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GENETIC ANALYSIS OF RIBOSOME STALLING AND RESCUE

by

Douglas R. Tanner

A dissertation submitted to the faculty of

Brigham Young University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Chemistry and Biochemistry

Brigham Young University

August 2009

BRIGHAM YOUNG UNIVERSITY

GRADUATE COMMITTEE APPROVAL

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As chair of the candidate's graduate committee, I have read the dissertation of Douglas R. Tanner in its final form and have found that (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

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## ABSTRACT

### GENETIC ANALYSIS OF RIBOSOME STALLING AND RESCUE

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Doctor of Philosophy

In eubacteria, ribosome stalling on broken messenger RNA transcripts can lead to cell death. The *trans*-translation quality control mechanism rescues many of these stalled ribosomes. In this process, tmRNA enters stalled ribosomes by mimicking a transfer RNA, accepting the stalled nascent peptide. The ribosome then releases the broken mRNA and resumes translation on a coding region within tmRNA itself. Translation of tmRNA marks the nascent peptide for destruction by the addition of a short proteolysis tag and the ribosome is released at a stop codon within the tmRNA open reading frame.

An intriguing aspect of *trans*-translation is that the ribosome synthesizes one protein from two RNA templates. How is the proper site chosen on tmRNA to resume translation? Do the conserved pseudoknot structures help set the reading frame? Using a genetic selection to assay libraries of tmRNA mutants, we found that stable hairpin structures can

functionally replace pseudoknot 1. We conclude that the role of pseudoknot 1 in tmRNA function is purely structural. Our results demonstrate that the inactivity of an RNA mutant designed to destroy a given structure should not be interpreted as proof that the structure is necessary for RNA function. Such mutations may only destabilize a global fold that could be formed equally well by an entirely different, stable structure.

Broken mRNAs are not the only cause of ribosome stalling; stalling can also result from nascent peptide interactions with the ribosomal exit tunnel that inhibit peptidyl-transferase activity. SecM, TnaC, and ErmCL all stall ribosomes to regulate the expression of downstream genes. What other peptide sequences can cause ribosome stalling? We modified our tmRNA-based selection to screen libraries of random peptides and identified a number of novel stalling peptides, including the sequence FxxYxIWPP. This sequence interacts with the exit tunnel differently than SecM and TnaC as seen in studies using mutant ribosomes. Like SecM, stalling occurs on this sequence with the next aminoacyl tRNA trapped in the A site but unable to react with the nascent peptide. These results show that a variety of peptides can interact in the exit tunnel and peptidyl-transferase center to regulate ribosome activity.

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## LIST OF ABBREVIATIONS

A site	Aminoacyl tRNA binding site
AB	antibody
cfu	colony forming units
E site	Ribosomal exit site
<i>E. coli</i>	<i>Escherichia coli</i>
EF	Elongation factor
EOP	Efficiency of plating
fMet	formylated methionine
GAC	GTPase associated center of ribosome
GDP	Guanosine Diphosphate
GST	Glutathione S transferase
GTP	Guanosine Triphosphate
His <sub>6</sub>	Six-histidine tag
IF	Initiation factor
MALDI	Matrix assisted laser desorption ionization
mRNA	messenger RNA
MS	Mass spectrometry
MS/MS	tandem mass spectrometry
nt	Nucleotides
P site	Peptidyl tRNA binding site of ribosome
PTC	Peptidyl transferase center of ribosome
RF	Release factor
rRNA	ribosomal RNA
S	Svedberg unit
SmpB	small protein B
tmRNA	transfer-messenger RNA
tRNA	transfer RNA
WT	Wild Type

## CHAPTER 1: INTRODUCTION TO RIBOSOME STALLING AND RESCUE

Ribosomes are large macromolecular complexes that catalyze the synthesis of every protein in a cell. Cells cannot live without properly functioning ribosomes. In fact, many antibiotics kill bacteria by binding to the bacterial ribosome and preventing proper protein synthesis. Through their central role in the expression of genetic information, ribosomes also participate in the regulation of a number of genes involved in various other aspects of protein synthesis.

The study of these essential protein synthesis machines has had significant impact on molecular biology. For example, our understanding of the role of mRNA and tRNA in protein synthesis was born of ribosome research (1). The recent determination of the atomic structure of ribosomes has taught us much about RNA structure. Many RNA structures in the ribosome have not been observed in any other context (2). Studying ribosomes has also answered many questions about the mechanism of antibiotic action as well as antibiotic resistance. Approximately 40% of antibiotic drugs target components of the ribosome or translation factors (3). Some antibiotics stall translation by binding to the catalytic peptidyl-transferase center while others do so by blocking the binding site of incoming tRNAs. Certain ligands, essential for translation, are also targeted by antibiotics to prevent them from assisting with the steps of translation.

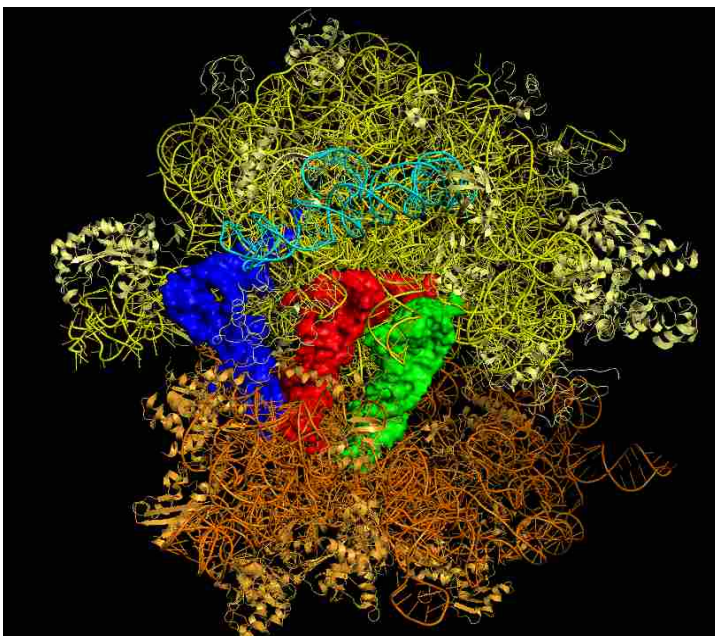
Despite these notable fruits of ribosome research and the fundamental cellular roles of the ribosome, there are still many aspects of ribosome function yet to be explored. Our research focuses on two of these aspects, ribosome

stalling and the rescue of stalled ribosomes. Following a general review of ribosome structure and function, these processes will be described in detail.

### *RIBOSOME STRUCTURE*

Ribosomes are nearly 200 Å in diameter and have a mass of ~2.5 megadaltons. A fully functional prokaryotic ribosome is assembled from two subunits to form a 70S complex. The larger of the two subunits, the 50S, is composed of over 30 proteins and two ribosomal RNAs (rRNAs), a short 5S RNA and the much longer 23S RNA (2904 nucleotides long). The smaller, 30S, subunit is made up of ~20 proteins and a 1542 nucleotide (nt) 16S RNA (4) (Figure 1-1). The rRNAs adopt complex structures that are stabilized by the ribosomal proteins; these proteins are mostly located on the surface of the ribosome.

The two subunits in a 70S ribosome play different roles in translation. The 50S subunit is primarily responsible for peptide synthesis and contains the peptidyl-transferase center (PTC) where peptide bonds are formed. It also



**Figure 1-1. Crystal structure of the *Thermus thermophilus* 70S ribosome with bound tRNAs.** The 50S subunit is composed of 5S rRNA (cyan), 23S rRNA and proteins (bright and pale yellow, respectively). The 30S is formed with the 16S rRNA and proteins (bright and pale orange, respectively). The A, P, and E-site tRNAs are green, red, and blue, respectively. (PDB acc. 2J01, 2J02) (4).

contains a region known as the GTPase associated center (GAC) where proteins bind to order and regulate the steps in translation. The 30S subunit selects proper tRNAs. This subunit contains a decoding center where tRNAs are paired with or “decode” mRNAs. This region binds mRNA and monitors the tRNAs entering the ribosome to ensure they are cognate or complementary to the mRNA. At the interface of the two subunits are three tRNA binding sites; the A or aminoacyl site, where aminoacylated tRNAs enter the ribosome; the P or peptidyl site, where the peptidyl-tRNA is bound; and the E or exit site, where deacylated tRNAs are held until they can be ejected from the ribosome. tRNAs bound in these sites interact with both subunits simultaneously.

### *RIBOSOME FUNCTION*

Ribosomes synthesize proteins in a three-step process of initiation, elongation, and termination (5, 6). During initiation, individual 30S and 50S subunits combine on an mRNA to form active 70S ribosomes. Then elongation ensues, where with the help of tRNAs and protein cofactors, the ribosome builds polypeptide chains one amino acid at a time. When a ribosome reaches a stop codon, release factors and recycling factors are recruited to terminate translation. Termination releases the newly synthesized polypeptide and then the subunits dissociate. These subunits are recycled and begin the translation process again on another transcript. Each of these steps is described below.

#### *Initiation*

The ribosomal subunits must be assembled properly at the right site on an mRNA to ensure correct frame selection. This alignment is achieved through the

interaction of a free 30S subunit with a consensus sequence in the mRNA called a Shine-Dalgarno sequence or ribosome-binding site (RBS). In an mRNA, the RBS (consensus AGGAGG in *E. coli*) is complementary to the 6 nt at the 3' end of the 16S rRNA and is positioned ~7 nt upstream of an initiation codon (usually AUG). Because of this spacing, the AUG codon is positioned in the P site of the ribosome when the RBS and rRNA are paired. This 30S-mRNA interaction is an essential part of initiation in prokaryotes.

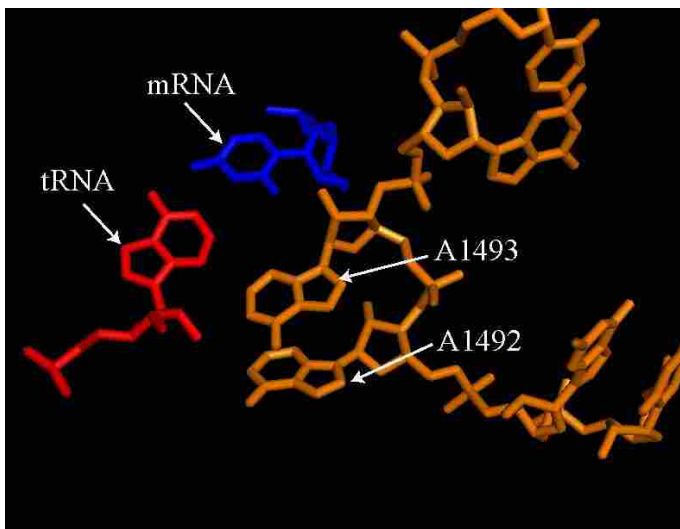
Also essential for initiation is the binding of a specialized tRNA and the 50S subunit to the 30S-mRNA complex. These binding interactions are coordinated by three initiation factor proteins, initiation factor (IF) 1, 2, and 3. The specialized tRNA, a formylated methionyl-tRNA (fMet-tRNA), is aligned with the mRNA start codon in the P site with the help of IF-2. IF-2 binds to fMet-tRNA and delivers it to the 30S subunit where it is tightly associated. IF-1 associates with the 30S subunit and blocks tRNAs from accessing the A site. IF-3 binds the 30S on the other side of fMet-tRNA, in the region of the E site, to prevent premature association of the 50S subunit. When the fMet-tRNA and initiation factors are properly associated with the 30S subunit, IF-3 is released from the ribosome and the 50S subunit binds. 50S binding triggers GTP hydrolysis by IF-2, resulting in the release of IF-1 and IF-2 from the complex. A 70S ribosome is now completely assembled on the mRNA. It has a bound P-site fMet-tRNA and an empty A site and is ready to begin elongation.

### ***Elongation***

Following formation of the 70S initiation complex, tRNAs are selected and incorporated in the process of elongation. Elongation of a polypeptide takes



place in a repetitive cycle of tRNA selection, peptidyl transfer, and translocation. During tRNA selection, many different aminoacyl-tRNAs diffuse into the A site; these tRNAs are part of a ternary complex with the elongation factor (EF)-Tu and GTP. EF-Tu•GTP binds to the aminoacylated 3' end of tRNAs to protect the amino acid from forming peptide bonds prematurely. The 30S subunit monitors these entering aminoacyl-tRNAs for proper base pairing between the mRNA codon in the A site and the anticodon loop on tRNA. In *E. coli*, the codons for each of the 20 natural amino acids are “decoded” by 49 different tRNAs (7), each with a unique anticodon sequence; proper codon-anticodon pairing ensures the addition of the correct amino acid to the polypeptide chain. The 30S subunit monitors these interactions through the movement of two adenine residues, A1492 and A1493, in helix 44 of the 16S RNA (Figure 1-2). When a non-cognate tRNA is in the ribosome, these residues are sequestered in an internal loop of the helix. When a cognate tRNA pairs with the mRNA, however, these bases flip out of the helix. When a cognate tRNA pairs with the mRNA, however, these bases flip out of the helix and interact with the minor groove of the codon-anticodon pair (4, 8). The repositioning of A1492 and A1493 in response to selection of a tRNA transmits a signal from the 30S subunit to the GTPase associated center in 50S



**Figure 1-2.**  
**Conformation of A1492 and A1493 when the correct tRNA decodes the A-site codon.**

Bases 1492 and 1493 of 16S RNA flip out of helix 44 (orange) when a correct codon:anticodon interaction is made. The red and blue residues are a representative base pair of a tRNA (red) that correctly decodes an A-site codon (blue) (PDB acc. 2J01, 2J02) (4).

subunit. This signal triggers EF-Tu hydrolysis of GTP and its dissociation from the ribosome, which, in turn, allows the aminoacyl-tRNA to rotate into a position where it is fully accommodated. Failure of a mismatched tRNA to alter the conformation of A1492 and A1493 prevents GTP hydrolysis and leads to its rejection by the ribosome.

After accommodation of the correct tRNA, the peptidyl-transferase center catalyzes the transfer of the nascent polypeptide from the P-site tRNA to the amino group on the A-site tRNA. The ribosome catalyzes amide bond formation in two ways. First, it catalyzes the reaction through entropic reduction by closely positioning the conserved 3' CCA stem of the A- and P-site tRNAs, which are attached to the amino acid directly. Secondly, the ribosome positions the 2'-OH of A<sub>76</sub> on the P-site tRNA so that the hydroxyl can shuttle protons in the reaction (9). Once the peptide has been transferred to the A-site tRNA, the P-site tRNA is uncharged and the ribosome is poised for translocation.

Translocation is the rearrangement that prepares the ribosome to select a new tRNA after peptidyl transfer. During translocation, the ribosome moves on the mRNA by one codon in the 5' to 3' direction. This repositions the mRNA and tRNAs within the ribosome. The A-site tRNA, now linked to the polypeptide, moves to the P site and the deacylated P-site tRNA advances to the E site. This movement is driven by GTP hydrolysis on EF-G, an elongation factor which binds to the ribosome following peptidyl transfer (10, 11). After translocation, EF-G•GDP dissociates from the ribosome, leaving an empty A site and the peptidyl-tRNA in the P site. Peptide elongation continues cycling through this three-step process of tRNA selection, peptidyl transfer, and translocation one

codon at a time until a stop codon is positioned in the A site. This signals the end of elongation and the beginning of termination.

### *Termination and Ribosome Recycling*

At a stop codon, the nascent peptide is released and the ribosome dissociates. In order for a nascent peptide to be released from the ribosome, the ester bond between the peptide and the P-site tRNA must be cleaved. This ester bond is hydrolyzed by a class I release factor, either RF1 or RF2. These proteins are specifically recruited by different stop codons; RF1 binds to UAG, while RF2 recognizes UGA stop codons in a similar manner (12). Both bind to UAA. Class I release factors free the nascent peptide by specifically positioning a water molecule to attack the ester. This molecule is held in place by a conserved GGQ motif in these enzymes (13). Hydrolytic release of the peptide allows the class II release factor, RF3, to bind to RF1 or RF2 and release them from the ribosome. The ribosomal subunits are then dissociated through the action of ribosomal release factor (RRF) in conjunction with EF-G.

### *RIBOSOME STALLING*

Failure of the ribosome to properly carry out any of the steps in elongation or termination leads to ribosome stalling. Often, ribosome stalling is due to damaged mRNAs. It can also be caused by insufficient levels of tRNAs. In such cases, stalling is not caused by problems with the ribosome but problems with its ligands. Ribosome stalling can also be caused directly, where ribosome function is inhibited; it is incapable of translation even in the presence of all the necessary components. Many antibiotics can directly prevent ribosome function and,

surprisingly, a handful of nascent peptide sequences are also capable of arresting ribosome function directly. Many of these peptides use ribosome stalling as a means for regulating gene expression. We will first present a few examples of indirect stalling, then direct stalling caused by stalling peptides and antibiotics will be discussed in detail.

### *Indirect Ribosome Stalling*

One cause of indirect ribosome stalling is the lack of a stop codon on an mRNA transcript. A non-stop mRNA is generated when RNA polymerase falls off a transcript prematurely or when exonucleases degrade its 3' end, including the stop codon. When a ribosome translates to the end of an mRNA without encountering a stop codon, it is arrested, waiting for a release factor. Without a stop codon, however, a class I release factor cannot enter the ribosome to release the nascent peptide that is bound to the P-site tRNA. Stalling on non-stop mRNAs is common in prokaryotic cells because translation begins on an mRNA while it is still being transcribed. Because transcription and translation are coupled, there is no mRNA quality-control step prior to translation. In eukaryotes, in contrast, ribosomes only begin translation in the cytoplasm following completion of several mRNA processing steps including splicing, addition of a 5' cap, and polyadenylation of the 3' terminus. Although eukaryotes do have safeguards against translating non-stop mRNAs, eukaryotic ribosomes do occasionally reach the 3' end of a transcript without encountering a stop codon. When this happens, a process known as "nonsense decay" is activated and the ribosome bound mRNA is rapidly degraded by a number of

exonucleases that form an exosome (14, 15).

Certain cellular conditions can also lead to ribosome stalling. A shortage of a given charged aminoacyl-tRNA, for example, can result from either amino acid starvation or the depletion of the tRNA itself. The *E. coli* genome contains a number of codons which are not highly represented. These rare codons are not often found in highly expressed genes. Often the tRNAs that decode rare codons are only expressed at low levels as well. When a rare codon is positioned in the A site but no cognate tRNAs are available, the ribosome pauses, waiting for the tRNA. The shortage of a particular amino acid leads to stalling for the same reasons as tRNA shortage. Low levels of an amino acid such as alanine, for example, result in shortages of alanyl-tRNA<sup>Ala</sup>. When the ribosome reaches an Ala codon, translation is paused until alanyl-tRNA<sup>Ala</sup> is accommodated. If there is little available, then this pause may be quite long. In these situations, accommodation of the proper tRNA even after a prolonged pause can return the ribosome to full function. This indirect type of stalling is due to a lack of resources and is not related to direct inhibition of the ribosome itself.

### *Stalling Through Direct Means*

A small number of nascent peptide sequences have been identified which directly prevent translation by interacting with specific regions of the ribosome. Three regions have been identified which play a major role in arresting ribosome function: the A site, the peptidyl-transferase center, and the exit tunnel for the nascent peptide. Interactions of charged tRNAs or free amino acids with the A site, in combination with nascent peptide interactions with the exit tunnel or PTC, can arrest the ribosome by inhibiting its peptidyl-transferase activity.

Three prokaryotic peptides, SecM, ErmCL, and TnaC, illustrate how nascent peptides, together with accessory molecules, can interact with each of these regions to inhibit peptidyl transfer. Each is a short leader peptide that regulates the expression of a downstream gene. This regulation is usually mediated through an mRNA attenuator hairpin. An attenuator is an RNA structure capable of either allowing or attenuating gene expression in response to a cellular signal. In these examples, the signal that regulates attenuation is ribosome stalling. Unless the ribosome stalls on the leader peptides, expression of the regulated genes is attenuated, or prevented. When the ribosome stalls, however, a change in RNA conformation allows the expression of the downstream gene. All three peptides, SecM, ErmCL, and TnaC, cause stalling through slightly different interactions with the ribosome, but each stalling event effectively regulates attenuation (16-18). After a brief look at the biological role of each of these regulatory peptides, we will look at the mechanism by which they inhibit ribosome function.

#### *Biological Relevance of Regulatory Stalling*

In *E. coli*, the secretion monitor (SecM) protein regulates the expression of *secA*, part of the secretory machinery. SecM contains a signal sequence, which, if the secretion machinery is functioning efficiently, allows the SecM peptide to be pulled out of the ribosome (18, 19). The ribosome always stalls when translating SecM, but if the concentration of SecA is high, the stall-inducing peptide is quickly pulled from the ribosome and translation continues. Rapid clearance of the stalling peptide favors the attenuation of *secA* expression. When the ribosome stalls on SecM and levels of SecA are low, however, the stalled

ribosome remains arrested. This stalling event alters the mRNA structure, increasing translation of *secA*.

ErmCL is a second leader peptide that uses ribosome stalling to turn on the expression of an attenuated gene (16, 20). As with SecM, ribosome stalling on ErmCL prevents attenuation. In this case, the regulated gene, *ermC*, encodes a methylase that confers cellular resistance to the antibiotic erythromycin.

Ribosomes stall while translating ErmCL in cells that have been exposed to sublethal levels of erythromycin, but they do not stall on the peptide in the absence of erythromycin. In this way, the resistance gene is only expressed when it is needed by the cell.

The third example of a regulatory stalling peptide is TnaC (17, 21). This leader regulates the expression of *tnaA*. *tnaA* encodes tryptophanase, an enzyme that breaks down tryptophan. Ribosome stalling on TnaC is dependent upon cellular levels of free tryptophan (Trp). When levels of Trp are high, free Trp binds in the A site of the ribosome and causes stalling on TnaC. Ribosomes stalled on TnaC prevent attenuation of *tnaA* expression, resulting in increased levels of tryptophanase. When Trp levels are low, tryptophanase is not needed and the ribosome does not stall on TnaC. Proper termination of TnaC without stalling allows attenuation of *tnaA* expression, lowering *tnaA* expression.

#### *How do these Peptides Cause Stalling?*

All three of these stalling peptides inhibit the peptidyl-transferase activity of the ribosome. This has been determined through use of the antibiotic puromycin to study stalled ribosomes. Puromycin, a structural mimic of aminoacylated tRNA, can bind to the A site of ribosomes. When bound in the A

site, puromycin acts as a peptide acceptor. Following peptidyl transfer to puromycin, however, the antibiotic does not remain associated with the ribosome. This results in the premature release of the nascent peptide. If the peptidyl-transferase activity of the ribosome is inhibited, then the nascent peptide will not be transferred to puromycin and it will remain bound to the P-site tRNA in the ribosome. The conclusion that ErmCL, SecM, and TnaC inhibit the peptidyl-transferase activity of the ribosome is based on the observation that, when stalled, none of these peptides is transferred to puromycin. Likewise, none of these peptides is transferred to the next amino acid or release factor in the wild-type context (16, 21, 22).

Although it is clear that ErmCL, SecM, and TnaC cause stalling by inhibiting the peptidyl-transferase activity of the ribosome, how they do this is a more difficult question to answer. The sequence of each peptide is quite different as are the cellular conditions that allow stalling in each context. But, despite these differences, each interacts with the peptide exit tunnel and the PTC. Stalling on SecM and TnaC is also supported by binding of specific ligands in the A site. While ErmCL also interacts with the exit tunnel and PTC it does not appear to have specific A-site requirements.

#### *The Sole of the A Site in Stalling*

The A-site substrate plays a role in stalling on SecM and TnaC. Stalling on SecM occurs during the elongation step of translation with the peptidyl tRNA bound to the P site and a prolyl-tRNA<sup>Pro</sup> in the A site. Although this proline (Pro166) is not incorporated into the nascent peptide, its identity is essential for stalling. Replacing Pro166 with alanine abolishes stalling on SecM. This



suggests that the prolyl-tRNA<sup>Pro</sup> makes specific interactions with the A site of the ribosome to contribute to stalling.

Like SecM, TnaC also shows notable sensitivity to the A site substrate. TnaC stalls during termination in the presence of high levels of free tryptophan. Translation of TnaC causes the A site of the ribosome to adopt a conformation that binds free Trp (23). The binding of Trp to the PTC inhibits RF2-mediated peptidyl transfer to water and subsequent peptide release (21). Replacing the stop codon (UGA) of TnaC with a tryptophan (Trp25) leads to constitutive stalling on TnaC, independent of free tryptophan levels. But replacing the stop codon with a proline instead of tryptophan abolishes stalling (17). The encoded tryptophanyl-tRNA<sup>Trp</sup> appears to interact with the A site in the same way as free Trp. It is clear from this finding that stalling on TnaC is highly sensitive to changes in the A site.

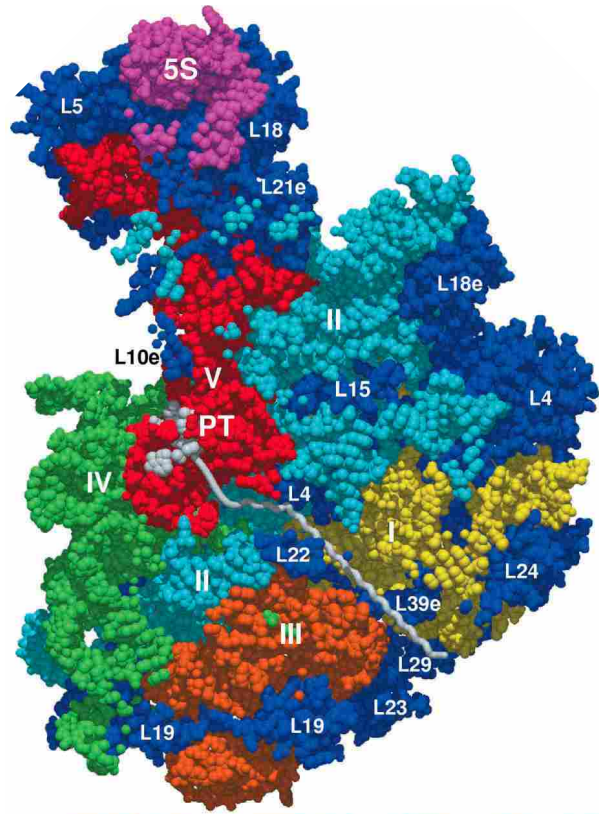
Unlike these examples, stalling on ErmCL is independent of A-site interactions. ErmCL stalls during elongation with the nascent peptide bound to the P-site tRNA, but the A-site tRNA does not contribute to this stalling event. The efficiency of ribosome stalling on the wild-type ErmCL sequence, with Ser-tRNA in the A site, is no different from the efficiency of stalling on a mutant peptide with alanine in place of serine (16). This is evidence that stalling on regulatory peptides occurs through different mechanisms, one of which does not rely as heavily upon the identity of the A-site substrate.

#### *Regulatory peptides interact with the exit tunnel*

In addition to the A site, the polypeptide exit tunnel also contributes to the efficiency of stalling on regulatory peptides. This tunnel allows nascent

**Figure 1-3. Cross section of the 50S subunit showing the peptide exit tunnel.**

The ribosome exit tunnel extends from the peptidyl transfer center (PT, red) to the outside boundary of the 50S subunit. L4 and L22 (dark blue) protrude into the tunnel to create a constriction where the tunnel bends. A nascent peptide (gray) must also bend at this constriction to transit the tunnel. Reprinted with permission from Nissen *et al.* (20).



peptides, as they are synthesized, to travel from the PTC through the 50S subunit  $\sim 100$  Å to the outside boundary of the ribosome (Figure 1-

3). Assuming an extended

conformation, this is long enough to accommodate a polypeptide between 30 and 40 residues long (24). The exit tunnel is lined primarily with RNA, permitting virtually all peptide sequences to pass through uninhibited (25, 26). It is because of the inert nature of the exit tunnel that the ribosome can synthesize every combination of amino acid sequences required to produce the proteins necessary for life. There is one region of this tunnel, however, where two ribosomal proteins protrude from opposite sides: these proteins, L4 and L22, form a narrow constriction near the only bend in the tunnel (26). Although the exit tunnel is quite inert, these regulatory peptides have been found to make specific contacts with both protein and RNA residues in the tunnel. These interactions help inhibit the peptidyl-transferase activity of the ribosome to cause ribosome stalling.

There is little homology between the stalling regions of the regulatory stalling peptides. SecM is 170 amino acids long, but a region of only 17 amino acids is sufficient to cause stalling: **F**<sub>150</sub>xxxx**WI**xxxx**GIRAGP**<sub>166</sub> (18). In this and each of the following peptides, only the residues specified are required for stalling; the spacing between the essential residues must be maintained, however, for maximum efficiency of stalling. The segment of TnaC that is required for stalling (13 of its 24 amino acids) is quite different from that of SecM. This sequence is **W**<sub>12</sub>xxx**D**xxxxxxx**P**<sub>24</sub>(**UGA**) (27). Regulation of *ermC* expression only requires the first nine residues of the 19 amino acid leader peptide, **M**<sub>1</sub>xxxx**IFVI**, and erythromycin (16). Met1 cannot be mutated to another residue, due to the need for a start codon in this position, so it is not clear if methionine is essential for stalling. When modeled in the exit tunnel, however, the formylated methionine of the nascent peptide appears to extend to the narrow region of the exit tunnel where it could make specific contacts (16). The only major similarities between the sequences are tryptophans Trp155 of SecM and Trp12 of TnaC and the isoleucines Ile6 of ErmCL and Ile162 of SecM, which are found similar distances from the PTC (16, 17).

<b>SecM</b>	<b>F</b> xxxx <b>WI</b> xxxx <b>GIRA</b>	<u><b>G</b></u>	<u><b>P</b></u>
<b>TnaC</b>	<b>W</b> xxx <b>D</b> xxxxxxx	<u><b>P</b></u>	<b>*</b>
<b>ErmCL</b>	<b>M</b> xxxx <b>IFV</b>	<u><b>I</b></u>	<u><b>S</b></u>

**Table 1-1. Alignment of stalling peptide sequences.**  
 The regions highlighted in green are near L4/L22 while those in blue are close to the PTC. The underlined blue letters are located in the P site and the underlined black letters are in the A site.

Alignment of these peptides indicates that the position of the essential residues may be more significant than their identity. The essential residues of each stalling peptide are clustered in two regions. The first region is near the L4/L22 constriction of the exit tunnel (Table 1-1). The second region consists of the residues proximal to the P-site tRNA of the stalled peptides. These residues are expected to interact with the ribosome in the region of the peptidyl-transferase center rather than the L4/L22 region.

If ribosome stalling is dependent upon the identity of the nascent peptide residues near the PTC and constriction of the exit tunnel, then there should also be essential ribosomal residues in these same regions. Genetic screens on libraries of mutant ribosomes have revealed that this is indeed the case. In fact, a pattern of reactivity for each peptide has been identified that extends from the constriction back to the PTC. Notably, however, each stalling peptide has a different profile of interactions with the ribosome.

The constricted region of the exit tunnel is where the most mutations have been identified that prevent stalling. A number of mutations in L22 can prevent stalling on ErmCL, TnaC and SecM. The most significant L22 mutation that relieves stalling on ErmCL and SecM is the deletion of Met82-Arg84 ( $\Delta$ MKR) (16, 28). SecM stalling is also relieved by Gly91Ala and Ala93Ser. Curiously, these mutants have no effect on TnaC-induced stalling (29). Likewise, an interaction unique to TnaC, Lys90, is absent in SecM and ErmCL stalling. An L22 Lys90Trp mutation relieves stalling on TnaC, but SecM still stalls efficiently in its presence (29). These differences support the hypothesis that each of these stalling peptides functions through a slightly different mechanism from the others.

Despite these differences, however, it is clear that each peptide makes contacts with this protein.

Among the 23S rRNA mutations that affect stalling near the constriction of the exit tunnel are an A insertion at base 751, which relieves stalling on both TnaC and SecM (29), and an A2058G mutation. The latter has notable and variable affect on the stalling peptides. Stalling on ErmCL and SecM is completely relieved by this mutation, whereas it actually increases the stalling efficiency on TnaC (16, 29). Again, this evidence supports the hypothesis that the PTC is regulated through interactions with distal regions of the tunnel.

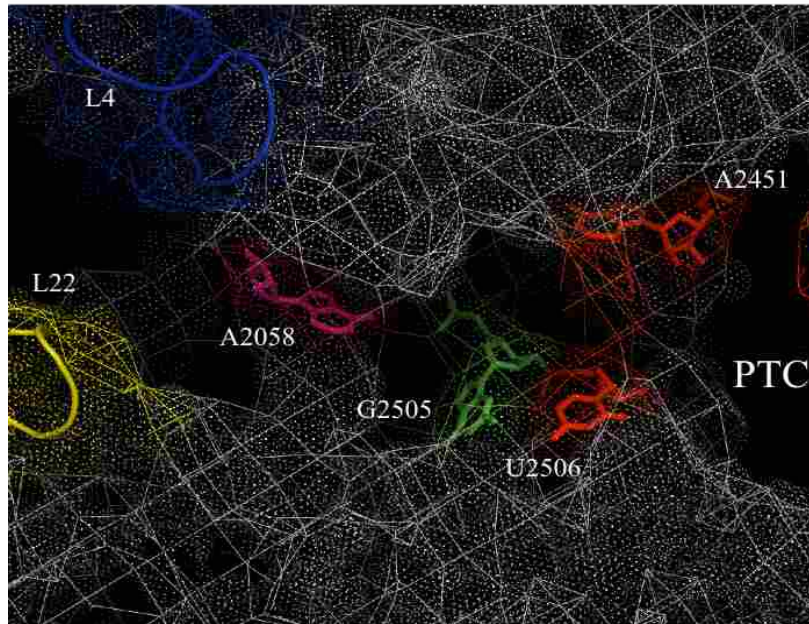
Moving from the constriction towards the PTC, we find more points of interaction between the rRNA and the stalling peptides. Among those with the most notable impact on stalling efficiency in this region is U2609C. This mutation completely abolishes stalling on TnaC (29). A second mutation in this mid-region of the tunnel is residue U2062A. This was shown to relieve stalling caused by the ErmCL peptide (16). The peptide interactions with this mid-region of the tunnel are likely to work in concert with those at the more distal end of the tunnel as well as at the PTC.

Although no stall peptides have been tested against mutations in the PTC, as these are lethal, this region is certainly involved in peptide-mediated stalling. The best evidence for this is the essential identity of the P-site substrate in each stalling peptide. The identity of the residue in the P site is important in each case, but it is notable that each is different: the P site of stalled TnaC has a Pro while it this a Glycine in SecM and an Ile in ErmCL. The unique interactions of each stalling peptide with the PTC, the mid-region and constricted region of the exit tunnel are evidence that the ribosome can be stalled through different

mechanisms. There are common patterns in each case, which likely involve a network of interactions with the exit tunnel, but the differences are significant enough to suggest that the ribosome can be regulated by a number of different peptide sequences.

It is apparent that a network of peptide interactions with the exit tunnel do contribute to stalling, but the constriction of the exit tunnel is  $\sim 25 \text{ \AA}$  away from the PTC. How does this interaction regulate the PTC from such a distance? It has been suggested that the nascent peptide can trigger a cascade of conformational rearrangements in the rRNA, which allosterically regulate the peptidyl transferase activity of the ribosome. This hypothesis is supported by the work of Mitra *et al.* in which cryo-electron microscopy was used to visualize structural differences between stalled and non-stalled ribosomes (30). The stalled ribosomes were generated on a SecM transcript. Comparison of the stalled and non-stalled ribosomes showed a dramatic rearrangement of rRNA throughout the 23S rRNA. These changes included the rearrangement of intersubunit bridges and the modification of the peptidyl-transferase region as indicated by the repositioning of tRNAs bound to the ribosome (30).

Conformational changes in the peptidyl-transfer center have been reported before. The Steitz lab determined the crystal structure of the large subunit complexed with peptidyl-tRNA mimics. In the different conformational states obtained with several analogs, it was found that rRNA bases U2585 and U2506 are repositioned in an induced-fit mechanism upon correct binding of a tRNA mimic (31). These bases protect the peptidyl-tRNA from hydrolysis by water and must rotate to allow peptidyl transfer or peptidyl release by water during termination. The two bases are among the four conserved bases in the



**Figure 1-4. Cross section of the 50S subunit showing potential signaling network.** Base A2058 (pink) in the exit tunnel is important for ribosome stalling. One potential mechanism for signal transmission from A2058 to the PTC is through base-backbone interactions with G2505 (green). Movement of G2505 would be transmitted to its adjacent nucleotide U2506 (red). This is one of the inner shell bases (red) that is essential for peptide bond catalysis in the ribosome (PDB Acc. 2AWB) (32, 33).

peptidyl transfer center known as the “inner shell” of nucleotides. The other two, besides U2585 and U2506, are A2602 and A2451.

Building on these observations, Beringer has proposed a specific mechanism for how peptides in the exit tunnel can regulate the PTC (33). The base A2058 in the 23S RNA affects stalling of all three regulatory peptides. He suggests that peptide interactions with this base transmit a signal to the PTC via an intermediate base. Specifically, base 2058 interacts with the peptide backbone of G2505. Movement of G2505 is directly transmitted to its “inner shell” neighbor U2506 (Figure 1-4). As was noted by Steitz, U2506 must be repositioned upon substrate binding in the A site in order to protect the ester bond between the tRNA and nascent peptide (31). Disrupting the movement of this or any of the other inner shell nucleotides may be sufficient to prevent

peptidyl transfer. Though this signaling network has yet to be verified, it is likely that such a cascade of RNA interactions is involved in regulating the PTC from the essential residues of the exit tunnel.

### *Regulatory Peptides Interact with the PTC*

In characterizing the nature of stalling on SecM and ErmCL, it was found that extensive interactions with the exit tunnel are not necessary for stalling. Using truncated versions of ErmCL (MSIFVI) and SecM (IRAGP) that are too short to reach the constriction of the exit tunnel, stalling still has been observed, though at a much lower efficiency (16, 34). Stalling on these sequences must be due to interactions with different regions of the exit tunnel than are involved in stalling on full-length peptides. It is likely that this interaction may even be limited to the peptidyl-transferase center itself and not rely upon the exit tunnel. A related phenomenon of stalling during termination compares to these short peptides and supports the hypothesis that stalling can be caused through direct interactions with the peptidyl-transferase center.

This unusual phenomenon involves ribosome stalling on the YbeL protein. Unlike the other stalling events described, stalling on YbeL occurs at the C-terminus of the full-length protein. YbeL is not a leader peptide and stalling on the gene plays no known regulatory or other biologically significant role. What is most notable about stalling on YbeL is not related to the gene itself, but to the sequence that causes stalling: Glu-Pro-Stop. This dipeptide followed by a stop codon has been found to cause stalling when added to the end of any gene. Similar sequences, Asp-Pro-Stop and Pro-Pro-Stop, have also been shown to cause ribosome stalling with high efficiency (35).



How can ribosomes be stalled at termination by only two amino acids? It has been shown that this is not an indirect stalling event caused by rare codons; the identity of the Pro codon has no effect on the levels of stalling on Glu-Pro-Stop. If stalling is caused directly by the peptide, any interactions between Glu-Pro-Stop and the exit tunnel must be minimal; as in the case of IRAGP and MSIFVI, stalling on Glu-Pro-stop would have to be the result of a more direct interaction with the PTC. In support of this hypothesis is the finding that the identity of the C-terminal amino acid proline is the key to this stalling event. Replacing proline in the peptide with the structurally similar azetidine-2-carboxylic acid decreased ribosome stalling markedly, whereas incorporation of 3,4 dehydroproline increased the level of ribosome stalling at termination (35). It is probable that proline is interacting with the PTC to regulate its function.

Interestingly, in addition to the role proline may play in regulating the PTC, it appears there are also A site requirements for this type of stalling. Early studies of termination revealed that the efficiency of termination with a C-terminal Pro is affected by the identity of the release factor (36, 37). This ribosome sensitivity to the A-site substrate during termination indicates that the A site may still play a role in stalling on Glu-Pro-Stop.

The results of the Glu-Pro-Stop research combined with the analysis of stalling on IRAGP and MSIFVI indicate that ribosomes can be stalled even without exit tunnel involvement. It is likely that these also interact specifically with the peptidyl-transferase center of the ribosome, but such a conclusion will be difficult to confirm through mutational analysis. Many of the 23S RNA bases in the region of the PTC are highly conserved; mutating these is often lethal. Although *in vivo* systems using orthogonal ribosomes have been developed to

study the effects of ribosome mutations, these have not yet been applied to studies of PTC mutations (38). There are many questions about stalling caused by interactions with the PTC that have yet to be answered.

### *Antibiotics Cause Ribosome Stalling*

As was mentioned previously, many antibiotics kill cells by inhibiting translation (3). Studying these effects of antibiotics on ribosome function may help explain stalling on nascent peptides. Chloramphenicol and erythromycin prevent peptide bond formation when bound to the ribosome. Chloramphenicol does so by binding to A2451, a nucleotide in the inner shell of the peptidyl transfer center. Erythromycin interacts with the exit tunnel near protein L22, likely blocking the passage of the nascent peptide, but this mechanism is unclear (39, 40). Other antibiotics inhibit translation by preventing tRNAs from accessing the A site. Among these are tetracycline, which binds to the 16S RNA to prevent general access of tRNAs, and streptomycin, which binds the 16S RNA and specifically prevents fMet-tRNA from binding to the ribosome (1). The interactions of each of these antibiotics with the ribosome give insight into what ribosomal residues may be important points of interaction or signaling during stalling on nascent peptides.

Regardless of how ribosome stalling is caused, whether by truncated mRNAs, rare codons, regulatory stalling peptides, C-terminal prolines, or antibiotics, the effect of this event can be very costly for a cell. Ribosome synthesis requires significant input of cellular resources and energy. Often several ribosomes are found translating a single transcript simultaneously; stalling of just one of these ribosomes could prevent recycling of several others

on the same transcript. To minimize the potentially lethal effects of high levels of ribosome stalling, bacteria have evolved several mechanisms to free stalled ribosomes. Among these are frameshifting (41), peptidyl drop-off (42) *trans*-translation(43). Although each of these may play a role in many stalling events, we have focused only on *trans*-translation, which not only frees stalled ribosomes but also destroys problematic peptides and mRNAs.

### *RIBOSOME RESCUE: TRANS-TRANSLATION*

How does a cell rescue stalled ribosomes? Rather than creating a new mechanism for termination and recycling, eubacteria have evolved a way to reactivate translation by stalled ribosomes. This mechanism, known as *trans*-translation, is carried out through the unique functions of two molecules: transfer-messenger RNA (tmRNA) and its protein cofactor small protein B (SmpB). These molecules, which are conserved in all eubacterial genomes, allow stalled ribosomes to be recycled through the canonical termination and recycling mechanisms (reviewed in (43, 44)).

tmRNA rescues stalled ribosomes by acting first as a tRNA mimic then as an mRNA. In the first step of *trans*-translation, alanylated tmRNA bound to SmpB enters the A site of stalled ribosomes. The nascent peptide is then transferred to the alanyl moiety of tmRNA and the peptidyl-tmRNA•SmpB complex is translocated to the P site. During this translocation step the ribosome actually switches templates: the stalled mRNA is ejected from the ribosome and translation resumes on an open reading frame (ORF) in tmRNA (hence the name *trans*-translation). Translation of the ORF of tmRNA adds a protease recognition sequence to the C-terminus of the nascent peptide. At the end of the ORF is a

stop codon that frees the ribosome and nascent peptide through the canonical steps of termination and ribosome recycling. The aborted protein is then degraded by cellular proteases such as ClpXP.

### *tmRNA Rescues Stalled Ribosomes*

Fully processed *E. coli* tmRNA is 363 nucleotides long and consists of four pseudoknots (pk1-4), a tRNA-like domain (TLD), and an open reading frame (ORF) (45, 46). Pseudoknots are stable tertiary RNA structures composed of at least two stem-loops in which one loop forms a stem with a second loop. Pseudoknots 2-4 of tmRNA can be individually replaced with single stranded RNA without destroying its function (47). Early studies of pseudoknot 1 reported that the structure was necessary for tmRNA function (48) but that finding has been challenged in recent reports (49, 50). The ability of tmRNA to function without specific pseudoknot structures suggests that they do not play a role in *trans*-translation, but they are more likely involved in maintaining the overall structure of tmRNA and protecting it from degradation (51). Unlike the pseudoknots, the TLD and ORF of tmRNA are essential for tmRNA function.

Another essential component of the *trans*-translation system is the protein SmpB (52). This 160 amino acid long protein (53) has a  $\beta$ -barrel core that binds to the TLD of tmRNA (54). The TLD of tmRNA includes a T arm, D loop and modified bases as well as the 3' CCA amino acid acceptor stem (45, 46). SmpB binding stabilizes the TLD of tmRNA, allowing its 3' end to be recognized and aminoacylated by alanyl-tRNA synthetase (53). SmpB also interacts with the

ribosome. These interactions likely help the ribosome resume translation in the proper frame on tmRNA (55, 56).

Even after tmRNA is bound by SmpB and alanylated, *trans*-translation cannot begin until the ribosome is ready. The major requirement for tmRNA and SmpB binding to stalled ribosomes is an empty A site (57, 58). The A site must not only be free of tRNA, but it must also be free of mRNA. The efficiency of tmRNA entry to the ribosome is directly related to the length of the mRNA. The longer the 3' end of an mRNA extends from the P site into the A site and beyond, the less efficiently tmRNA is bound to the ribosome (34). The requirement for an empty A site is automatically met in ribosomes stalled on non-stop mRNAs, but when a ribosome is stalled on a full-length transcript, the mRNA must first be cleaved before tmRNA can enter. The mRNA in stalled ribosomes is either cleaved in the A site by an unidentified endonuclease or at the 3' boundary of the ribosome by exonucleases (58, 59). This cleavage event eliminates steric constraints between the mRNA and the tmRNA•SmpB complex, allowing the *trans*-translation machinery to access and act upon the stalled ribosome.

Once tmRNA is alanylated and the A site is cleared of mRNA, *trans*-translation may begin. This starts with tmRNA entry into the ribosome. The aminoacylated TLD of tmRNA is recognized by EF-Tu, which delivers the tmRNA•SmpB complex to the ribosome. Once the tmRNA•SmpB complex enters the A site, it must bind the ribosome in a specific conformation that will allow GTP hydrolysis then peptide bond formation.

How is a stalled ribosome induced to hydrolyze GTP in the absence of any codon-anticodon interactions? There is no mRNA codon for tmRNA to

recognize in the A site; tmRNA does not even contain an anticodon region. It has been proposed that the C-terminal region of SmpB forms a helix that interacts with the decoding center to activate GTP hydrolysis by EF-Tu. The sites of interaction between SmpB and the ribosome have recently been mapped; the C-terminal region of SmpB interacts with the ribosome in the mRNA channel in the 30S region of the A and P sites (56). This finding suggests that SmpB may activate GTP hydrolysis through the same mechanism as a canonical codon-anticodon interaction, transmitting a signal from the region of 1492 and 1493 in the 30S subunit to the GTPase associated center in the 50S subunit.

After tmRNA•SmpB is bound to the ribosome and GTP is hydrolyzed by EF-Tu, the nascent peptide is transferred from the P-site tRNA to alanyl-tmRNA. After the P-site tRNA is deacylated, EF-G binds to the ribosome and promotes translocation of the peptidyl-tmRNA to the P site. Movement of tmRNA•SmpB into the P site causes the problematic mRNA to be rapidly released from the ribosome, promoting its degradation (60, 61).

During the translocation of tmRNA from the A site to the P site, the ribosome switches templates and begins translating tmRNA. The most notable part of this template-swapping event is that the ribosome resumes translation in the correct frame without any Shine-Dalgarno sequence or start codon (AUG). How the ribosome recognizes the correct resume codon is an area of active investigation. It has been shown that the nucleotide sequence just upstream of the resume codon is important for this process (62).

Translation of the ORF of tmRNA in *E. coli* results in the addition of an 11 amino acid tag to the C-terminus of the nascent peptide; this includes the Ala from tmRNA and the ten amino acids encoded in the ORF. In *E. coli* this region,

positioned between pk1 and pk2, encodes the sequence ANDENYALAA. This sequence is specifically recognized by ClpXP, Lon, and other cellular proteases that degrade any peptide bearing it (63); addition of this tag ensures that the malformed protein will be rapidly destroyed. Although the wild-type ORF is essential for tmRNA to carry out its biological role, the sequence can be modified without destroying tmRNA activity (50, 64); a number of different species encode variations of length and peptide sequence in the ORF of tmRNA found in *E. coli* (65).

The tmRNA ORF ends with a stop codon. When the ribosome reaches this stop codon, canonical termination and ribosome recycling occur. This effectively marks the completion of ribosome rescue by *trans*-translation. In fulfilling the essential task of releasing stalled ribosomes, *trans*-translation also leads to the destruction of the defective mRNA and aborted polypeptide.

### ***Regulatory Ribosome Stalling***

Many of the known causes of ribosome stalling are the result of problems with translation such as premature transcriptional termination and broken or decayed mRNA (from the 3' end), but not all ribosome-stalling events are harmful to the cell. As illustrated earlier, ribosome stalling may be used to regulate gene expression; in such cases *trans*-translation would destroy the regulatory mechanism. As was mentioned, tmRNA does not rescue stalled ribosomes with occupied A sites. It appears that blocking tmRNA access to the A site is a common means of protecting regulatory peptides and regulated mRNAs from destruction by tmRNA. Ribosomes stalled during elongation that still contain an mRNA transcript cannot be rescued by tmRNA until the mRNA

is cleaved. In chapter three we will address questions related to *trans*-translation and regulatory ribosome stalling.

## CONCLUSION

Although the mechanisms of many steps of translation have been resolved at the molecular level, numerous questions still remain unanswered. The research presented in chapter two focuses on determining how the ribosome resumes translation in the correct position on the ORF of tmRNA. It was hypothesized that pk1 plays a role in positioning the resume codon and is thus essential for tmRNA function. Our work challenges this hypothesis; it shows that tmRNA can function at nearly wild-type levels even when pk1 is replaced with an unrelated structure.

In chapter three we move our focus from ribosome rescue to ribosome stalling. This research was driven by two questions: What peptide sequences can cause ribosome stalling and how do they do this? Using a genetic selection to screen a randomized peptide library, we have identified a number of novel peptide sequences that cause ribosome stalling. Three of these peptides were characterized to determine how they stall ribosomes. This characterization reveals notable similarities as well as significant differences between the mechanism of stalling on regulatory peptides and those we have identified.



## CHAPTER 2: GENETIC ANALYSIS OF THE STRUCTURE AND FUNCTION OF TMRNA PSEUDOKNOT 1

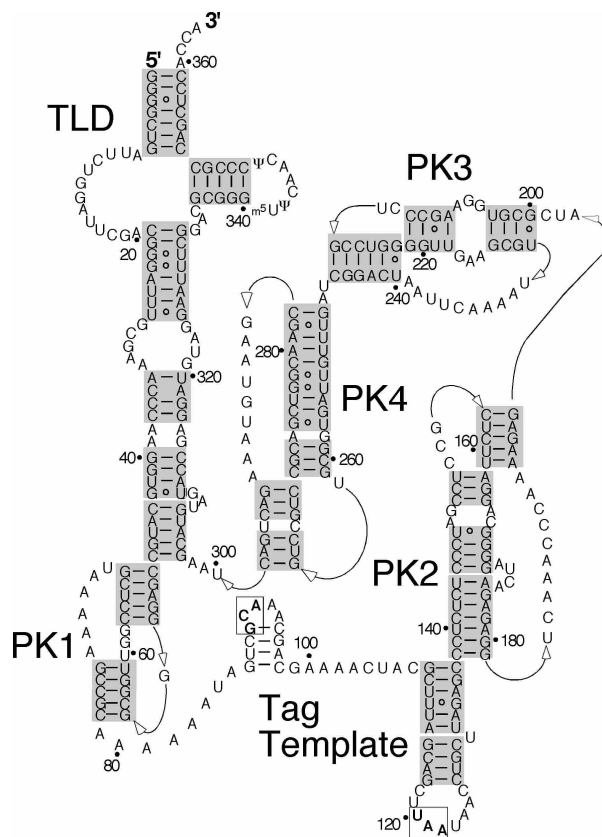
Adapted from: Tanner, D.R., Dewey, J.D., Miller, M.R. & Buskirk, A.R. Genetic analysis of the structure and function of transfer messenger RNA pseudoknot 1. *J Biol Chem* **281**, 10561-6 (2006).

### ABSTRACT

tmRNA rescues stalled ribosomes in eubacteria by forcing the ribosome to abandon its mRNA template and resume translation with tmRNA itself as a template. Pseudoknot 1 (pk1), immediately upstream of this coding region in tmRNA, is a structural element that is considered essential for tmRNA function based on the analysis of pk1 mutants *in vitro*. pk1 binds near the ribosomal decoding site and may make base-specific contacts with tmRNA ligands. To study pk1 structure and function *in vivo*, we have developed a genetic selection that ties the life of *E. coli* cells to tmRNA activity. Mutation of pk1 at 20% per base and selection for tmRNA activity yielded sequences that retain the same pseudoknot fold. In contrast, selection of active mutants from  $10^6$  completely random sequences identified hairpin structures that functionally replace pk1. Rational design of a hairpin with increased stability using an unrelated sequence yielded a tmRNA mutant with nearly wild-type activity. We conclude that pk1's role in tmRNA function is purely structural and that it can be replaced with a variety of hairpin structures. Our results demonstrate that in the study of functional RNAs, the inactivity of a mutant designed to destroy a given structure should not be interpreted as proof that the structure is necessary for RNA function. Such mutations may only destabilize a global fold that could be formed equally well by an entirely different, stable structure.

## INTRODUCTION

tmRNA plays an important role in quality control of protein synthesis in eubacteria (66, 67). This dual-function RNA acts as both tRNA and mRNA to rescue ribosomes stalled on broken templates or at certain nascent peptide sequences (35). Aminoacylated tmRNA enters the A-site of stalled ribosomes and transfers alanine to the growing peptide chain as would a normal tRNA.



**Figure 2-1. Secondary structure of *E. coli* tmRNA.** Four pseudoknots (pk1-4) dominate the global tmRNA structure. The tag template lies between pk1 and pk2, with the resume and stop codons marked with white boxes. TLD = tRNA-like domain. Adapted from (68).

The ribosome then resumes translation with tmRNA as the template, adding a ten amino acid tag to the nascent polypeptide. As a result of tmRNA action, stalled ribosomes are released and recycled and the aborted protein product is marked for destruction by proteases (66, 67).

In the trans-translation model of tmRNA function described above, the ribosome switches templates while synthesizing a single

polypeptide. How does tmRNA position itself inside the ribosome for translation to resume in the correct frame? The global fold of tmRNA places the first codon near the decoding center of the ribosome; a short single-stranded sequence immediately upstream of this initial codon determines the

precise frame (69-71). The global structure of tmRNA is dominated by several pseudoknot structures surrounding the tag-encoding sequence (45, 72). The *E. coli* tmRNA, for example, has four pseudoknots (Figure 2-1).

Only 11 nucleotides upstream of the resume codon, pseudoknot 1 (pk1) may be involved with positioning the template in the ribosomal A site. The cryo-EM structure of the 70S ribosome bound to tmRNA and its partner protein SmpB reveals that while pk2-4 are looped around the beak of the 30S subunit without forming extensive contacts with the ribosome, pk1 is bound intimately between the beak and the decoding center on the 30S subunit (57). Valle *et al.* speculate that pk1 is pulled toward the decoding center as tmRNA transitions from the initial binding complex visualized by cryo-EM to full accommodation in the A-site, resulting in Ala transfer and tmRNA translocation to the ribosomal P-site (57).

*In vitro* studies support the conclusion that pk1 plays a crucial role in tmRNA function. Although pk2-4 are dispensable for trans-translation *in vitro*, replacing pk1 with single-stranded sequence destroys tmRNA function (47). Mutations designed to disrupt the base-pairing of pseudoknot 1 helices reduce tmRNA-mediated tagging dramatically (48). Alteration of single-stranded loop sequences lowers activity, leading to proposals that these nucleotides form Mg<sup>2+</sup> binding sites (48) or make base-specific contacts with the ribosome (73).

One limitation in the study of the trans-translation system has been the lack of a robust *in vivo* selection for tagging activity. The *ssrA* gene that encodes tmRNA in *E. coli* is not essential for growth under laboratory conditions (67). One report of a genetic selection takes advantage of tmRNA-mediated tagging and proteolysis of the Arc repressor, allowing derepression of the *kanR* gene and

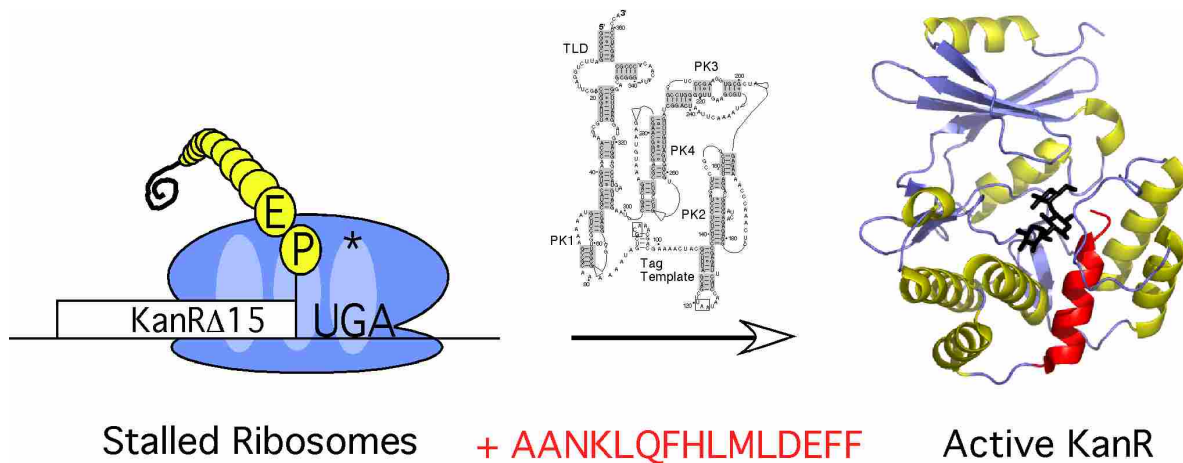
survival on kanamycin (69). The authors characterized 2451 colonies by replica plating and reported high background (~0.1%); this screen is not robust enough to search through large collections of mutants (libraries) of tmRNA or other components of the tagging machinery.

To extend the study of tmRNA structure and function to a relevant *in vivo* context, we have created a genetic selection that ties tmRNA function to the life of an *E. coli* cell. This selection allows the characterization of millions of mutants of tmRNA in a single experiment. Here we describe the application of this selection to identifying the structural and functional requirements for pseudoknot 1. How does the pk1 sequence determine its structure and what role does pk1 play in positioning tmRNA correctly inside the ribosome for resumption of translation on tmRNA?

## RESULTS

### *Development of a Selection for tmRNA Activity*

The function of tmRNA *in vivo* is to tag mistranslated proteins with an eleven amino acid sequence. If this tag could be altered to confer protein function rather than proteolysis, the activity of the tagged protein would reflect the level of tmRNA function. To provide a genetic selection, the protein must be required for cell survival and have essential residues in its C-terminal ten to twenty amino acids. In the absence of tmRNA activity, the truncated protein would be inactive and the cells would die; in the presence of an active tmRNA variant, the protein could be tagged with the essential amino acids to form a functional enzyme and confer cell survival (Figure 2-2).



**Figure 2-2. Genetic selection for tmRNA activity.**

Ribosomes stall on a truncated *kanR* template (*kanRΔ15*) at the Glu-Pro-(Opal) sequence. Active tmRNA molecules with a mutant template sequence add the final 15 amino acids of KanR (shown in red) to the nascent polypeptide (yellow). These C-terminal 15 residues form a structurally critical helix in the crystal structure of the homologous Aph(3')-IIIa protein (74). tmRNA function is linked to KanR activity and cellular survival, yielding  $10^6$ -fold enrichment of active sequences.

The selection protein we chose is the *E. coli* kanamycin resistance protein (KanR). Analysis of the homologous Aph(3')-IIIa : kanamycin co-crystal structure (Figure 2-2) reveals that the C-terminal helix of 15 residues (shown in red) plays an important role both structurally and catalytically in binding the substrate (74). Deletion of these residues leads to loss of function: cells expressing the KanR C-terminal deletion (*kanRΔ15*) show no more kanamycin resistance than cells lacking the *kanR* gene.

Natural tmRNA sequences encoding the peptide tag vary in length and composition and can be altered without loss of function (64, 75). We replaced the natural tag template with a sequence encoding ANKLQFHLMLDEFF and expressed tmRNA from its own promoter from the *tmRNA-K1* plasmid. Following transfer of Ala from the tmRNA itself, the addition of these 14 residues encoded by tmRNA restores the missing 15 amino acids that make up the C-terminal helix of KanR. We found that the identity of the resume codon was an important factor: GCA was significantly more active than AUG, the

wild-type *kanR* codon (data not shown). All known tmRNA template sequences contain GNN resume codons (75). Presumably G at this position is important for the recognition of the proper frame for reinitiation of translation by the ribosome.

In order for the altered tag to complete the KanR protein correctly, ribosomes must stall at exactly the right position on the *kanRA15* mRNA. Ribosomes stall during termination at inefficient stop codons such as the opal codon (UGA) when the protein sequence terminates in proline (35). The *E. coli* YbeL protein, for example, ends in Glu-Pro-(Opal) and is tagged with good efficiency (up to 40%) by tmRNA precisely at the position of the stop codon (35). Two mutations in *kanRA15*, Asn255Glu and Met257Opal, create a Glu-Pro-(Opal) sequence in the *kanR* mRNA that acts as a signal to induce ribosome stalling, yielding the *kanR-SEP* selection gene. Based on the structure of Aph(3')-IIIa, the KanR protein is expected to have a surface-exposed loop of five residues, ending in Pro256, preceding the C-terminal helix (74). This loop permits the introduction of the Glu-Pro-Ala scar introduced by these changes and transfer of Ala from aminoacylated tmRNA without affecting KanR function.

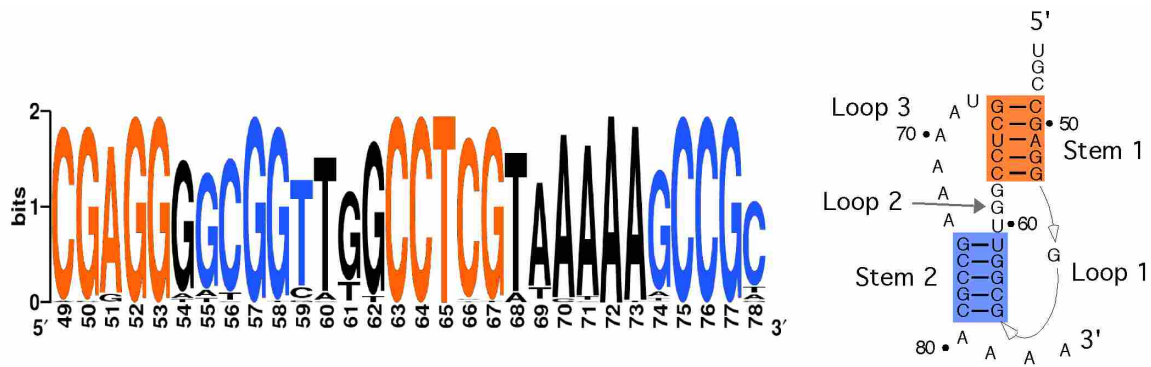
Tagging of the truncated KanR polypeptides on stalled ribosomes by the altered tmRNA generates full-length, functional KanR protein, conferring kanamycin resistance to cells. We co-transformed an *E. coli* strain lacking tmRNA with the *kanR-SEP* and *tmRNA-K1* plasmids. When plated onto media containing 15  $\mu\text{g}/\text{mL}$  kanamycin, all of the co-transformants survive at 37 °C. Under the same conditions, only 5 out of  $10^7$  bacteria with *kanR-SEP* but lacking the modified *tmRNA-K1* survive. The enrichment ratio is roughly a factor of  $10^3$  better than the previous genetic selection for tmRNA function (69).

### *Conservative Mutagenesis of pk1*

This genetic selection for trans-translation provides a method to rapidly identify active tmRNA sequences from libraries of millions of mutants. Conserved bases in the sequences of active mutants reveal which positions contain information required for function. This strategy of mutagenesis and selection was used to characterize the relationship of the pk1 sequence to its structure and function.

In creating a library of pk1 mutants, we biased the sequences towards the wild-type pk1 sequence in order to retain the overall pk1 fold thought to be required for tmRNA function. We randomized the sequence of pk1 at the rate of 20% per base (see Materials and Methods). Analysis of mutants prior to selection revealed that an average of 7 mutations were introduced at random positions in the 30 nucleotides of pk1. The library of  $\sim 4 \times 10^7$  tmRNA mutants was introduced with the selection gene into an *E. coli* strain lacking tmRNA. Following selection at 25 °C on 15  $\mu\text{g}/\text{mL}$  kanamycin,  $10^4$ - $10^5$  colonies survived.

The comparison of 67 pk1 sequences from the selected tmRNAs gives a clear picture of what positions are tolerant to mutation. The average number of mutations per pk1 sequence was 1.4, significantly lower than the average of 7 in pre-selection sequences. This was expected given the sensitivity of the pk1 structure to mutation. The results in Figure 2-3 indicate the information required at each position by the total height (e.g. position 61 has low information content and is very tolerant of mutations). Since the greatest possible mutagenesis at each position is 20%, the sequence is heavily biased towards the wild-type sequence. The information content is therefore artificially inflated and the



**Figure 2-3. Consensus sequence of active pseudoknot 1 mutants.**

The height at each position indicates the informational content required for function. The wild-type sequence was mutated at 20% mutagenesis per base, so differences in height and frequency are more significant than they appear, as the sequence is heavily biased towards wild-type. Stem 1 is colored orange and stem 2 blue. Created by Weblogo (76).

differences are more significant than they appear.

The two helical regions, stem 1 (nucleotides 49-53 and 63-67) and stem 2 (nucleotides 55-59 and 74-78), are not equally important in the pk1 structure (Figure 2-3). Stem 1 is absolutely invariant, with all the selected clones maintaining all five possible base pairs. The wobble pair introduced by the mutation A51G is active; likewise, mutations at the other positions are always accompanied by covarying mutations that re-establish the base pair (e.g. G50C and C66G). In contrast, only three of the five base pairs of stem 2 are conserved; uncompensated mutations of the outer G55-C78 and U59-G74 base pairs are tolerated.

Contrary to conclusions based on *in vitro* work (48, 73), loop 2 (nucleotides 60-62) can be mutated *in vivo* without affecting trans-translation. The earlier finding that loop 2 mutants form pseudoknots but display inhibited activity led to speculation that these bases form an  $Mg^{2+}$  or ribosome binding site. The strict requirement for G or U at position 61 and G at 62 *in vitro* was not observed in our *in vivo* assay: G61C and G62U mutants were viable.

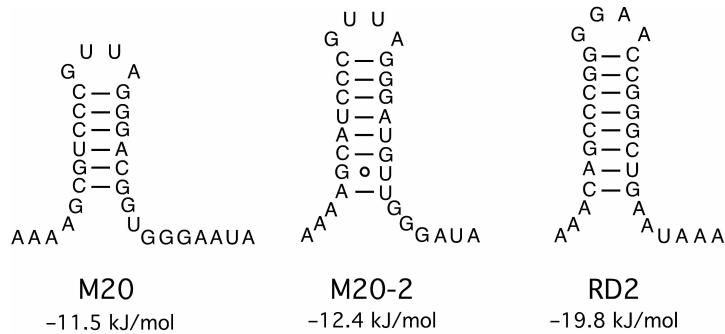


There is disagreement in the literature whether or not the first nucleotide in loop 2, U60, pairs with A73 to extend stem 2 by one base pair (45, 48, 72). Chemical probing showed that U60 is accessible but A73 protected from modifications (48). Similarly, our findings suggest that U60 can be mutated to the other bases without loss of function, but A73 is highly conserved. We propose that U60 is unpaired and is part of loop 2, while A73 forms interactions elsewhere.

The conservation of the adenosines in loop 3 (nucleotides 69-73) is striking, particularly at the 3'-end of that sequence. Analysis of the pseudoknot database shows that adenosine is highly enriched in loop 3 of pseudoknots (63.9% A) particularly at the 3'-end (77). These adenosines may make A-minor motif interactions (78) with the stem 1 helix, as seen in the high-resolution structures of several pseudoknots (53, 79). Such interactions are consistent with the *in vitro* finding that mutations in loop 3 destabilize stem 1 in tmRNA pk1 (48). Formation of A-minor motif interactions may place constraints on the sequence of stem 1, particularly the G52-C64 and G53-C63 pairs. These positions are invariant in all of our analyzed sequences. Construction of a covariant mutant (G<sub>49</sub>U<sub>51</sub>C<sub>53</sub>-G<sub>63</sub>A<sub>65</sub>C<sub>67</sub>) that alters these base-pairing patterns in stem 1 did not restore activity *in vitro* (48).

### ***Random Mutagenesis of pk1***

Having learned what the requirements are for pk1 to fold correctly, we wondered if more radical mutations could form alternate structures that function in place of pk1. To isolate active sequences far away in sequence space from wild-type pk1, we created a library of  $1 \times 10^6$  mutants in which the entire pk1



**Figure 2-4. Secondary structures of active pk1 replacements.**

The M20 mutant was selected from a randomized sequence (N30) replacing the wild-type pk1 sequence 49-79. 20% mutagenesis of the M20 sequence and selection at higher stringency yielded M20-2. RD2 was rationally designed using an unrelated sequence to form a hairpin structure with increased stability. Structures and thermodynamic stabilities were predicted by the mfold algorithm at 37 °C (81).

sequence was completely randomized (N30). This library covered but a tiny fraction of the theoretical diversity of  $4^{30}$  or  $\sim 10^{18}$ . Following selection at 25 °C on 15  $\mu\text{g}/\text{mL}$  kanamycin, roughly 50 colonies survived. Sequencing of tmRNA sequences revealed that these mutants bear no resemblance to the wild-type pk1 sequence. None form pseudoknot structures by inspection or analysis by the prediction software *pknotsRG-mfe* (80). Instead, the mutant structures are predicted by the mfold algorithm (81) to form simple hairpin-loop structures (e.g. mutant M20, Figure 2-4). All mfold free energy calculations and structural predictions were performed at 37 °C.

The activity of mutants like M20 that replace pk1 with a hairpin shows that pk1 is not required for tmRNA activity. M20 is significantly less active than wild-type tmRNA, however, failing to confer resistance to high kanamycin concentrations (50  $\mu\text{g}/\text{mL}$ ) or at higher temperatures (37 °C)—conditions under which cells containing wild-type tmRNA display 100% survival. In a second generation library, we mutated the M20 sequence at 20% per base over the 30 nucleotides replacing the pk1 sequence. A library of  $2 \times 10^6$  M20 derivatives was

subjected to selection on plates containing 50 µg/mL kanamycin (kan50) at 25 °C. Of the 8 surviving clones, M20-2 is the most active, with 100% survival on kan50 at 25 °C and 50% survival on kan15 at 37 °C. The predicted secondary structure of M20-2 contains two more base pairs than its M20 parent and is predicted to be slightly more stable thermodynamically (-12.4 kJ/mol vs. -11.5 kJ/mol respectively).

### ***Quantification of pk1 Mutant Activity***

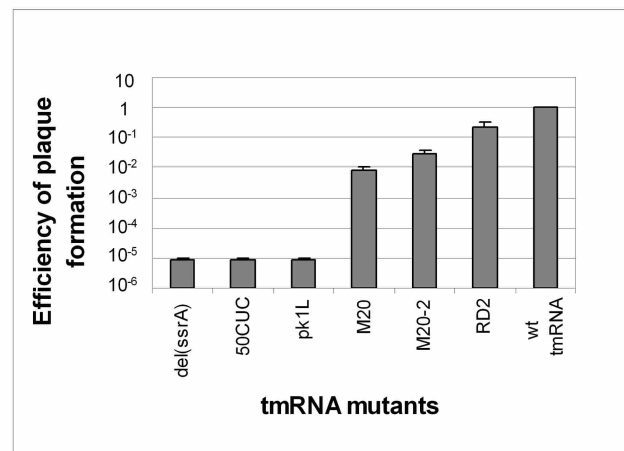
Our initial analyses of M20 and M20-2 function were performed with the KanR assay and in the tmRNA context (a mutated tag sequence) in which the mutants were evolved. In order to correlate our assay with others in the literature and to obtain more quantitative measures of the mutants' activity, we introduced the evolved pk1 sequences into otherwise wild-type tmRNA (containing the natural template encoding ANDENYALAA). tmRNA activity was measured by the efficiency of plaque formation by the hybrid bacteriophage *λimmP22 c2-dis*. This phage only forms plaques on bacteria with intact trans-translation systems (67, 82): cells expressing wild-type tmRNA from a low copy plasmid (pKW11) support the growth of  $\sim 1 \times 10^5$  more plaques than cells expressing no tmRNA.

In addition to the wild-type and  $\Delta$ ssrA controls, we assayed two pk1 mutants that were characterized previously *in vitro*. The first, pk1L, replaces pk1 with a single-stranded sequence CGAGGGCCGC. This mutant is from the study reporting that although pk2, pk3, and pk4 were dispensable for tmRNA function *in vitro*, pk1 was essential (47). The second, 50CUC, maintains the pk1 sequence

but is designed to destroy the folding of stem 1 by preventing the core three bases from pairing. The 50CUC mutant displays a ten-fold reduction in activity *in vitro* (48). Efficiency of plating (EOP) assays reveal that these two mutants are indistinguishable from the tmRNA knockout,  $\Delta$ ssrA (Figure 2-5). As measured by this assay, a missing or misfolded pk1 region renders tmRNA completely inactive *in vivo*.

The M20 clone evolved from random sequence is ~ 1000-fold more active than the missing or misfolded pk1 controls, suggesting that its hairpin structure is sufficient to restore tmRNA stability and function (Figure 2-5). M20 is, however, ~ 100-fold weaker than wild-type tmRNA. The M20-2 second-round clone shows an activity 4-fold higher than M20. The cutoff for survival in the high-stringency KanR assay must therefore lie between M20 and M20-2 activity.

The fact that the improved M20-2 mutant has more predicted base pairs and thermodynamic stability led to the hypothesis that further improvements in stability of the hairpin could improve activity. If a hairpin structure is all that is required to replace the wild-type pk1, perhaps there is nothing special about the hairpins that we evolved, and any stable hairpin structure could functionally



**Figure 2-5. Analysis of mutant tmRNA activity.** The hybrid bacteriophage  $\lambda$ immP22 *c2-dis* only forms plaques on cells expressing active tmRNA (82). Data are expressed as efficiency of plating (EOP) with wild-type tmRNA taken as EOP = 1. The  $\Delta$ (ssrA) mutant lacks tmRNA; mutants pk1L (47) and 50CUC (48) destroy pseudoknot 1 folding and tmRNA function *in vitro*. Error bars represent the standard deviation of four independent experiments.

replace pk1. To test these hypotheses, we rationally designed an eight-base pair stem with a tetraloop, utilizing a sequence substantially different from the evolved M20 and M20-2 clones. This rationally designed hairpin, RD2, is predicted to be significantly more stable ( $-19.8$  kJ/mol vs.  $-11.5$  kJ/mol) than the M20 hairpin (81). EOP assays show that RD2 is nearly as active as wild-type tmRNA, 29-fold more active than M20 (Figure 2-5). These findings suggest that the stability of the structure replacing pseudoknot 1 plays an key role in the overall function of tmRNA mutants, but that the specific sequence used is not as important.

## DISCUSSION

Previous *in vitro* studies suggest that tmRNA pseudoknot 1 (pk1) is essential to the tagging function in *trans*-translation. It must form a pseudoknot structure (47, 48), may make specific contacts to the ribosome (73), and may play a role in positioning the template region of tmRNA near the decoding center for proper continuation of translation (57), although the precise choice of frame is determined by the single-stranded sequence between the resume codon and pk1 (69-71).

To study the requirements for pk1 structure and its relation to tmRNA function *in vivo*, we developed a genetic selection that ties the life of an *E. coli* cell to tmRNA activity. KanR polypeptides lacking an essential C-terminal sequence are stalled on ribosomes; if rescued by an altered tmRNA that codes for the missing amino acids, the ribosomes produce full-length, functional KanR and the cell survives on kanamycin plates. Cells lacking tmRNA activity are killed. This selection provides the means to identify rare active mutants among large

libraries of tmRNA, giving  $10^6$ -fold enrichment of active sequences. Work is also underway to use this genetic selection to characterize other components of the *trans*-translation process.

The generation of two libraries with different levels of mutagenesis yields apparently contradictory conclusions. The first library, generated by a conservative mutagenesis scheme (20% per base), supports the previous *in vitro* findings that mutations which destabilize the pseudoknot structure of pk1 destroy tmRNA activity. Very few mutations were tolerated: the stem 1 region allowed covarying mutations only, maintaining all five of its base pairs, whereas stem 2 allowed the loss of its outer two base pairs. The U<sub>60</sub>G<sub>61</sub>G<sub>62</sub> bases of loop 2 are tolerant to mutation, suggesting that they do not form critical Mg<sup>2+</sup> binding sites or bind external ligands such as ribosomal components as previously proposed (48, 73). U60 does not pair with A73; instead, A73 and other conserved adenine bases at the 3'-end of loop 3 probably form A-minor motif interactions with the end of stem 1. These findings about the sequence requirements of pk1 structure add a new layer of detail to what was previously known, but are largely consistent with previous *in vitro* findings. They reinforce the idea that a well-folded pseudoknot 1 structure is essential to tmRNA function.

In contrast, results from the second library, in which pk1 was replaced by completely random sequence (N30), support the conclusion that any stable secondary structure is sufficient. Rare mutants were isolated that substitute hairpin-loop structures in place of pk1. A second round of mutagenesis and selection improved the activity of the best of these clones, M20, by increasing hairpin stability (mutant M20-2). Rational design of an even more stable hairpin, using an unrelated sequence, created a mutant (RD2) which is nearly as active as

wild-type tmRNA. The sequence of the hairpin appears to be much less important than its thermodynamic stability. These surprising results were confirmed with a standard efficiency of plating (EOP) assay using phage *λimmP22 c2-dis*. This assay demonstrates that our results are not due to an artifact of the KanR assay or the altered tmRNA tag sequence used in the selection.

What is the resolution of these apparently paradoxical conclusions? Low levels of mutagenesis of the wild-type pk1 sequence either destroys or retains the pk1 fold, but total randomization allows alternate stable structures far away in sequence space to be identified. We propose that the only requirement for the pk1 region of tmRNA is that it forms a stable structure that prohibits other deleterious global misfolding events. Alternate stable conformations were detected by NMR and denaturation profiles previously *in vitro* (48, 73); the role of stable pseudoknots is probably to prevent these structures from folding. A pseudoknot structure is not necessary, however.

This finding has important consequences for mutational analyses of structures in functional RNAs. The inactivity of a mutant designed to destroy a given structure does not logically require that the structure is necessary for function, as is too often assumed. The mutation may cause global folding problems that in turn render the molecule inactive. This does not prove that the given structure could not be replaced by a stable structure quite different from itself. In our case, the inactivity of a mutant pseudoknot does not prove that the structure has to be a pseudoknot; it only demonstrates that the region in question must form a stable fold. Stability and global folding should therefore be an important consideration in designing mutations to test hypotheses about the role

of specific structures in RNA and in interpreting such studies. Our findings also highlight the importance of analyzing a large number of mutants and the power of genetic approaches to do so in an efficient manner.

#### ACKNOWLEDGEMENTS

We thank Robert Sauer and co-workers for the pKW11 plasmid and the X90 *ssrA:cat* strain and David Friedman for the *λimmP22 c2-dis* phage. This research was supported by funds from Brigham Young University.

#### MATERIALS AND METHODS

DNA oligonucleotides were synthesized on an ABI Expedite 8909 or purchased from Sigma-Genosys. Enzymes were purchased from New England Biolabs.

##### *Plasmid Construction*

The *tmRNA-K1* plasmid expresses the altered tmRNA gene from its own (*ssrA*) promoter on a low-copy (p15A) origin with a tetracycline resistance marker. It was created by altering the tag sequence (ANDENYALAA) to code for the last 14 amino acids of KanR (ANKLQFHLMLDEFF). Nucleotides 90-137 of tmRNA, from the resume codon to the end of helix 5, were replaced with the sequence GCAAATAAACTGCAGTTTCATTTGATGCTCGATGAGTTCTTCTAATAACAGAATCTCATC. This sequence creates a stem-loop structure similar to helix 5 following the tag template. It contains a *Pst*I cleavage site for cloning mutant *pk1* regions immediately upstream. The *tmRNA-K1* plasmid was created by PCR amplification of pKW11 (64) using the primers



GAGCATCAAATGAAACTGCAGTTTATTTGCGACTATTTTTTGCGGCTT  
TTTAC and GATGAGTTCTTCTAATAACAGAATCTCATCCCTCTC  
TCCCTAGC CTCC, followed by PNK phosphorylation and blunt end ligation.

Truncated *kanR* constructs were expressed from the *araBAD* promoter on a multicopy (pBR322 origin) vector with a  $\beta$ -lactamase marker. The first version of the selection, used in the 20% mutagenesis of wild-type *pk1*, induced stalling with the sequence Pro255-Pro256-Opal. The *kanR-DPP* plasmid was cloned by the amplification of the *kanR* gene with primers

CATATGGCTAGCATGAGCCATATTCAACGG GAAAC and  
CGGCTTTTTCAAAAATATGGTATTGATCCGCCTTGAGAATTC GAGCTC,  
digestion with *NheI* and *EcoRI*, and ligation into the pBAD-GFP vector.

To combine the two plasmids into a single selection vector, the tmRNA expression cassette was PCR amplified from *tmRNA-K1* with primers  
CCGCTACGGTCCGAGAACTGTGAATGCGCAAACC and  
TAGCGAAGATCTT AAATCCTGGTGTCCCTGTTG. The *kanR-DPP* plasmid was PCR amplified with primers CGACCGAGATCTTCGCTACGTGAC  
TGGGTCATG and AGTTCTCGGAC CGTAGCGGAGTGTATACTGGCTTAAC. These fragments were digested with *BglIII* and *RsrII* and ligated together. Quickchange mutagenesis and removal of *SphI* and *PstI* sites from the resulting plasmid, pBad-KT2, allow these unique sites surrounding the pseudoknot 1 sequence to be used for cloning. The sequence GAATAGAGGCCTTC  
AACTCCGCGGATACTA was then inserted between *SphI* and *PstI*, replacing wild-type *pk1* and making tmRNA inactive. This “dummy insert” also provides *StuI* and *SacII* sites to assist in library creation.

A second, more active version of the selection vector was generated by replacing the C-terminal amino acids Asp254-Pro255-Pro256 (*kanR-DPP*) with Ser254-Glu255-Pro256 in the truncated *kanR* gene. This *kanR-SEP* sequence was discovered by cloning 6 random codons after Ile253 of the truncated *kanR $\Delta$ 15* gene in pBad-KT2 and selecting for kanamycin resistance under more stringent conditions: 37 °C and 15  $\mu$ g/mL kanamycin. Addition of a chloramphenicol resistance cassette into the *Bgl*III site provided a second antibiotic marker to reduce contamination, yielding the plasmid p16Dum-Cat.

tmRNA expression vectors for the bacteriophage assays were created by PCR amplification of the pKW11 plasmid and blunt-end cloning. The procedure was similar to that described above to make *tmRNA-K1*, except that no change was made to the tag template: the only difference from wild-type tmRNA is the pseudoknot 1 sequence. The  $\Delta$ ssrA control was made by removing the entire tmRNA sequence by digestion of pKW11 with NcoI and EcoRI, followed by treatment with the Klenow fragment of DNA polymerase I to create blunt ends for religation. The pk1 sequences are as follows:

50CUC, CCTCGGGCGGTTGGCCTCGTAAAAAGCCGC;

pk1L, CGAGGGCCGC;

M20, AAAAGCGTCCCGTTAGGGACGGTGGGAATA;

M20-2, AAAAGCATCCCGTTAGGGATGTTGGGATA;

RD2, AAACAGCCCGGGGAACCGGGCTGAATAAA.

### *Selection of Mutant pk1 Libraries*

The conservative mutagenesis of pk1 was accomplished by mutating the 30 nt of pk1 at 20% per base. The oligonucleotide CAAGGTGCATGCCGAGGGGCGGTTGGCCTCGTAAAAAGCCGCAAAAAAT AGTCGCAAATAAACTGCAGTTTCAT was synthesized with mixed phosphoramidites incorporated into the positions underlined. These mixes included 80% of the wild-type base and ~7% each of the other three bases. A short primer bound to a constant 3' region was extended by the Klenow fragment of DNA polymerase I to generate double-stranded DNA. This DNA was cleaved by *SphI* and *PstI*, ligated into the pBad-KT2 vector, and the plasmid library introduced into DH10B by electroporation. Following plasmid purification, the resulting library was introduced into the selection strain, X90 *ssrA::cat* (83). The cells were induced with 2% arabinose for 3 h at 30 °C, plated onto media containing ampicillin, 2% arabinose, and 15 µg/mL kanamycin, and grown for 48 h at 25 °C.

The second library, with fully-randomized pk1 sequences, was generated in a similar manner except it incorporated N30 (25% each base) in place of the underlined sequence above. This library was cloned into the second-generation selection vector p16Dum-Cat and selected as above. The third library, with 20% mutagenesis of M20, replaced the underlined sequence with mixed phosphoramidites as above but based on the M20 sequence. The mutant inserts were cloned into p16Dum-Cat and selected on media containing ampicillin, 2% arabinose, and 50 µg/mL kanamycin at 25 °C. Surviving clones from these libraries were recloned, transformed into fresh cells, grown up from single

colonies, and reassayed under selection conditions to ensure that the tmRNA mutant in question is responsible for the tagging activity.

### *Phage Efficiency of Plating (EOP) Assays*

X90 *ssrA::cat* cells carrying a pKW11-derivative expressing *pk1* mutants of tmRNA were grown overnight in media with tetracycline at 37 °C. The cells were washed in 2xYT media, diluted, and grown in 2xYT with 10 mM MgSO<sub>4</sub> for three hours at 37 °C to an OD<sub>600</sub> of about 1. The cells were then washed with 10 mM MgSO<sub>4</sub>, resuspended to an OD<sub>600</sub> of 0.5, and measured out into 200 µL aliquots. 1 µL of phage *λimmP22 c2-dis* at various dilutions was added to an aliquot, incubated at 37 °C for 15 minutes, and mixed with 3 mL of top agar (2xYT, 10 mM MgSO<sub>4</sub>, and 0.7% agarose), and grown overnight at 37 °C. Plaques on tmRNA mutants were smaller than those formed on wild-type tmRNA-containing cells as reported previously (82). The number of plaques were counted for four different trials as reported in Figure 2-5.

CHAPTER 3: IDENTIFICATION AND CHARACTERIZATION OF NASCENT  
PEPTIDE SEQUENCES THAT INDUCE RIBOSOME  
STALLING DURING ELONGATION

*ABSTRACT*

TnaC and SecM are short peptides that are capable of inhibiting the peptidyl-transferase activity of the ribosome to stall translation. Although these peptides each cause stalling by interacting with the same three regions of the ribosome, they do so through unique interactions. Intrigued by the lack of homology between the sequences and the unique mechanisms by which they inhibit the peptidyl-transferase activity of the ribosome, we sought to identify new peptides which could also inhibit peptidyl transfer. To identify novel stalling peptides we created a genetic selection that would tie the life of the cell to ribosome stalling. This selection takes advantage of a modified tmRNA carrying out its natural function of recognizing stalled ribosomes and adding a tag to the stalled peptides. With this selection we have screened a library of randomized hexamers. Those sequences that caused stalling were tagged by tmRNA and survived on a selective medium. From the survivors of the selection we have identified three classes of stalling peptides: peptides containing a C-terminal Pro, peptides similar to SecM, and peptides with the novel stalling sequence FxxYxIWPP. Further characterization of the latter class has revealed a similar mechanism for stalling as used by SecM and TnaC, but with distinct requirements. We conclude that ribosome stalling may be caused by numerous sequences and is likely much more common than previously believed.

## INTRODUCTION

The ribosome efficiently synthesizes an enormous diversity of peptide sequences without regard to their chemical properties. This generality is not universal, however. Several peptides interact with the ribosome and induce stalling during their own translation, either in the elongation or termination steps (20, 84). Such translational pauses may affect protein folding before the protein fully dissociates from the ribosome (85). Programmed stalling events also regulate the expression of genes in both prokaryotes and eukaryotes (20, 84, 86).

In two well-characterized examples from *E. coli*, ribosome stalling on a leader peptide increases the expression of a downstream gene on the same mRNA. The secretion monitor peptide SecM, for example, regulates *secA* in response to changes in protein export activity. If export activity is low, ribosome stalling on the SecM peptide alters the secondary structure of the mRNA and upregulates the translation of *secA*, a key component of the secretory machinery (87, 88). When export activity is high, the SRP-Sec translocation system binds the signal peptide in SecM and pulls it from the stalled ribosome. A second example is the regulation of *tnaA*, an enzyme that breaks down tryptophan, by its upstream leader peptide TnaC in response to cellular levels of tryptophan. When tryptophan concentrations are high, ribosome stalling on TnaC blocks a transcriptional terminator, increasing expression of *tnaA*. Lower tryptophan levels do not support ribosome stalling and lead to attenuation of the transcript.

Stalling at these peptides is the result of three interactions: the binding of the nascent peptide to the ribosomal exit tunnel and the peptidyl-transferase center, and the binding of an effector in the ribosomal A site. The peptide exit

tunnel in the large ribosomal subunit is 100 Å long and 10-20 Å wide (26). Mostly made of RNA, it provides very few hydrophobic surfaces for elongating proteins to bind, accounting for their ability to pass through unhindered. A significantly constricted portion of the tunnel is formed by loops in proteins L4 and L22. SecM and TnaC interact with the tunnel near this constriction, using critical Trp residues 10-12 amino acids upstream of the stalling site. Ribosomal mutations that reduce stalling map to the exit tunnel, implicating A751, A2058, and U2609 in the 23S rRNA and specific residues in the L22 protein in the stalling mechanism (18, 29). A cryo-EM study of the SecM-stalled ribosome revealed a network of conformational changes in 23S rRNA emanating from the exit tunnel (30).

Nascent peptides also interact directly with the peptidyl-transferase center (PTC) to induce stalling. In the case of SecM, the identity of the final five residues is critical for stalling at the FxxxxWxxxxGIRAG<sub>165</sub> sequence. Likewise, the C-terminal Pro residue in TnaC is essential for stalling at the sequence WxxxDxxxxxxxP\* (17). These amino acids must be acting within the PTC to inhibit its catalytic activity, either peptidyl transfer in SecM or peptidyl hydrolysis in TnaC. In some cases, the peptide sequence in the PTC is sufficient to induce stalling without exit tunnel interactions. A C-terminal Pro residue in the YbeL protein inhibits termination, especially when preceded by the amino acids Asp, Glu, or Pro.

In addition to nascent peptide interactions with the exit tunnel and the PTC, the binding of an effector molecule in the A site is also required for stalling at SecM and TnaC. SecM stalls during elongation with unreacted Pro-tRNA bound in the A site (22, 89). Mutation of this Pro codon to Ala alleviates stalling.

Likewise, TnaC stalling requires binding of free tryptophan near the PTC (17). The action of free tryptophan can be mimicked by Trp-tRNA if the *tnaC* stop codon is mutated to a Trp codon. Other aminoacyl-tRNAs (Phe, Met, Pro) do not induce stalling (17). Binding of the amino acid Trp creates a PTC conformation that leads to stalling on the TnaC peptide.

Although nascent peptide sequences that induce ribosome stalling interact with the exit tunnel and PTC, they share little sequence similarity. This led us to hypothesize that there are additional, unknown peptide sequences that might inhibit peptidyl transfer or hydrolysis. Here, we report the development of a genetic selection to identify stalling peptides from random libraries and the characterization of peptides that stall at high efficiency during elongation. To our knowledge this is the first systematic identification of peptides that cause stalling through direct binding to the ribosome.

## RESULTS

### *A Genetic Selection for Novel Stalling Peptides*

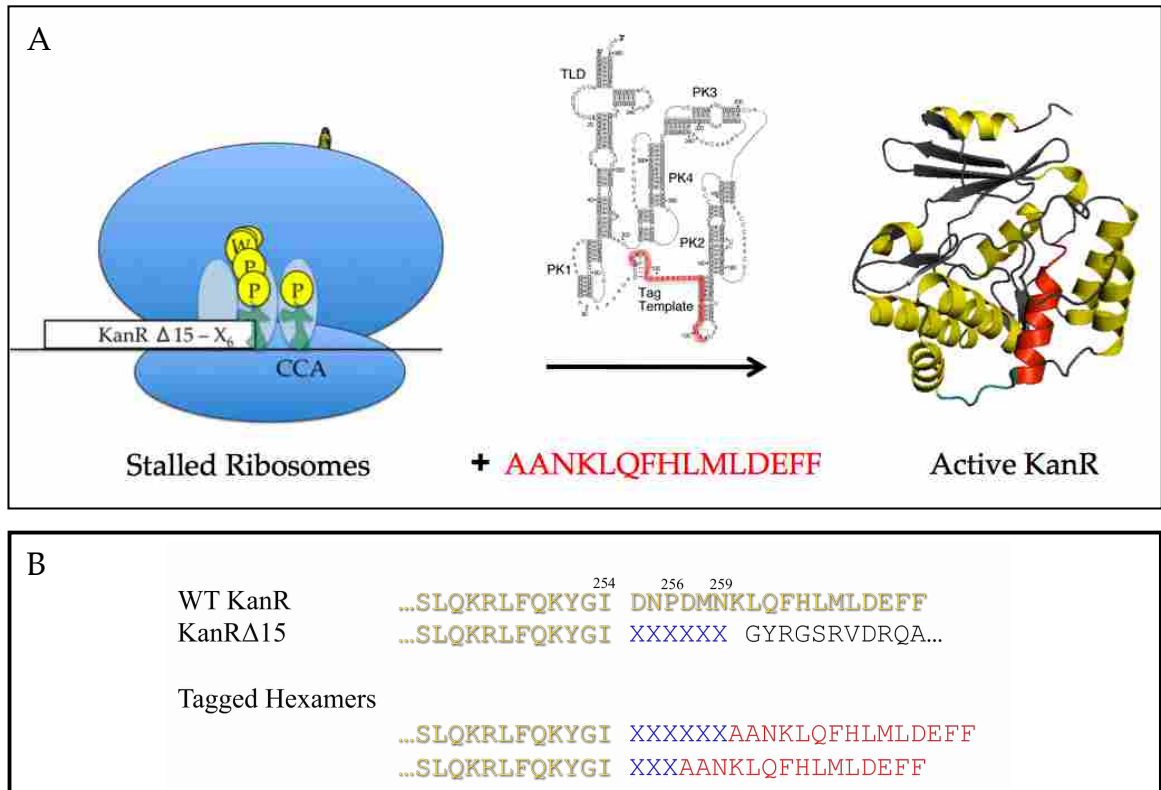
We set out to systematically identify peptide sequences like SecM and TnaC that interfere with peptidyl-transfer or hydrolysis and induce ribosome stalling. To identify stalling peptides from random libraries, we modified a genetic selection that we developed previously to link ribosome stalling to the life of the cell (50). In this selection, stalled ribosomes are recognized by transfer-messenger RNA (tmRNA), a small, stable RNA found in eubacteria that is part of a quality control system for protein synthesis. tmRNA's natural function is to release stalled ribosomes and tag the aborted nascent peptides for destruction (35, 64). Acting as a transfer RNA, tmRNA enters the empty A site of the



ribosome and adds Ala to the nascent polypeptide chain. tmRNA then serves as a template, encoding a short peptide tag that is recognized by cellular proteases. After this tag is translated, the ribosome is released at a stop codon within tmRNA and the aborted protein product is degraded. For the purposes of our selection, it is important to note that although tmRNA was first characterized as rescuing ribosomes stalled on mRNAs lacking stop codons (83), recent studies demonstrate that it can also act on ribosomes stalled by nascent peptides (35, 90).

To create a genetic selection for ribosome stalling based on this ribosome rescue machinery, we altered tmRNA so that instead of tagging proteins for proteolysis, it completes the synthesis of an essential protein, linking stalling to the life of the cell. The kanamycin resistance protein (KanR) from Tn10 has a C-terminal helix of 15 amino acids that is structurally critical; truncation of this helix leads to loss of activity. To complement the truncated KanR protein, we changed the tmRNA template sequence to encode the last 14 residues of KanR (ANKLQFHMLDEFF). Together with the Ala from aminoacylated-tmRNA, these residues complete the KanR protein and restore KanR activity—but only if the ribosome stalls at exactly the right site. This serves as the basis for our selection: peptide sequences that stall the ribosome at the end of a truncated KanR protein can be easily identified in random libraries because they recruit tmRNA, complete KanR, and confer resistance to kanamycin (Figure 3-1).

How can stalling be induced at the end of the KanR protein without interfering with the final structure and activity of KanR? We previously showed that two mutations, Asn255Glu and Asp257Opal, create a Glu-Pro-(Stop) sequence that induces stalling during translational termination. Expression of



**Figure 3-1. Genetic selection for stalling peptides.**

A. When the hexamers at the end of the *kanRΔ15* gene cause ribosome stalling, the KanRΔ15 protein (yellow) is tagged by tmRNA-K1. The KanR tag (red) forms a helix that restores the function of KanR. Only those library sequences that cause stalling will be tagged and survive on kanamycin. The active KanR structure is adapted from the crystal structure of the homologous Aph(3′)-IIIa protein (74). B. The random hexamer (blue) was inserted after I254 of KanR. The tagged sequence contains two alanine residues then the sequence matches from N259 to the end of the protein. Tagging on the random hexamer could occur at any point within the randomized region.

this truncated *kanR-SEP* construct and the altered tmRNA, *tmRNA-K1*, allow cells to survive equally well on selective (15 μg/mL kanamycin) or non-selective plates at 37 °C. Under the same conditions, bacteria lacking the modified *tmRNA-K1* gene survive at the rate of 5 colony forming units in 10<sup>7</sup> (50). These results demonstrate that the introduction of the Glu-Pro-Ala “scar” from the stalling and tagging process does not destroy KanR activity. Analysis of the crystal structure of the homologous Aph(3′)-IIa protein suggests that the C-terminal helix in KanR is preceded by a surface-exposed loop of poorly conserved residues (Ile253 through Pro256). We anticipated that this loop region

might tolerate a variety of sequences that induce stalling and tagging while maintaining robust KanR function.

Nascent peptide sequences that induce ribosome stalling were isolated from a library of random hexamers fused to the truncated KanR protein. Eighteen amino acids were deleted from KanR, including the C-terminal helix and three residues in the preceding loop. Random hexamers were cloned at the C-terminus of the truncated protein beginning with KanR residue 254. No stop codon was specified. We generated a library of  $5 \times 10^6$  KanR mutants, corresponding to roughly 10% of the theoretical diversity of a peptide hexamer library. We transformed the library, together with *tmRNA-K1*, into an *E. coli* strain lacking wild-type tmRNA ( $\Delta$ *ssrA*) and selected for survival on plates containing 15  $\mu$ g/mL kanamycin at 37 °C. Roughly 1 in  $10^4$  colonies survived—a substantial fraction—suggesting that a variety of different peptide sequences can induce ribosome stalling.

### ***Three Classes of Stalling Peptides***

Sequencing of the surviving clones revealed three classes of peptides (Table 3-1). The most common cause of stalling, found in over 90% of the clones, is inefficient termination at the sequence Pro-Stop. The Pro residue is found almost exclusively at position three of the random hexamers, corresponding to native KanR residue Pro256. While there is no significant codon bias for any particular Pro codon, the opal stop codon (UGA) is highly overrepresented (23/29 clones). There is also selection for the (-2) residue just upstream of Pro: Glu is overrepresented (16/29) in the Pro-Stop clones and Asp, Pro, and Gly are each seen several times.

A second class of peptides induce stalling during elongation, not termination. These clones contain two consecutive Pro codons, most commonly at positions three and four, with no nearby stop codon. The majority of these clones were found by lowering the selection stringency by plating at 25 °C. When tested individually, they showed poor survival at 37 °C, roughly 1-10%, much weaker than the 100% survival seen with the Pro-Stop sequences above. An exception to this rule is clone 46-36 which contains a sequence (GIRAPP) that

<b>Table 3-1. Sequences of library clones representing different modes of stalling.</b>		
<b>Class I: sequences containing Pro-Stop</b>	<b>Clone</b>	<b>Comments</b>
Y G I S E P * TATGGTATT <u>TCTGAACCGTGAGAAAGT</u> GGTACC	37s-16	This group consisted of greater than 90% of all the sequences identified in the selection.
Y G I K D P * TATGGTATT <u>AAGGATCCTTAGGACTAG</u> GGTACC	37s-7	
Y G I W P P * TATGGTATT <u>TGGCCACCTTGACTAACC</u> GGTACC	46-1	
<b>Class II: sequences with Pro-Pro</b>		
Y G I R A P P H C G T TATGGTATT <u>AGGGCGCCTCCCCATTGC</u> GGTACC	46-36	In those clones containing Pro-Pro without Trp, the prolines were in positions three and four of the random hexamer.
Y G I A D P P C A G T TATGGTATT <u>GCTGACCCACCTTGTGC</u> AGGTACC	618-6	
Y G I R S P P N S G T TATGGTATT <u>AGATCTCCACCGAATAGT</u> GGTACC	618-19	
Y G I L D P P G M G T TATGGTATT <u>CTGGATCCTCCAGGCATG</u> GGTACC	618-20	
<b>Class III: sequences with Trp-Pro-Pro</b>		
Y G I W P P W Y R G T TATGGTATT <u>TGGCCCCCTGGTATAGG</u> GGTACC	250-36	These sequences all stalled with high efficiency. In each of the Trp-Pro-Pro peptides, the prolines were in positions two and three of the random hexamer.
Y G I W P P D V * TATGGTATT <u>TGGCCTCCGGATGTATGA</u> GGTACC	37s-20	
Y G I W P P P S I G T TATGGTATT <u>TGGCCACCGCCATCGAT</u> TGGTACC	46-20	

closely resembles the stall site of the SecM peptide (GIRAGP).

A third class of clones contain the sequence Trp-Pro-Pro without a stop codon immediately following. Like the other Pro-Pro sequences, these peptides must also stall during elongation rather than termination. Unlike the second class of clones, however, the two consecutive Pro codons appear at positions two and three rather than three and four. Clone 46-5, for example, contains the hexamer WPPWYR. Another difference is that WPP-containing clones survive robustly (100%) in the KanR selection at 37 °C when characterized individually. Further experiments on these peptides are described below.

The sequence Pro-Stop occurs commonly and stalls at high levels; to prevent such clones from overwhelming other novel sequences, we created a second library of random hexamers in which stop codons were prevented from occurring at positions four through six. This was done by allowing only C, G, and A at the first nucleotide of these codons, also eliminating Phe, Tyr, Cys and Trp at positions four through six. We screened an  $8 \times 10^6$  member library at high stringency, obtaining colonies at a 0.01% survival rate. 21/23 sequenced clones contained the sequence WPPP at the first four positions. This result confirms that WPP-containing sequences are robust inducers of stalling, particularly when coupled with a third Pro codon.

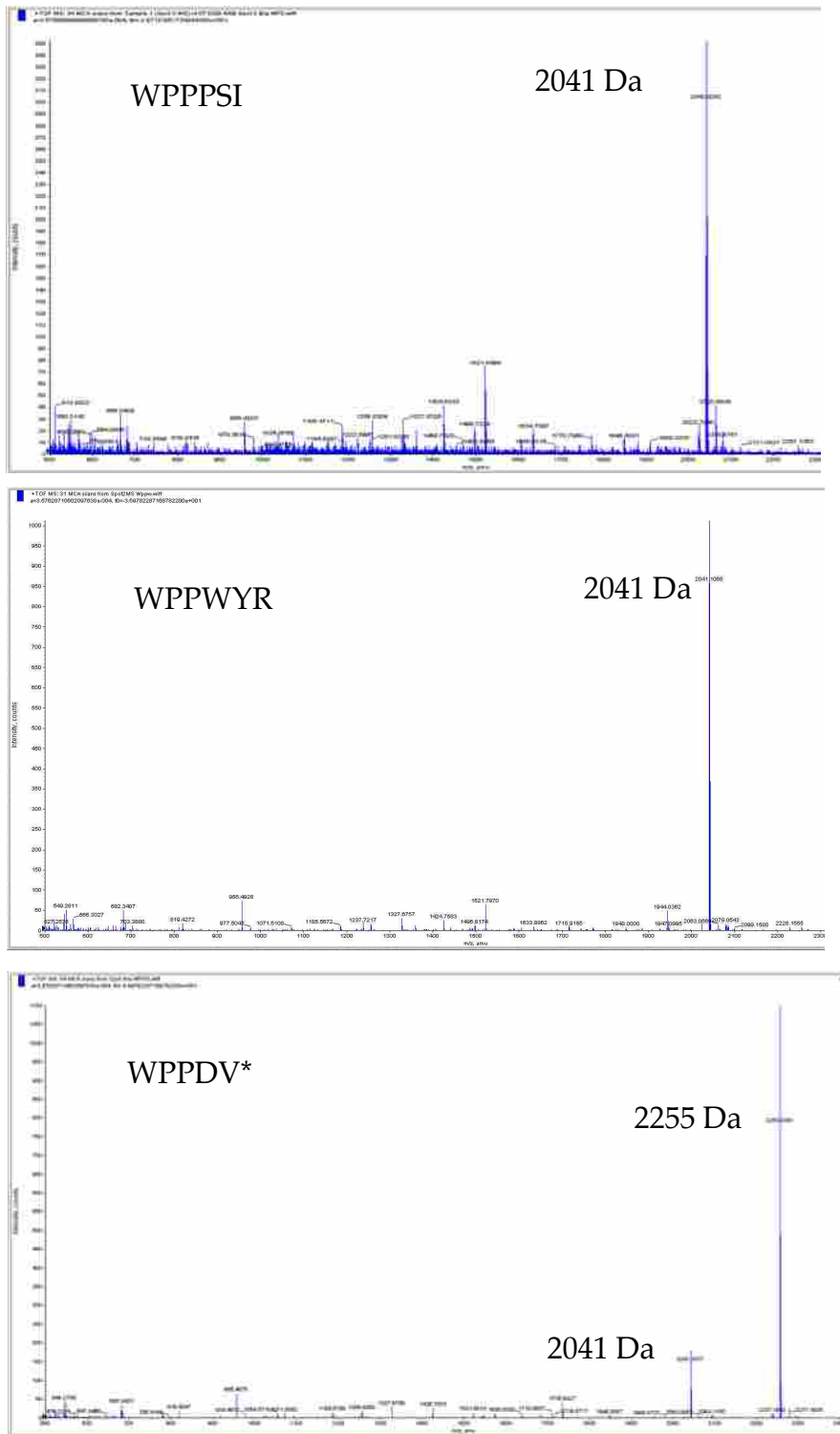
Selection of this second library at low stringency yielded much higher levels of survival (0.25%). Nearly all of these clones fall into the second class of stalling peptides, with two consecutive Pro codons at hexamer positions three and four. An alignment of 46 of these sequences reveals that Arg or His are strongly preferred at the first position, with Ala, Asp, Ser, and Pro at the second

position. Including the constant Gly-Ile upstream, the consensus sequence becomes GI(R/H)xPPxx.

### ***Stalling and Tagging Occur Following WPP***

The peptide sequences in class three (containing WPP) show high levels of activity in the KanR assay and stall translation during the elongation step. We chose to further characterize three sequences: WPPPSI, WPPDV\*, and WPPWYR. Where does stalling occur in these sequences? Where is the tag added by tmRNA? To determine the point of tagging using mass spectrometry, we first transferred the stalling sequence to the C-terminus of the GST protein. This full-length, stable protein served as a scaffold enabling overexpression. Some of the KanR protein context was fused to GST as well, from 12 amino acids upstream of the random hexamer through the first stop codon downstream: SLQKRLFQKYGIWPPPSIGYRGSRVDRQAWLFWRMREDFQPDTD\*. To isolate proteins tagged by tmRNA, we used a modified tmRNA encoding six His residues in its template sequence (*tmRNA-H*). GST-fusions tagged by *tmRNA-H* were purified by affinity chromatography using Ni-NTA resin and digested with trypsin. From this tryptic digest, the C-terminal tagged peptide was purified again with Ni-NTA resin.

The C-terminal peptide contains both the stall sequence from KanR and the tmRNA tag; determining its mass and peptide sequence by MALDI-MS revealed the site of stalling and tagging by tmRNA. A single large peak in the mass spectra for the WPPPSI and WPPWYR C-terminal tagged peptides corresponded to a mass of 2041 Da. This is the mass expected if the tmRNA tag is added after the second Pro (YGIWPPAANDH<sub>6</sub>D, Figure 3-2). The mass



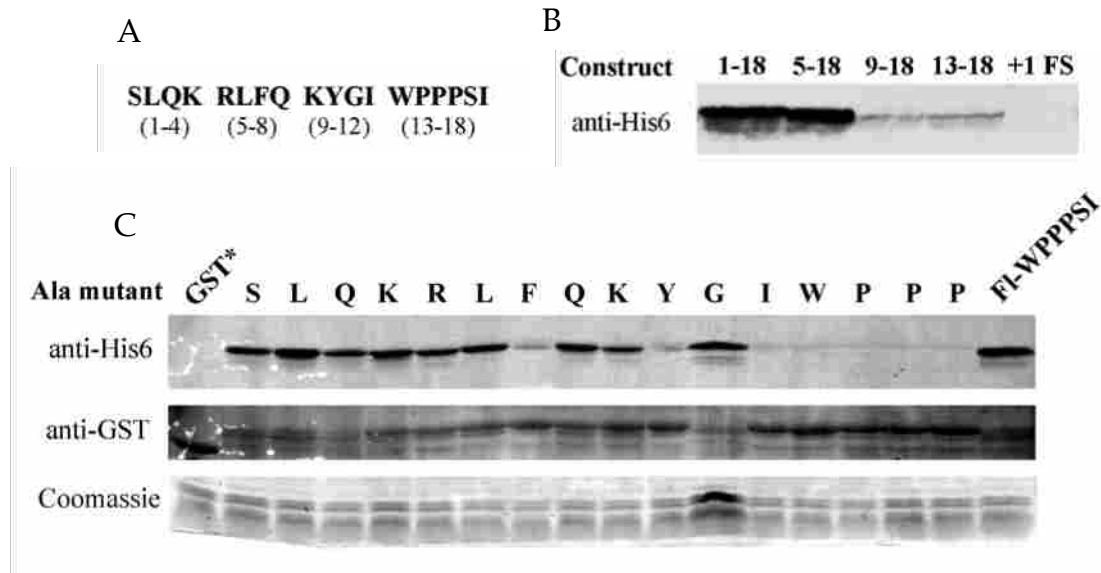
**Figure 3-2. Mass spectra of stalled class three peptides.** Stalled peptides are tagged with tmRNA-H, digested with trypsin and purified. The mass of each fragment indicates the point of stalling and tagging. Stalling occurs on WPPPSI and WPPWYR after the second Pro, as indicated by a mass of 2041 Da. WPPDV\* stalls after the second Pro, but actually stalls more efficiently at termination on Val.

spectrum of the WPPDV\* peptide fragment contained the same peak at 2041 Da together with a more abundant peak at 2255 Da corresponding to the peptide YGIWPPDVAANDH<sub>6</sub>D. In the WPPDV\* clone, stalling occurs both after WPP and during termination at the stop codon. Peptide fingerprinting by tandem MS/MS was performed on all four of these peptides to confirm the amino acid sequence directly.

### *Determination of Residues Necessary and Sufficient for Stalling and Tagging*

The MS data indicate that stalling occurs immediately after WPP in these three clones. What amino acids cause this stalling event? In the case of SecM and TnaC, residues essential for the highest levels of stalling are found upstream and interact with the exit tunnel. For this reason we included 12 amino acids (SLQKRLFQYGI) from KanR along with the hexamers in making the GST-fusions. To assay for stalling and tagging in the GST-fusions, we detected the tag added by *tmRNA-H* with anti-His<sub>6</sub> antibodies. High levels of tagging were detected for the full-length GST-WPPPSI fusion (1-18: 12 residues from KanR followed by the hexamer) (Figure 3-3 B). Deletion of the first four amino acids had little or no effect (5-18), but removal of the first eight nearly eliminated tagging (9-18). We conclude that residues upstream of the WPPPSI sequence play a critical role in high-efficiency tagging. Interestingly, some minimal activity resides in the hexamer sequence alone (13-18) with no KanR upstream sequence. Analysis of the GST fusions using anti-GST antibodies reveals that stalling is indeed efficient; far higher levels of full-length GST are seen when tagging and stalling are lost.





**Figure 3-3. Determining essential components of stall peptide.**

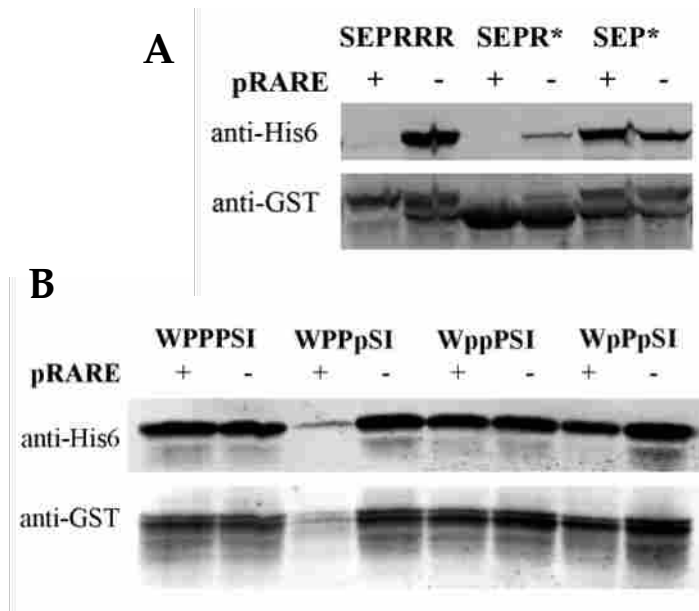
Immunoblots of full-length (Fl) and truncated constructs of the WPPPSI library selectant. **A.** The stall peptide was divided into four regions, numbering from the N-terminus of the library sequence. **B.** Removing 4 amino acids at a time from the N-terminus of the stall peptide indicated where the residues essential for stalling were located. A +1 frameshift (FS) was also assayed for tagging. **C.** Individual alanine mutations were made in each position of the Fl stall peptide WPPPSI. The amino acid replaced in each mutant is listed above its representative lane. A coomassie stain served as a loading control for each of the different mutants.

To identify how each residue contributes individually to stalling, we performed alanine scanning on the full-length stalling peptide (1-18). Residues 1 to 16 were individually mutated to alanine and assayed by immunoblot. Consistent with the truncation results, mutating residues 1-4 had little or no effect on tagging (Figure 3-3 C). Alanine substitutions for Arg5, Leu6, Gln8 and Lys9 likewise made little difference in the level of stalling. In contrast, the Phe7Ala and Tyr10Ala mutants dramatically decreased tagging levels. The requirement for Phe7 explains why truncating residues 1-8 strongly lowers tagging above. Stalling was eliminated by mutating Ile12 or of any residue in the WPP sequence to Ala. Notably, tagging was also eliminated by the Pro16Ala mutation. This is surprising because the MS data shows that the third Pro in WPPPSI is not incorporated into the stalled peptide.

tmRNA rescues ribosomes stalled on broken or defective mRNA templates; perhaps these tagging events arise from RNA synthesis defects in the KanR mRNA or post-transcriptional nucleolytic cleavage. To prove that tagging requires translation of the peptide sequence, we created a mutant of the GST-WPPPSI fusion in which a single nucleotide is added upstream of the full-length stall peptide. The resulting +1 frameshift changes the identity of every amino acid in the stalling sequence except for Phe7 and Lys9 while retaining the same nucleotide sequence. Immunoblot analysis of this mutant revealed that tagging was completely abolished, demonstrating that stalling on the GST-WPPPSI fusion is due to the amino acid sequence and not the nucleotide sequence (Figure 3-3 B).

### *The role of Codon Usage*

We anticipated at the outset of our KanR selection experiments that we would isolate stalling sequences with rare codons. The three tRNA<sup>Arg</sup> isoacceptors decoding the CGG, AGA, and AGG codons are present at low levels in *E. coli* (91). Overexpression of proteins containing consecutive rare codons induces high levels of stalling and tagging by tmRNA (92). Why do such sequences not survive the KanR selection? To address this question, we measured tmRNA tagging levels for a GST-fusion construct containing SEPR\* and SEPRRR encoded by the rare Arg codon AGG. SEPR\* stalling was barely detectable, much lower than SEP\*, while SEPRRR tagged at very high levels in the immunoblot assay. Tagging at both sequences was completely alleviated by overexpression of the cognate tRNA<sup>Arg</sup> from a plasmid (pRARE) (Figure 3-4 A). The same SEPR\* and SEPRRR sequences were then cloned in place of the



**Figure 3-4. Immunoblot showing effects of rare codons.** Cells were grown with or without the pRARE plasmid, which overexpresses a rare Arg codon and a rare Pro codon. A. Stalling on the sequence SEP\* is unaffected by pRARE, but it is completely relieved on a rare Arg sequence with supplemented tRNA<sup>Arg</sup>. B. The rare proline codon that is complemented by *proL* on pRARE does not affect stalling on WPPPSI except when placed in the position of the third Pro. This dramatically reduces the overall expression of GST (equal amounts of total protein were verified by coomassie staining).

randomized cassette of the *kanR* selection plasmid. The sequence SEP in the first three positions is known to be compatible with KanR activity; in the SEP\* context it conveys 100% survival. Cells expressing these plasmids survived no better than an empty vector control under low stringency conditions (data not shown). These results show that tagging activity at rare codons is either insufficient or incompatible with restoring KanR function.

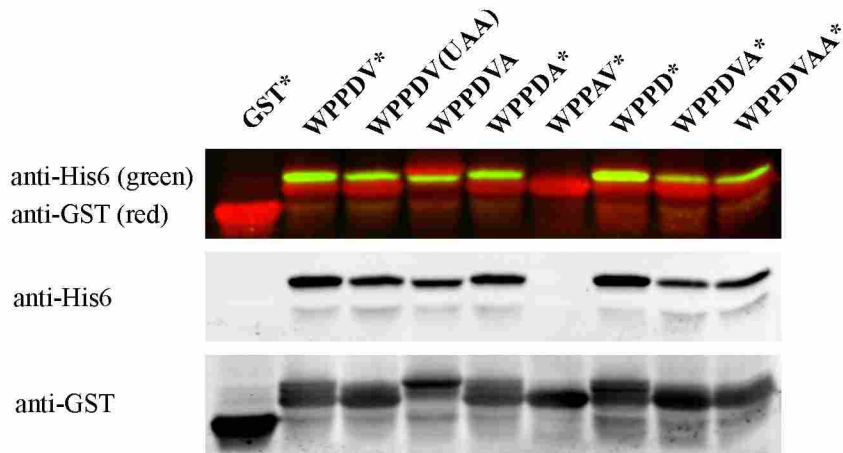
What is the role of codon usage in the WPP-containing sequences isolated in the KanR selection? All four Pro codons were found in the sequences encoding these clones. There was no obvious selection for individual codons in the WPPP sequences isolated in the second library. Nevertheless, it is possible that stalling or tagging at WPPPSI requires depletion of Pro-tRNA<sup>Pro</sup> in the cell; if so, then overexpression of tRNA<sup>Pro</sup> should alleviate stalling or tagging. To test this hypothesis, we altered the WPPPSI coding sequence to include one or more CCC codons. CCC is decoded by only one tRNA, Pro2, which also recognizes CCU (91). The original WPPPSI sequence contains neither CCC nor CCU; we

altered it to include CCC at the first two Pro codons (WppPSI), the third (WPPpSI) or the first and third (WpPpSI).

The immunoblot assay was used to visualize the tagging levels of these GST-fusions with or without overexpression of Pro2 from the *proL* gene on the pRARE plasmid (Figure 3-4 B). Tagging of the original WPPPSI sequence lacking CCC codons was unaffected by overexpression of *proL*. Likewise, little or no change in tagging occurred when the first two Pro residues were encoded by CCC (WppPSI). In contrast, when the third Pro codon was CCC, tagging was sharply reduced by *proL* overexpression. In addition to the loss of tagging, the overall expression of the GST-WPPpSI and WpPpSI fusions was dramatically reduced. *proL* had no effect on GST levels in WPPPSI or WppPSI. These results show that depletion of the tRNA decoding the third Pro codon is necessary for tagging. When that tRNA is abundant, tagging does not occur and, paradoxically, GST expression is dramatically reduced.

#### ***Tagging at Termination in WPPDV\****

The MS data show that tagging occurs in the WPPDV\* sequence both immediately after the WPP sequence and during termination at the stop codon after WPPDV. To further understand the effect on termination, we measured tagging levels for a series of GST-WPPDV\* variants in the immunoblot assay. Mutation of the opal (UGA) stop codon to the more efficient ochre (UAA) codon reduced tagging slightly; replacing the stop codon altogether with an Ala codon reduced it even further. We propose that the substantial tagging that remains in the WPPDVA variant likely represents stalling directly after the WPP, as seen in the MS data.



**Figure 3-5. Immunoblot analysis of mutations on WPPDV\*.**

Ribosomes stall on WPPDV\* after the second Pro and at termination after Val. Replacing the opal codon with the more efficient ochre codon slightly decreases stalling efficiency. Removing the stop codon completely also diminishes the level of tagging. Stalling is completely abolished when Asp is removed. The spacing between the stop codon and Asp also appears to play a role in stalling efficiency.

If the WPPDV sequence is interfering with termination, how far downstream does this effect carry? An opal stop codon immediately following WPPD tagged at the same level as the original WPPDV\* sequence. Moving the stop codon one or two codons downstream by inserting Ala residues, however, reduces the tagging levels to those lacking a stop codon altogether (WPPDVA). These results show that the stop codon must be only one or two codons downstream of WPPD for stalling to occur during termination.

We next examined the role of the Asp and Val amino acids immediately upstream of the stop codon. Val is not known to inhibit termination when found at the C-terminus of proteins; indeed, the Val17Ala mutant showed no loss of tagging. The Asp16Ala mutation, however, completely alleviated tagging (WPPAV\*). The Asp residue must therefore be critical for tagging after WPP as well as after WPPDV during termination. This role is consistent with the critical

nature of the third Pro residue in WPPPSI even though neither is incorporated into the tagged protein.

### *The Residue after WPP is Critical for Stalling*

Alanine-scanning mutagenesis of both the WPPDV\* and WPPPSI clones demonstrates that the residue after WPP, either Asp or Pro, plays a key role in stalling ribosomes. To identify which amino acids might satisfy this requirement, we created a library of peptide trimers following WPP in the KanR selection (WPPXXX), constrained as above to prevent stop codons. Following selection at high stringency, sequencing the surviving clones revealed that ~80% contained the sequence WPPPxx and another ~20% the sequence WPPDxx. No selection was apparent for the final two amino acids. To obtain a more quantitative picture, we created mutants of the GST-WPPPSI fusion expressing all of the possible amino acids after WPP (i.e. WPPxSI). These were subjected to immunoblot analysis with *tmRNA-H* (Figure 3-6). Confirming the genetic data, the Pro, Asp, and Trp mutants showed high levels of tagging, while the other 17 amino acids showed much lower levels of tagging.



**Figure 3-6. Immunoblot of WPPxSI.**

Immunoblots were performed on WPPxSI variants encoding each of the 20 amino acids in the third position of the hexamer. An anti-His6 antibody was used to detect *tmRNA-H* tagged peptides. Stalling is reduced to virtually nothing unless the third position is a Pro, Trp, or Asp.

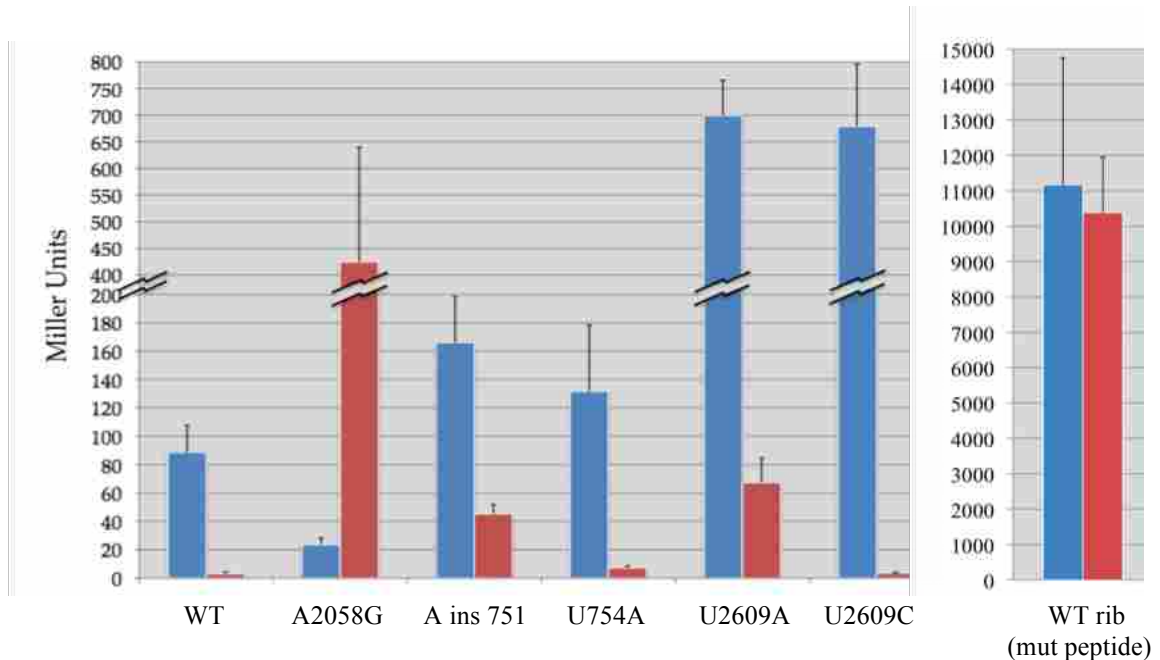
### ***Ribosomal Interactions Necessary for Stalling***

Both the KanR and the *tmRNA-H* immunoblot assays rely on tmRNA function to measure levels of ribosome stalling. To detect stalling directly, we inserted the WPPPSI 18mer after residue nine of *lacZ* and assayed for the activity of  $\beta$ -galactosidase. Our stall peptides were compared to SecM and a non-stalling SecM control that has an Ala substitution of the C-terminal Pro (GIRAGA). As shown by Nakatogawa and Ito (18), the SecM peptide dramatically inhibits  $\beta$ -galactosidase expression which is 1300-fold higher in the non-stalling Ala mutant. The 18-mer WPPPSI peptide also reduced *lacZ* expression, though not as well as SecM (116 versus 8 Miller Units of remaining activity). Mutation of the second Pro residue, shown above to result in loss of tagging, likewise resulted in 96-fold higher LacZ activity. These results show that our selected peptide sequences induce stalling with high efficiency using an assay that measures stalling directly rather than relying on tmRNA-mediated tagging.

Ribosomal RNA mutations that map to the exit tunnel have been shown to affect stalling at SecM, and TnaC. Does WPPPSI interact with the same ribosomal RNA nucleotides? Using  $\beta$ -galactosidase assays, we measured the effect of several 23S rRNA mutations on stalling at the WPPPSI 18-mer. 23S rRNA mutants known to affect stalling on other peptides were overexpressed in the presence of wild-type ribosomes. Consistent with the findings of Nakatogawa and Ito (18), the efficiency of stalling on SecM was markedly decreased (78-fold) when translated by ribosomes with the 2058G mutation (Figure 3-7). Likewise, efficiency of stalling on SecM was decreased, though to a lesser extent (six-fold), in the presence of ribosomes with an A insertion at

nucleotide 751. We also analyzed SecM-stalling in the presence of three rRNA mutants that were studied in connection with TnaC. We found that the U2609A mutation reduced stalling somewhat (eight-fold) while the U2609C mutation had no effect.

While the stalling efficiency at WPPPSI is not as great as SecM, stalling was further reduced by the both the U2609A and U2609C mutants (seven-fold). The U754A and A751 insertion mutations, in contrast, showed no significant effect. Another notable result is the different effect of the A2058 mutation on stalling at SecM and WPPPSI. This mutation was the most effective at relieving stalling on SecM but actually increases stalling on WPPPSI (eight-fold). These results show that WPPPSI interacts with some of the same rRNA nucleotides as



**Figure 3-7. Effects of 23S RNA mutations on stalling of WPPPSI and SecM.**

The efficiency of stalling on SecM (red) or WPPPSI (blue) in the presence of 23S mutants was measured using a  $\beta$ -galactosidase reporter assay. The original stalling peptides were expressed with the noted 23S mutants. The two columns on the right are mutant stalling peptides WPAPSI (blue) and GIRAGA (red) of SecM expressed in cells with wild-type 23S RNA. Note the different scale for the mutant peptides.



SecM or TnaC but that a unique pattern of exit tunnel interactions is required for each peptide.

## DISCUSSION

We performed a genetic selection to identify novel peptides that inhibit peptidyl-transferase activity during their own synthesis. The selection is based on the ability of tmRNA to recognize and rescue stalled ribosomes. When stalling occurs at the C-terminus of a truncated KanR protein, tmRNA encodes the missing amino acids to complete the protein and restore KanR activity.

By far the most common source of stalling that we identified is inefficient termination at Pro-Stop sequences. Several components need to be present to cause high-efficiency stalling during termination. First, the opal (UGA) stop codon was strongly preferred over the other two stop codons in the selection. UGA is the least efficient stop codon, leading to readthrough and recoding events, such as the programmed frameshift at an opal codon in the RF2 gene (93). Pro-opal sequences in particular cause strong +1 frameshifting at CCC\_UGA sequences (94) and significant levels of stalling and tagging by tmRNA. These phenomena may derive from the lower rate of peptide release by RF2, the factor recruited by UGA codons. As seen in previous studies, the residue upstream of Pro is also critical for maximal stalling efficiency (35). In particular, Glu, Asp, and Pro were overrepresented in the -2 position (e.g. Glu-Pro-opal) in our selectants. These results validate our selection and demonstrate that survival in the KanR assay requires high levels of ribosome stalling.

A second set of selectants show weaker activity (surviving only at low stringency) and contain the consensus sequence GI(R/H)xPP. It seems likely

that these peptides are subtle variants of the SecM sequence GIRAGP<sub>166</sub>. The GIRAPP clone that matches SecM the most closely survives even at high stringency. The only difference with SecM is that Pro replaces Gly in the ribosomal P site upon stalling. This suggests that some alterations in this critical SecM sequence are tolerated. The following mutations result in substantial though lesser KanR activity: Arg163His or replacing Ala164 with Asp, Ser, or Pro. It is interesting to note that the GI residues were not part of the random hexamer library; by chance, these were the two amino acids just upstream. Like the Pro-stop sequences above, the first Pro codon in GIRAPP is in position three in the hexamer where by analogy with SecM, stalling occurs. Such sequences are easily found in low stringency selections because the consensus sequence (R/H)xPPx is easily specified. There is no reason to suspect that these residues were selected solely for KanR function based on the alignment of proteins homologous to KanR.

Overexpression of SecM has been reported to result in high levels of tagging by tmRNA, though such tagging is probably not biologically relevant (43, 89). Tagging occurs even with a shortened SecM sequence, IRAGP, lacking the Trp residue that interacts with the exit tunnel near the L4/L22 constriction. It is likely, however, that tagging by tmRNA is an artifact of overexpression. In order to properly regulate and respond to SecA levels, stalled SecM peptides must be removed by the secretory machinery (95). Furthermore, tagging requires the removal of mRNA downstream of the stalled ribosome—the very *secA* cistron upregulated during SecM stalling. In these ways, the rescue of stalled SecM peptides by tmRNA would interfere with its biological function.

The presence of aminoacyl-tRNA<sup>Gly</sup> in the A site likely blocks tmRNA from acting on stalling peptides that regulate gene expression. TnaC and ErmCL, a third regulatory stalling peptide, are not tagged by tmRNA because the A site of the ribosome is occupied by either RF2 or Ser-tRNA, respectively (16, 21). Likewise, SecM stalls with unreacted Pro-tRNA in the A site. Hayes *et al.* showed that SecM overexpression depletes Pro-tRNA, creating a subset of ribosomes stalled on SecM with empty A sites. Following a period of prolonged stalling, the downstream mRNA is degraded and tmRNA can enter the stalled ribosomes and tag SecM. These authors also report that overexpression of Pro-tRNA eliminates this tagging (89).

Like these stalling peptides, our WPP-containing selectants stall with peptidyl-WPP-tRNA in the P site and unreacted aminoacyl-tRNA in the A site. In the case of the WPPPSI clone, for example, the mass spectrometry data show that stalling occurs after WPP. Yet the Ala scanning data show that the next residue (the third Pro) is required for stalling and tagging, even though it does not react with the nascent peptide. We conclude that it is the peptidyl-transferase activity that is inhibited in WPP-containing peptides, not another step in the translation process, such as translocation. This implicates changes in the conformation of the PTC in the stalling mechanism. Like SecM, tagging at WPPPSI cannot occur when the A site is occupied. Overexpression of the tRNA decoding the third Pro codon abolishes tagging. At the same time, tRNA overexpression actually lowers GST expression because stalling is more robust with the tRNA in the A site, and no GST-stalled ribosomes are released by tmRNA.

The amino acid Pro plays two different roles in the WPPPSI clone. First, Pro-tRNA acts as a poor peptidyl acceptor in the A site. *N*-alkylamino acids such as Pro have been shown to act as slow nucleophiles in the peptidyl-transferase reaction (96). Using full-length tRNAs, Pavlov *et al.* demonstrated that Pro-tRNA<sup>Phe</sup> reacts 23-fold slower with fMet-tRNA<sup>fMet</sup> than Phe-tRNA<sup>Phe</sup> does. They propose that this is due to steric effects and reduced nucleophilicity of the secondary amine. Interestingly, the rate of Pro reactivity is accelerated by the natural tRNA<sup>Pro</sup> isoacceptor; Pro-tRNA<sup>Pro</sup> only has a three to six-fold defect. The slow reactivity of Pro is used in natural stalling sequences: Pro-tRNA must bind in the A site for stalling to occur on SecM (22). Pro-tRNA plays a similar role in stalling on 2A peptides in viral genomes. 2A peptides stall at the Gly residue in the sequence D(V/I)ExNPGP, terminating protein synthesis after Gly(97, 98). We propose that the reduced rate of peptidyl-transfer to Pro in the WPPPSI sequence gives the nascent peptide time to interact with the exit tunnel and PTC, shifting the 23S rRNA to an inactive conformation.

While Pro-tRNA is known to react slowly in the A site, our work suggests that other aminoacyl-tRNAs may play a similar role. WPPD and WPPW sequences were also isolated from our peptide libraries. Replacing the Asp residue with Ala abolished tagging at WPPDV\*. Immunoblot analysis revealed that tagging only occurs if the residue following WPP is Pro, Asp, or Trp; the other 17 amino acids show much lower levels of tagging. This interaction is probably specific for the amino acid, not the codon or tRNA alone. The amino acid is the key component of Pro-tRNA in SecM; the Pro analog azetidine dramatically reduces stalling. Likewise the binding of free tryptophan or Trp-

tRNA<sup>Trp</sup> is required for stalling at TnaC. Trp binding in the A site must be based on ribosomal interactions with the amino acid itself.

The second role of Pro in WPPPSI stalling is that it acts as a poor peptidyl donor. Slow reactivity of nascent peptides with Pro in the P site has also been observed previously. In a purified translation system, peptides ending in Pro react with puromycin slower than peptides ending in any of the other 20 amino acids (99). C-terminal Pro residues also inhibit release factor function, as seen above in the Pro-Stop clones, TnaC, and the UL4 gene of the mammalian virus CMV (ending in IPP) (84). It seems likely that the cyclic Pro residue interferes with conformational changes in the PTC that are required for both elongation and termination.

The sequence context has a great effect—upstream peptide sequences are required for high-efficiency stalling. Stalling in the WPPPSI clone requires the consensus peptide sequence FxxYxIWPP. Phe7 and Tyr10 are aromatic residues that may bind rRNA in the exit tunnel. Phe7 is nine residues away from the P site, the same length as the ErmCL peptide. This provides sufficient length for the peptide to interact with the L22 constriction. Closer to the PTC, the Ile four residues away from the P site is necessary for stalling in both ErmCL and SecM as well as WPP-containing peptides, presumably because of hydrophobic interactions with rRNA nucleotides in the entrance of the nascent peptide exit tunnel (16, 18). The ErmCL peptide probably binds the antibiotic as well as the PTC; the additional sequence requirements of SecM, TnaC, and FxxYxIWPP peptides are required to bind the exit tunnel directly.

The WPP-containing peptides that we discovered stall ribosomes robustly: in the lacZ assay, the WPPPSI 18-mer sequence reduced activity nearly 100-fold

over the WPAPSI mutant. This result is also important because it demonstrates stalling without using tmRNA-mediated tagging. In tagging assays, full-length GST is expressed at far higher levels in non-stalling clones (e.g. the 9-18 truncation).

Analysis of stalling levels with mutant ribosomes reveals nucleotides that are required for efficient stalling on FxxYxIWPP peptides. A2058 is near the L4/L22 constriction; the A2058G mutation reduces stalling at SecM by nearly 80-fold, but it actually increases stalling on the WPPPSI clone by eight-fold. This increased stalling efficiency on A2058G ribosomes has also been observed with TnaC when wild-type ribosomes are also present in the cells (29). Likewise, mutation of U2609 has different effects on these three peptides. In TnaC, the U2609C mutant completely abolished stalling while U2609A only affected it partially (29). SecM stalling is more reduced by the A mutant, and stalling at WPPPSI is reduced by either the C or A mutant equally. These data show that the FxxYxIWPP peptide binds with the exit tunnel at similar sites and in a similar mechanism as these other peptides, though the specifics of each interaction are slightly different.

Given that these interactions between rRNA nucleotides and key residues in the nascent peptides are the basis for stalling, it is difficult to explain how stalling occurs during termination at the sequence WPPDV\*. The exit tunnel interactions must be lined up properly with the second Pro codon in the P site and Asp-tRNA in the A site. How then is termination inhibited when the peptide has moved two amino acids further into the tunnel, with peptidyl-tRNA<sup>Val</sup> in the P site and RF2 in the A site? While we cannot answer this question fully, we can state that the MS data definitively shows tagging after

WPPDV, that the Asp residue is required for stalling but that the Val residue is not, and that the stop codon must be one or two codons downstream from WPPD for stalling to occur. It is tempting to speculate that this distance limitation is due to plasticity of the peptide binding inside the exit tunnel.

We recognize that some peptides that induce stalling were missed in our selection because they were either too long or incompatible with the structure and activity of KanR. We were surprised that consecutive rare codons, known to induce tagging, were not isolated in the selection. We demonstrated by immunoblot that tagging does occur at SEPR\* and SEPRRR, but these sequences did not support KanR rescue by tmRNA. In the case of SEPR\*, tagging is probably at too low a level to support robust KanR activity. While SEPRRR induces higher levels of tagging, the tag is probably not added with the precision required to restore the KanR protein sequence properly. Alternatively, depletion of low abundance tRNAs may be too taxing for cells. Immunoblot analysis of tagging is performed after a brief period of strong overexpression. In contrast, our genetic selection requires overexpression and tagging of KanR over long periods of cell growth and division.

A second bias in our selection results is possible requirement for Pro at the third position of the hexamer library. All three cases of selectants have Pro at this position and stall with this Pro in the ribosomal P site. This may reflect the length requirements of the loop before the KanR C-terminal helix; adding amino acids to this loop may destabilize the protein structure. It seems likely that KanR function requires Pro at this site; the corresponding residue in the natural KanR sequence is Pro256.

Like SecM and TnaC, WPP-containing sequences stall due to nascent peptide interactions in the PTC and exit tunnel, with an effector bound in the A site. This stalling involves inhibition of the peptidyl-transferase activity, probably the result of conformational changes in the 23S rRNA as seen in the cryo-EM reconstruction of ribosomes stalled on SecM. How might these conformational changes inhibit ribosome function? Structural studies reveal that residues U2506 and U2585 in the peptidyl-transferase center protect the peptidyl-tRNA from hydrolysis. These nucleotides are displaced upon the binding of the incoming A-site tRNA, allowing the amino acid to enter and react. The PTC also changes conformation as prompted by release factors to promote hydrolysis at termination (31). A conformation that locks these nucleotides in place could prevent both elongation and termination. SecM, TnaC, ErmCL, and FxxYxIWPP peptides interact with different ligands (tRNAs or antibiotics) and different exit tunnel features to achieve this common end. The lack of similarity in the residues required for stalling in these peptides suggests that many solutions may exist, and that regulation of gene expression by nascent peptides may be more common than these few examples suggest.

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## MATERIALS AND METHODS

DNA oligos were purchased from Sigma-Genosys or synthesized on a BioAutomation MerMade 6 synthesizer. Enzymes were purchased from New England Biolabs.

### *Plasmid Creation*

Creation of the KanR tmRNA has been described previously (50). Following validation of the selection on a two-plasmid system, the tmRNA-kanR was cloned into the kanRA15-lib vector, to create pBAD-KT2. Randomized library plasmids were created by PCR amplification using degenerate oligonucleotide primers. Degenerate bases in the oligos were made from a mix of 25% of dA, dT, dG, and dC phosphoramidites. PCR primers were Pep6: CGAAAGGGTACCN<sub>18</sub>ATTACCATATTTTTGAAAAAGCCGTTTCTG and Kan5: CATATGGCTAGCATGAGCCATATTCAACGGGAAAC.

### *Selection of stall peptide library*

Library plasmids were ligated and introduced by electroporation into DH10B cells, quantitated and grown in 500 mL overnight in ampicillin with shaking at 37 °C. The amplified library was then introduced into X-90 *ssrA::cat* by electroporation. After 30 minutes, ampicillin was added and kanRA15-lib was induced for 2 hours with 2% arabinose. Cells were then plated on 2% ara, amp, and 15 µg/mL kanamycin at 25 or 37 °C. Selectants were grown, sequenced and recloned into fresh vector for verification. Testing of the recloned mutants in

fresh cells is an important control to ensure that the colony is surviving because of the altered peptide sequence and not a mutation elsewhere in the plasmid or the cells.

The low stringency, constrained libraries were both done essentially the same as the original library, but with the following reverse primers Vlib:

WPPNNV: AGACAAGGTACCN<sub>8</sub>BGGGGGGCCAAATACCAT

ATTTTTGAAAAAGCCGTTTCTG.

WPPVVV: AGACAAGGTACC(NNB)<sub>3</sub>N<sub>6</sub>AATACCAT

ATTTTTGAAAAAGCCGTTTCTG. The B mix consisted of equal volumes of dT, dG, dC phosphoramidites.

### *Mass Spectrometry*

The pGEX-3X vector (GE Healthsciences) was amplified with inverse PCR to create NheI and PstI cloning sites and remove the C-terminal linker. The library cassette +12 residues from *kanR* was PCR amplified with PstI and NheI and cloned into the pGEX-3X vector to create pGEX-WPPP, WPPDV\*, WPPWYR vectors. X-90 cells bearing the plasmid pCH201 (tmRNA-H) were transformed with each pGEX-WPP vector and grown overnight. Then, 500mL cultures were inoculated to an OD<sub>600</sub> = 0.05 and grown to OD<sub>600</sub> = 0.5 and induced with 1 mM IPTG for 2.5 h. Cells were pelleted for 20 min in a GS3 rotor at 4,000 rpm. Pellets were resuspended in 20 mL B-PER (Thermo-scientific) and incubated with stirring at 23 °C for 20 min. Cell lysate was cleared in an SS-34 rotor at 15,000 rpm for 20 min. Supernatant was incubated on ice then His-tagged GST was directly loaded and purified at 4 °C on a Ni<sup>2+</sup>-NTA agarose resin (Qiagen). 50 µg

of protein was acetone precipitated and digested with Trypsin (Promega) for 14 h with agitation at 37 °C. Tryptic fragments were purified in a Ni<sup>2+</sup>-NTA slurry at 4 °C, protein was loaded on a reverse-phase ZipTip (Millipore) then spotted on a MALDI-plate and overlaid with an alpha-CHC matrix (G2037A, Agilent Technology). Samples were analyzed with a QSTAR Pulsar quadrupole orthogonal time-of-flight mass spectrometer following MALDI and analyzed with Analyst QS.

### *Immunoblot Assays*

Each site-directed mutation for the immunoblot assays was cloned by PCR as described above with the specific mutations incorporated in the forward primer. The GST vector used for mass spectrometry sample preparation was used as the backbone for all of these mutant vectors. pGEX-mutant plasmids were transformed into X-90 *ssrA::cat* with tmRNA-H and grown overnight. 2 mL cultures, diluted to OD<sub>600</sub>~0.1, were grown and induced at OD<sub>600</sub>~0.5 with 1mM IPTG for 2-2.5 h then pelleted and frozen on dry ice. Samples were resuspended in water and diluted 1:1 in SDS-lysis buffer and boiled 5 min. Lysates were quantitated with DC Protein Assay (BioRad) and loaded equally on a 12% SDS-PAGE gel and run in a Laemmli buffer for ~60 min at 200 V. Proteins were then transferred to PVDF in a BioRad transfer apparatus at 300 mA at 4 °C. Membrane was blocked 30 minutes in 5% milk-TBS then incubated overnight with rocking at 4 °C in 20 mL 5% milk-TBS-tween with 1:1000 dilution of mouse monoclonal anti-His6 (27E8 Cell Signaling Technology) and 1:1000 dilution of rabbit monoclonal anti-GST antibodies (91G1 Cell Signaling Technology).

Samples were rinsed and incubated 30 min in 5% milk-TBS-tween with 1:1000 dilution of both goat anti-mouse (LiCor IRDye 800CW) and goat anti-rabbit (IRDye 680) secondary antibodies. Images were obtained on a Licor-Odyssey IR scanner.

### *Miller Assays*

Plasmids were created by ligating the same inserts from GST immunoblot vectors after the ninth codon of full-length *lacZ* (derived from pNH122) (18). AD16 cells (18) bearing a *lacZ* plasmid and a ribosomal mutant plasmid were grown to saturation then diluted to  $OD_{600} \sim 0.1$ , grown to  $OD_{600} \sim 0.5$  and induced with 1mM IPTG for 40 min. 0.8 mL of cells was pelleted and resuspended in 1 mL Z-buffer with  $\beta$ -mercaptoethanol. Cells were lysed with 0.01% SDS and chloroform then incubated at 30 °C with ONPG (4 mg/mL in 0.1 M phosphate buffer, pH 7.0). The reaction was quenched with 1 M  $Na_2CO_3$  and pelleted at 13,000 rpm for 10 min. The supernatant was removed and the  $OD_{420}$  was recorded with a Beckman Coulter DU730 UV/Vis spectrophotometer.

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