore bind and react slowly. Stalling can also occur during translational termination when release of the nascent polypeptide is inefficient. For example, tmRNA tags the full-length YbeL protein in *E. coli*; the C-terminal Glu-Pro sequence in YbeL inhibits release factor activity⁷. When combined with an inefficient opal stop codon (UGA), a C-terminal Pro residue can lead to levels of stalling so high that 40% of the full-length protein is tagged by tmRNA⁷.

We have taken a genetic approach to the study of ribosome stalling and rescue, developing genetic selections for and against tmRNA function. While tmRNA is essential in some bacterial species¹⁶, deletion of the tmRNA gene *ssrA* in *E. coli* yields only minor phenotypic changes²⁴. We previously reported a KanR-based selection that ties the life of the cell to tmRNA function²⁰. This selection has proved valuable in studying mutants of tmRNA and SmpB to understand how the ribosome resumes translation on tmRNA^{20, 36}. Here we report two novel selections in which tmRNA function leads to cell death. To our knowledge, these are the first genetic selections developed against tmRNA activity. We apply these negative selections to identify ribosomal RNA mutations that inhibit tagging at two different stalling sequences. Our goal is to understand which ribosomal elements are specifically required for *trans*-translation and the role of the ribosome in the rescue process.

MATERIALS AND METHODS

Plasmid construction—The bar-R selection plasmid was generated by amplifying the barnase gene with PCR using the primers 3483 CATATGGCTAGCCAGGTTATCAACACGTTTGAC and 3600 GAGCTCGAATTCGTCACCTTGTTTTGTAAATCAGCCAGTCGC. The PCR

product was cloned downstream of the *araBAD* promoter in pBAD-GFP using the NheI and EcoRI restriction sites. The bar-P selection was cloned in a similar manner, except that barnase was amplified with primer 3483 and 3722

GAGCTCGAATTCGTCAGGGTGAGTAAAGAATCCGGTCTG. The tmRNA-B1 expression plasmid was generated by amplifying the pKW11 plasmid⁴⁸ by inverse PCR using the primers 3486 GATCCGCTAATAAAGGATTCTTGGTCCTCTCTCCCTAGCCTCC and 3487 TTGGTAAAGGTCTGATAATGGTCGACTATTTTTTGCGGCTTTTTAC. Following phosphorylation with polynucleotide kinase, the blunt ends of the PCR product were ligated together. The tmRNA-B2 plasmid was cloned in a similar manner with primer 3486 and 3721 TTGGTAAAGGTCTGATAATGGTCTGTTGTTTTGTAAATCAGCCAGTCGACTATTTTTT GCGGCTTTTTA.

To create the ptRNA-BT selection plasmids, the *tetR* and *ssrA* genes were amplified from the tmRNA-B1 or B2 plasmids above with the following primers

TTCGCCTCCGGATTGACAGCTTATCTTCGATTAGCTTTAATGCGGTAGTTTATC and ACTCACTCCGGAAAATAAATCCTGGTGTCCCTG and the PCR product was inserted into the BspEI site in ptRNA-67⁴⁹. Into the HindIII site of the resulting plasmids we inserted the *araC* gene and *bar-R* or *bar-P* expression cassettes amplified with the primers ACCAGCAAGCTTTTCACCGTCATCACCGAAAC and TAAACACAAGCTTCGCAGAAAGGCC.

The maltose binding protein (MBP) vectors are derivatives of pMAL-C2G (New England Biolabs) in which the *lacI* coding sequence was deleted and MBP is expressed constitutively from the *lacI* promoter. We combined this MBP expression cassette with a pCDF origin and TetR marker, creating pCDF-MBP. To add stalling sequences, the 3'-end of the MBP gene was

amplified with the primer TAACAAAGATCTGCTGCCGAACC and either GGAAGCCTGCAGTCAGGGTGAAGTCTGCGCGTCTTTCAGGG (Ser-Pro-opal) or GGAAGCCTGCAGTCACCTCCTCCTCCTCCTCCTCCTCCTAGTCTGCGCGTCTTTCAGG GC (eight rare AGG Arg codons) or GGAAGCCTGCAGTCACCTAGTCTGCGCCTCTTTCAGGGC (Arg-opal) or ACAAGGCTGCAGAAAAAAGCCCGCTCATTAGGCGGGCTGCGGTCTGCGCGTCTTTC AGGG (non-stop, adding the *trpA* terminator). These PCR products were digested with BglII and PstI and ligated into the expression vector.

The GST recoding vectors were created by inverse PCR of pCDF-MBP with the primers CCCTACTGCAGGCAAGCTTGGCACTG and TTGCTAGGTACCATTACACCCTGAATTGACTC to add PstI and KpnI sites. GST was amplified from pGEX-3 (GE Healthcare) using the forward primer TTGCTAGGTACCATGTCCCCTATACTAGGTTATTG and one of the following reverse primers:

GGAAGCCTGCAGTTATTACTTATCGTCATCGTCCTTATAGTCTTGTCAGGGAGAGAG GATCCCACGACC (readthrough),

GGAAGCCTGCAGTTATTACTTATCGTCATCGTCCTTATAGTCTTGGTCAGGGAGAGA GGATCCCACGACC (plus one frameshifting), or

GGAAGCCTGCAGTTATTACTTATCGTCATCGTCCTTATAGTCTTGGGTCAGGGAGAG AGGATCCCACGACC (minus one frameshifting). The GST amplicons were digested and ligated into the PstI and KpnI sites. *araC* and the *araBAD* promoter were amplified with the primers AGATCCACTAGTACTGACAGATCTTCGCTACGTGACTGGGTC and

GGACATGGTACCCATATGTATATCTCCTTCTTAAAGTTAAAC and inserted between SpeI and KpnI sites upstream of GST to drive its expression.

Table 2-1. Bacterial strains and plasmids.

Material	Genotype or Description	Reference
SQ171	<i>ArrnG ArrnA ArrnD ArrnE ArrnH ArrnB ArrnC recA⁻ ptRNA67 pKK3535</i>	49 and Squires
X90 ssrA::cat	F' <i>lacI^d lac' pro' / ara Δ(lac-pro) nalA argE(am) rif^r thi-1 ssr::cat</i>	44
pbar-R	pBR322 derivative, expresses truncated barnase (Δ10) from <i>araBAD</i> promoter, with rare Arg-opal at the C-terminus	This study
pbar-P	pBR322 derivative, expresses truncated barnase (Δ18) from <i>araBAD</i> promoter, with Pro-opal at the C-terminus	This study
ptmRNA-B1	Tet ^r and p15A origin, expresses tmRNA with 10 residue barnase tag	This study
ptmRNA-B2	Tet ^r and p15A origin, expresses tmRNA with 18 residue barnase tag	This study
ptRNA67	Spc ^r p15A origin, expresses tRNAs missing from the genome of SQ171	49
ptRNA-BT1	Derivative of ptRNA67, Tet ^r and expressing the <i>bar-R</i> and <i>tmRNA-B1</i>	This study
ptRNA-BT2	Derivative of ptRNA67, Tet ^r and expressing the <i>bar-P</i> and <i>tmRNA-B2</i>	This study
pTS-rrnC	Expresses wild-type rRNA; temperature-sensitive SC101 origin	This study
pCDF-MBP	Tet ^r and CDF origin, expresses MBP constitutively with various stalling sequences at the C-terminus	This study
pCDF-GST	Tet ^r and CDF origin, expresses GST from <i>araBAD</i> promoter with Pro-opal followed by the FLAG epitope in various frames	This study
rRNA libraries	Derivatives of pLK45 with mutant rRNA cloned in targeted segments of either the 16S or 23S genes	50

Selection—*rRNA* libraries generously provided by Alexander Mankin⁵⁰ were amplified and introduced into the SQ171 strain carrying either the ptRNA-BT1 or -BT2 selection plasmids. The cells were rescued for 2 h at 30 °C in 2xYT with 0.2% glucose then added to 400 mL of 2xYT, 0.2% glucose, and 100 μg/mL ampicillin. The culture was grown 20 h at 37 °C to allow for loss of the wild-type ribosome plasmid, ribosome turnover, and the synthesis of the new mutant ribosomes. The culture was then plated onto media containing ampicillin and 2%

arabinose to induce barnase expression. Plasmid DNA was extracted from the pool of surviving cells and resubjected to the selection procedure.

Immunoblots—Tagging of the MBP protein was assayed by immunoblot as described³⁵ except that expression of MBP was constitutive, not induced with IPTG. Analysis of recoding events on GST was performed similarly, except that expression was induced with 2% arabinose for 2 h and readthrough or frameshifting was detected with a monoclonal anti-FLAG antibody (Sigma).

RESULTS

DEVELOPMENT OF A SELECTION AGAINST tmRNA TAGGING AT RARE ARG CODONS

To isolate ribosomal RNA mutations that prevent tmRNA function, we created a genetic selection that links tagging to cell death. We altered tmRNA so that instead of tagging proteins for proteolysis, it completes the synthesis of a toxic protein. Barnase is a 110-residue ribonuclease that is highly toxic; it has been used previously in negative selections with excellent results^{51,52}. A catalytic base, His102, is required for activity⁵³. We removed this critical residue by deleting the last 10 amino acids in the barnase protein. This truncated protein is harmless. We created a tmRNA mutant (tmRNA-B1) that rescues ribosomes stalled during barnase synthesis and completes the protein by encoding the last ten amino acids, DHYQTFTKIR (Figure 2-1A). To induce stalling at the proper site, we mutated Thr100 to the rare Arg codon AGG, followed by the opal stop codon UGA, yielding the *bar-R* construct. As shown previously, inefficient release at the opal stop codon traps the cognate Arg-tRNA, leading to further depletion of this rare tRNA and stalling at this Arg codon⁴⁶. Upon rescue, the nascent barnase protein is transferred to Ala-tmRNA and the ribosome switches to the tmRNA-B1 open

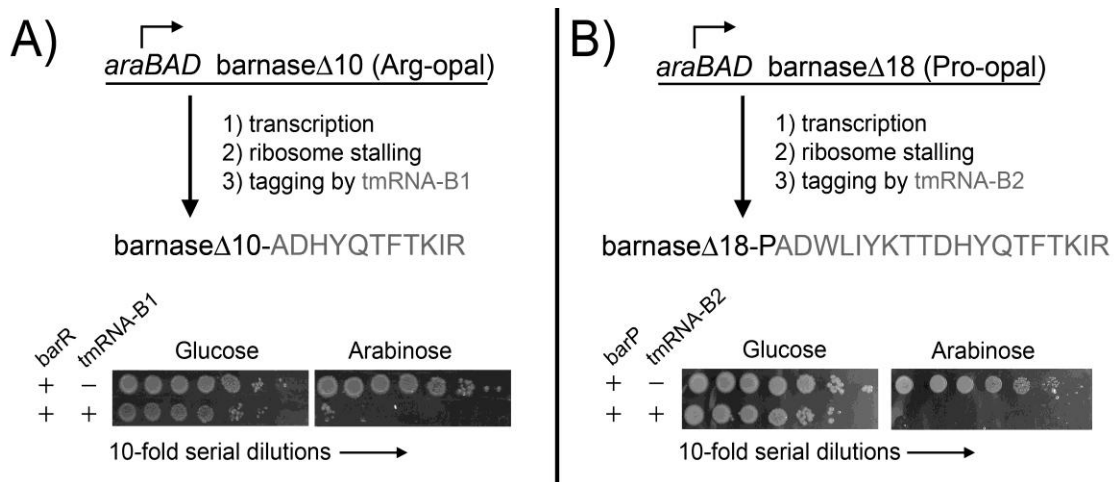


Figure 2-1. GENETIC SELECTION AGAINST TRANS-TRANSLATION

tmRNA directs the completion of the toxic ribonuclease barnase, leading to cell death. A) Transcription of a truncated barnase gene ending in Arg-opal is driven by the arabinose-inducible *araBAD* promoter. Ribosomes stall at the 3'-end of barnase at the rare Arg codon AGG due to sequestration of the rare cognate tRNA. tmRNA with a modified tag sequence (tmRNA-B1) rescues the stalled ribosomes and encodes the missing residues, after which full-length barnase kills the cell. Expression of the barnase construct (*bar-R*) is only toxic when co-expressed with tmRNA-B1 and induced with 2% arabinose (bottom). B) A second, similar selection in which stalling is induced by inefficient termination at a Pro-opal sequence at a different site in the barnase protein, which is then tagged by tmRNA-B2.

reading frame, completing the toxic protein. Barnase synthesized through the tagging process contains only one mutation, Thr100Ala.

To validate this negative selection, we transformed a strain lacking tmRNA (X90 *ssrA::cat*) with a plasmid encoding *bar-R* driven by the arabinose-inducible *araBAD* promoter. Plating of serial dilutions reveals that *bar-R* alone is non-toxic, as cells grow equally well on glucose or arabinose (Figure 2-1A). In contrast, fewer than 1 in 10^4 cells containing both *bar-R* and *tmRNA-B1* survive on media containing arabinose (Figure 2-1A). These results show that stalling and rescue of the barnase protein by tmRNA-B1 leads to cell death.

DEVELOPMENT OF A SELECTION AGAINST tmRNA TAGGING AT PRO-OPAL

We also created a second barnase selection in which stalling occurs at Pro-opal rather than Arg-opal. We anticipated that such a selection might identify mutants that inhibit stalling, perhaps by suppressing the inefficient termination at C-terminal Pro residues⁷. Like the *bar-R* selection described above, the *bar-P* selection relies on stalling at a truncated barnase gene and rescue by a modified tmRNA to complete barnase and kill the cell (Figure 2-1B). We truncated barnase by 18 amino acids by mutating Ser92 to Pro and introducing an opal stop codon at codon 93. The tmRNA template was altered to encode the missing barnase residues DWLIYKTTDHYQTFTKIR (hereafter referred to as *tmRNA-B2*). Two mutations occur in the final barnase protein: the Ser92Pro mutation and insertion of Ala between residues 92 and 93. As these changes occur in a surface-exposed loop between two β -strands⁵⁴, this scar was not anticipated to affect barnase function. Analysis of cells containing *bar-P* and *tmRNA-B2* confirms the toxicity upon induction with arabinose, while *bar-P* alone is non-toxic. Fewer than 1 in 10⁶ cells survive the *bar-P* selection on arabinose (Figure 2-1B).

SELECTION FOR 16S rRNA MUTANTS THAT INHIBIT TAGGING AT ARG-OPAL

The isolation of ribosome mutants that prevent tagging requires that each cell contain only one rRNA operon, since wild-type ribosomes would tag barnase and kill the cell. Squires and co-workers have generated *E. coli* strains in which each of the seven ribosomal RNA operons is genetically deleted (SQ171 *recA*-) (⁴⁹ and Selwyn Quan and Cathy Squires, personal communication). We introduced two plasmids into the SQ171 strain to adapt it to our selection. pTRNA-BT1 encodes tRNAs missing from the genome as well as the selection genes *bar-R* and *tmRNA-B1*. The second plasmid, pTS-*rrnC*, expresses the *rrnC* ribosomal RNA operon and has

a temperature-sensitive pSC101 origin. This plasmid is rapidly lost at 37 °C if the cells have another source of rRNA, allowing us to exchange it for plasmids encoding mutant rRNA operons. This approach ensures that mutant ribosomes function efficiently in all stages of translation because they are solely responsible for protein synthesis. In effect, this is a built-in positive selection for ribosome function added to the negative selection against stalling and tagging.

To isolate mutants that inhibit tagging at Arg-opal, we screened a library of 10^4 - 10^5 mutant 16S rRNA genes⁵⁰ in the SQ171 ptRNA-BT1 selection strain. The small subunit RNA was chosen because we were initially interested in how tmRNA and SmpB interact with the decoding center upon entering the A site of stalled ribosomes. After three rounds of selection and enrichment, nearly all of the transformed cells survived on selective media. Sequencing of the 16S rRNA in surviving colonies revealed three clones containing one mutation each: the point mutant A1150G or deletion of A1150 or U1123. It is impossible to tell which nucleotide in 1150-1152 (AAA) is deleted; the same is true of 1121-1123 (UUU). Since these two sequences pair together, we believe that all three mutations have the same structural consequence (see Discussion). The activity of all three clones was confirmed by retransformation and testing of single colonies in the barnase selection. Serial dilutions demonstrate robust survival of the representative A1150 Δ mutant upon induction of barnase with arabinose (Figure 2-2). Growth curves reveal that the A1150 Δ mutation causes a mild growth defect (doubling time of 65 min compared to the wild-type 59 min), consistent with a general defect in translation.

23S rRNA MUTANTS THAT INHIBIT TAGGING PRO-OPAL

We also isolated ribosomal RNA mutations that inhibit tagging at Pro-opal. Initially we hoped to find rRNA mutants that would restore efficient termination. We therefore chose the 23S rRNA for mutagenesis because it contains the peptidyl-transferase center, the site where the peptide is hydrolyzed from tRNA during the termination reaction. We first tested 23S mutants in the *bar-P* selection that were reported in the literature to inhibit stalling on the peptides SecM and TnaC. These mutants include A2058G, U2609C, U754A, and A_{ins}751^{55,56}. None of them showed an increase in survival compared to wild-type rRNA (data not shown). We therefore set out to identify mutants from random rRNA libraries. A library of 10^4 - 10^5 23S mutants⁵⁰ was introduced into the SQ171 ptRNA-BT2 selection strain. After two rounds of enrichment, nearly all the transformants on the selection plates survived. Sequencing of the 23S rRNA genes revealed two clones, one with the single C889U mutation and the other with two mutations, C889U and U846C. The activity of these mutants was verified by retransformation and testing of single colonies in the barnase selection (for C889U, see Figure 2-2).

The C889U and U846C mutations are found in helix 38, known as the A-site finger or ASF⁵⁷. This helix forms intersubunit bridge B1a, crossing over the A site and contacting the S13 protein in the small subunit⁵⁸. The C889U mutation is found at the tip of the finger in the loop that contacts S13. Perhaps this mutation exerts its effect by disrupting bridge B1a. We tested this hypothesis by performing the *bar-P* selection on cells containing a 22 nt deletion in helix 38 (H38 Δ 22), effectively destroying the B1a interaction⁵⁹. No increase in survival was observed, indicating that disruption of the B1a interaction is insufficient to prevent tagging (Figure 2-2).

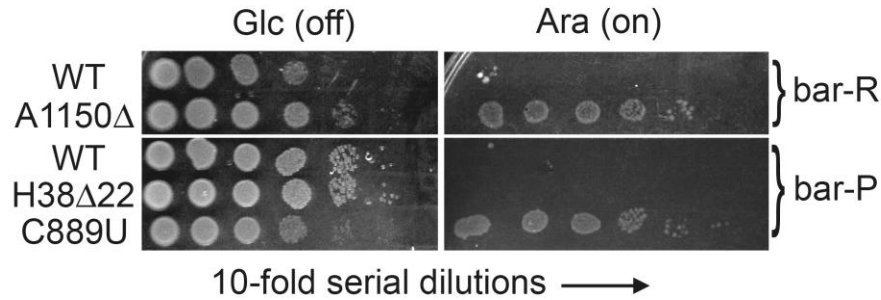


Figure 2-2. RIBOSOMAL RNA MUTANTS THAT SURVIVE THE BARNASE SELECTION

SQ171 cells expressing a single rRNA operon were plated on media containing either 2% arabinose to induce barnase expression or 0.2% glucose to repress it. Wild-type rRNA and a 16S mutant lacking A1150 were characterized in the *bar-R* selection (top). Two helix 38 mutants were compared to wild-type rRNA in the *bar-P* selection (bottom). 10-fold serial dilutions of the culture are shown from left to right.

Previous studies have shown a slight growth defect from truncating helix 38 and disrupting B1a⁵⁹⁻⁶¹. We measured growth rates for SQ171 cells expressing either wild-type or C889U mutant rRNA and found them to be identical (with a 59 min doubling time). Since there is only one ribosomal RNA operon in these cells, the mutant ribosomes must be capable of performing essential functions in translation as well as wild-type ribosomal RNAs.

THE SELECTED MUTATIONS INHIBIT TAGGING AT THEIR RESPECTIVE STALLING SEQUENCES

To confirm that the selected rRNA mutants rescue cells in the barnase selection by reducing the amount of tagged protein produced, we performed immunoblots to measure tagging levels directly. To test the 16S A1150Δ mutant isolated in the *bar-R* selection, we expressed the full-length maltose-binding protein (MBP) with a rare Arg-opal sequence at the C-terminus to induce stalling. We altered the tmRNA template to encode the sequence ANDHHHHHHD so that tagging can be detected by anti-His₆ antibodies³⁵. These changes in the tag also prevent rapid proteolysis of the tagged product⁴⁸. Analysis of the Arg-opal construct in SQ171 revealed

that tagging is reduced nearly three-fold in the mutant strain compared to wild-type (Figure 2-3, lanes 5 and 6).

Similarly, we tested whether our selected 23S mutants inhibit tagging using an MBP construct ending in Pro-opal. Using anti-His₆ antibodies to visualize the addition of the tmRNA tag, we see a four-fold decrease in tagging in the C889U and double mutant strains compared to wild-type (Figure 2-3, lanes 1-3). No reduction of tagging was seen in the H38Δ22 mutant (lane 4), as predicted by its inability to survive the *bar-P* selection. These immunoblot data show that both the A1150Δ and C889U mutations reduce tagging levels at their stalling sequences, Arg-opal and Pro-opal, respectively, confirming the genetic results above.

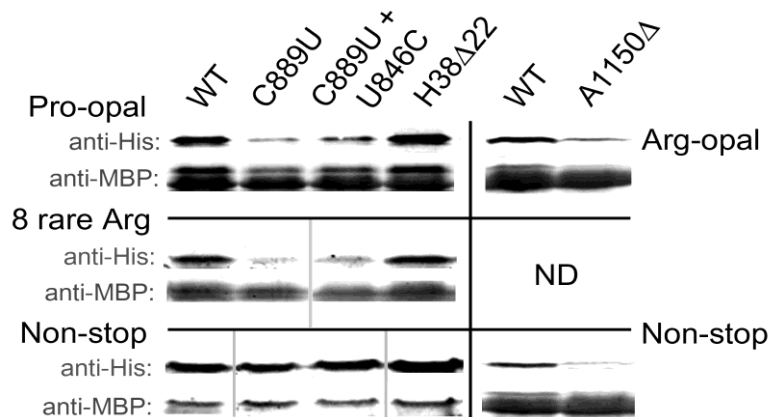


Figure 2-3. ANALYSIS OF TAGGING LEVELS IN MUTANT STRAINS

The maltose binding protein (MBP) was expressed with various stalling sequences at the C-terminus: Pro-opal, Arg-opal, a string of eight rare Arg codons (AGG), or a non-stop MBP construct containing the *trpA* transcriptional terminator. Tagging of MBP by a modified tmRNA encoding a His₆ tag was detected by anti-His₆ antibodies. Anti-MBP antibodies were used to control for protein expression and loading. Band intensities were quantified in triplicate to yield the ratios described in the text. Dark lines separate blots and lighter lines separate lanes cropped from the image of the same blot without other manipulation.

C889U INHIBITS TAGGING AT A CLUSTER OF RARE ARG CODONS

We also tested the mutants at other stalling sequences to determine which step in the stalling and tagging process is defective. We initially anticipated that the *bar-P* selection might yield 23S mutants that restore efficient termination at the sequence Pro-opal. If the C889U mutant acts in this manner, tagging should be inhibited only at Pro-opal and not when ribosomes are stalled for another reason, such as a string of rare Arg codons. We tested this hypothesis by inducing stalling at the C-terminus of MBP with eight rare Arg codons (AGG), measuring tagging in cells containing either wild-type or mutant rRNA. Tagging was dramatically reduced in the C889U and C889U / U846C mutants compared to the wild-type and H38Δ22 rRNAs (Figure 2-3). Since the inhibition of tagging is not specific to Pro-opal, it seems unlikely that the C889U mutant acts by increasing termination efficiency, which presumably is not relevant to stalling and tagging on the eight-Arg sequence. We were unable to test the 16S A1150Δ mutant with the eight-Arg MBP construct as its growth was severely inhibited. This increased sensitivity to Arg-tRNA depletion and ribosome stalling is consistent with a defect in the rescue process in the A1150Δ strain.

A1150Δ GLOBALLY INHIBITS TMRNA FUNCTION WHEREAS C889U DOES NOT

Since the C889U mutation inhibits tagging at both Pro-opal and a string of rare Arg codons—unrelated stalling sequences—it could be that it globally inhibits all tmRNA function. This would occur if the mutation prevents the ribosome from interacting productively with tmRNA or its associated protein, SmpB. To test this possibility, we measured tagging levels on constructs which stall at a non-stop mRNA. The *trpA* transcriptional terminator was cloned following the full-length MBP gene, creating an mRNA of defined length that lacks a stop

codon. Analyzing tagging at non-stop mRNAs allows us to study the trans-translation process alone, apart from stalling and other upstream events.

Analysis with anti-His₆ antibodies shows no loss of tagging in either the C889U or the C889U / U846C mutants on the non-stop construct (Figure 2-3). In contrast, tagging in the A1150Δ mutant is decreased roughly three-fold versus the wild-type strain. This level of reduction is similar to that seen with Arg-opal. We conclude that the A1150Δ mutant in 16S inhibits tmRNA function directly, while the selected 23S mutations do not inhibit the tagging process itself but some upstream step.

THE C889U MUTATION DOES NOT INDUCE RECODING EVENTS

The C889U mutation reduces tagging at two different stalling sequences (Pro-opal and eight-Arg) but does not inhibit tmRNA activity itself on a non-stop mRNA. One explanation of these results could be that it induces higher levels of recoding events. Stalled ribosomes can shift reading frame or read through stop codons, incorporating a suppressor tRNA. High levels of +1 frameshifting have been reported at the Pro-opal sequence CCC-UGA^{62, 63}. Recoding is an attractive explanation because it is downstream of stalling (i.e. slower than efficient termination) and upstream of tagging. Furthermore, deletion of the A-site finger is reported to cause an increase in +1 frameshifting levels⁵⁹.

To test this hypothesis, we measured readthrough and frameshifting levels for ribosomes stalled at Pro-opal on the full-length GST protein. A sequence encoding the FLAG epitope was cloned downstream of the stop codon so that the GST-FLAG fusion protein is synthesized if and only if a recoding event occurs. Three constructs were created: one tests stop codon readthrough, another tests +1 frameshifting, and the last -1 frameshifting (Figure 2-4A).

Synthesis of the GST-FLAG fusion was detected by anti-FLAG antibodies on protein extracted from SQ171 cells expressing either wild-type or C889U mutant rRNAs.

As expected, the sequence CCC-UGA led to very high levels of +1 frameshifting. -1 frameshifting is at a far lower level and readthrough is barely detectable when analyzed on the same blot at the same intensity (Figure 2-4B, bottom). When each recoding event is analyzed separately, we clearly see no significant changes in the level of readthrough or in the levels of +1 or -1 frameshifting in the C889U mutant strain. These results show that the reduction in tagging in the C889U mutant strain is not a result of an increase in recoding events.

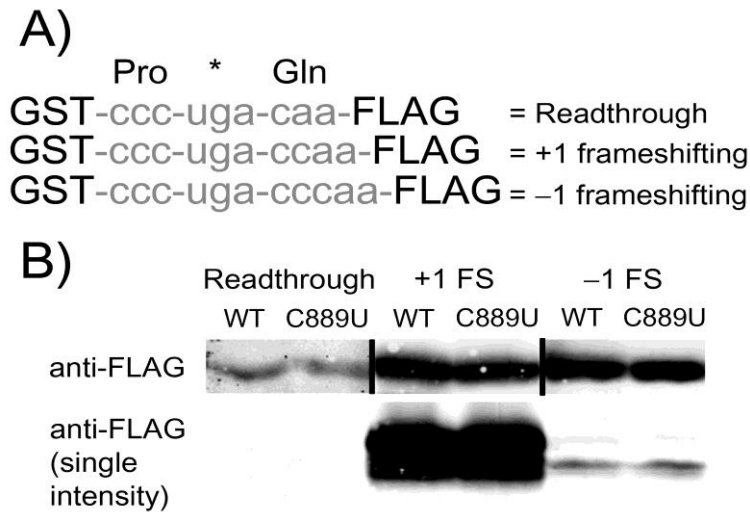


Figure 2-4. ANALYSIS OF RECODING EVENTS AT PRO-OPAL

A) The FLAG epitope was cloned after the stop codon in the GST-Pro-opal construct so that synthesis of the GST-FLAG fusion depends on readthrough, +1 frameshifting, or -1 frameshifting. **B)** Recoding events in SQ171 cells expressing wild-type or C889U rRNA were detected by immunoblot with anti-FLAG antibodies. The lower data are from a single blot at a single intensity, illustrating the relative levels of the three events.

DISCUSSION

To study ribosome stalling and rescue in *E. coli*, we created genetic selections that tie tmRNA activity to cell death. Stalling on a truncated barnase gene leads to rescue by a modified tmRNA and completion of the toxic protein. Stalling in the *bar-R* selection is induced by a rare Arg codon, while stalling in the *bar-P* selection is induced by inefficient termination at Pro-opal. These selections are powerful tools for identifying mutations in the translation machinery that prevent tmRNA function. In principle, they could also be used to identify small molecules that inhibit trans-translation. This would be of interest because the reduction of tmRNA activity sensitizes bacteria to antibiotics that target the ribosome⁶⁴.

We identified three separate 16S rRNA mutants that survive the *bar-R* selection: A1150G and deletion of either A1150 or U1123. The A1150 mutation inhibits tmRNA function not only at the Arg-opal sequence at which it was selected, but also on a non-stop mRNA. We conclude that this mutation interferes with tmRNA function directly, inhibiting the trans-translation process itself rather than an upstream step. All three likely work via the same mechanism; they map to a single site within helix 39 of 16S rRNA (Figure 2-5, right). Despite their distance in primary sequence, A1150 base pairs with U1123. These mutations are expected to destabilize pairing in the first stem of helix 39 near this base pair. Helix 39 forms a coaxial stack with helices 38, 36, and 35, stretching the whole length of the head of the 30S subunit⁶⁵. The S9 and S10 proteins bind helix 39; replacement of the A1152 phosphate with phosphorthioate inhibits 70S ribosome assembly⁶⁶, presumably because it interferes with proper S10 binding. It seems possible, then, that these mutations alter the conformation or dynamics of the head of the 30S subunit.

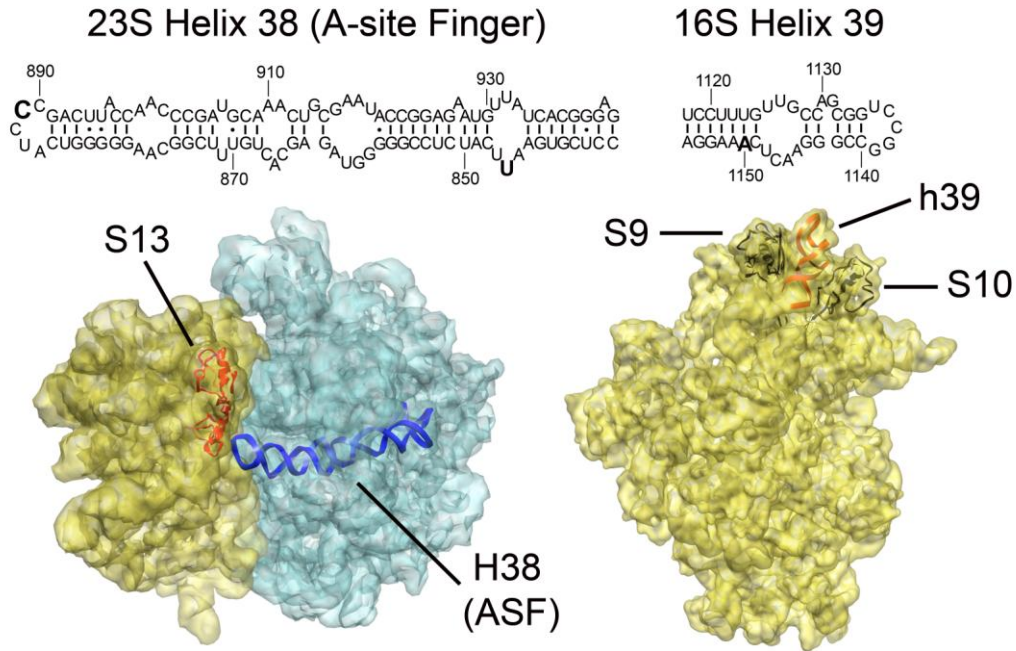


Figure 2-5. LOCATION OF THE SELECTED rRNA MUTANTS WITHIN THE RIBOSOME STRUCTURE²

Left: the U846C and C889U mutations lie within helix 38 of 23S rRNA. Helix 38, also known as the A-site finger (dark blue), lies in the central protuberance of the 50S subunit (light blue) and crosses over the A site where it binds to the S13 protein (red) in the head of the 30S subunit (yellow), forming intersubunit bridge B1a. Right: the A1150Δ mutation lies within helix 39 of 16S rRNA, as do the A1150G and U1123Δ mutations identified in the bar-R selection. Helix 39 (orange) lies between the S9 and S10 proteins (black) at the top of the head of the 30S subunit (shown from the cytoplasmic side). Rendered with Chimera.

We likewise isolated two 23S rRNA clones, C889U alone or in combination with U846C, that survive the *bar-P* selection and reduce tagging at Pro-opal. As shown in Figure 5, these mutations are found in helix 38, the A-site finger (ASF), so called because it contacts the A-site bound tRNA^{57,67}. The ASF interacts with the S13 protein in the small subunit to form intersubunit bridge B1a⁵⁸. The B1 bridges (B1a, b, and c) are the sole link between the head substructure in the 30S subunit and the 50S subunit. During the translation process, the B1a interaction is broken and the ASF changes its binding partner from S13 to S19⁶⁸. This motion is

part of the ratchet-like rotation of the head that occurs during translocation upon EF-G binding⁶⁸. The fact that the ASF binds A-site tRNA and plays a role in ribosome conformational dynamics may explain the role of C889U in ribosome stalling and rescue.

The C889U mutation lies immediately next to the nucleotides 886-888 in the loop of the ASF which interact with S13⁵⁸. We hypothesized that the C889U mutation might exert its effects by disrupting the B1a interaction. In support of this idea, deletion of 22 nt at the tip of helix 38 was reported to reduce the energetic barrier to translocation⁵⁹. We found, however, that this H38Δ22 mutant does not lead to survival in the barnase assay nor does it lower tagging levels in the Pro-opal or at a string of rare Arg codons in the immunoblot assay. If changes in B1a are responsible for the reduction in tagging, it must be more subtle than mere disruption, perhaps favoring one ratchet rotation state over the other (S13 / S19). All in all, it seems that B1a plays a minor role in the normal translation process, since ASF truncations and S13 mutations have only minor effects^{59-61, 69}.

The ASF has a kink-turn motif near its base that is predicted to play a role in its flexibility and motion⁷⁰. As the U846C mutation lies within this kink-turn motif, it is tempting to speculate that it affects the structural dynamics of the ASF. The U846C mutant was only identified together with C889U, however, and the single and double mutants do not show any detectable difference in activity in either the immunoblot assays or barnase selection. It is therefore unclear if this mutation conveys any additional advantage.

How does C889U reduce tagging? It is unlikely that C889U restores high-efficiency termination, thereby reducing stalling. Two findings support this conclusion. First, although the C889U mutant was selected for its ability to inhibit tagging at Pro-opal, immunoblots show that it also reduces tagging levels at a cluster of eight rare Arg codons. While we have not directly

ruled out that stalling is reduced at Pro-opal, it seems more likely that a step downstream of stalling is inhibited, explaining both the Pro-opal and eight-Arg results. Secondly, if termination rates increased, the level of readthrough and frameshifting should decrease in the mutant strain, since these recoding events occur because of inefficient termination. We found, however, that C889U does not alter readthrough or frameshifting levels.

In contrast to the A1150 Δ case, the C889U mutant does not inhibit trans-translation itself. Since neither stalling nor tmRNA function appears to be inhibited in the C889U mutant, it seems that some intermediate step between the two must be affected. One possibility is that this mutation may affect processing of the stalled mRNA. Before tmRNA and SmpB can enter the A site of stalled ribosomes, the downstream mRNA must be removed, either by an A-site endonuclease or by the action of 3'-5' exonucleases⁷¹. mRNA sequences longer than 12 nt downstream of the stalled P site prevent rapid release of ribosomes by tmRNA⁷². An effect on mRNA processing is an appealing explanation because tagging is reduced only when stalling occurs in the middle, not at the 3'-end, of an mRNA. We are currently characterizing of the molecular mechanism of action of the C889U and A1150 Δ mutants. Determining the effects of these mutations will shed light on how the ribosome interacts with tmRNA and SmpB to bring about the rescue of stalled ribosomes.

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