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The Role of SmpB in the Early Stages of Trans-Translation

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THE ROLE OF SMPB IN THE EARLY STAGES OF TRANS-TRANSLATION

by

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ABSTRACT

THE ROLE OF SMPB IN THE EARLY STAGES OF TRANS-TRANSLATION

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Master of Science

Ribosomes stall on defective messenger RNA transcripts in eubacteria. Without a mechanism to release stalled ribosomes, these cells would die. Transfer-messenger RNA (tmRNA) and small protein B (SmpB) reactivate stalled ribosomes in a process known as trans-translation. Together, tmRNA and SmpB mimic alanyl-tRNA, entering the A site of stalled ribosomes and accepting transfer of the stalled polypeptide. A portion of tmRNA is then positioned as a template for the ribosome to resume translating. The tmRNA open reading frame encodes a proteolysis tag to mark the aberrant polypeptide for degradation and a stop codon to release the ribosome.

How are tmRNA and SmpB allowed into stalled ribosomes? In normal translation, decoding mechanisms carefully monitor the anticodon of tRNAs entering the A site and select only those that are complementary to the mRNA codon. How do

tmRNA and SmpB get around the decoding machinery? It appears that interactions between the SmpB C-terminal tail and the decoding center are responsible. Using an *in vivo* tagging assay and an *in vitro* peptidyl-transfer assay, we monitored the effect of mutations in the SmpB tail on trans-translation. We found that mutations in SmpB that prevent helix formation are unable to support peptidyl transfer. We also found that while mutation of key nucleotides in the ribosomal decoding center severely inhibit peptidyl transfer to normal tRNAs, these mutations do not inhibit peptidyl transfer to tmRNA. We conclude that the SmpB tail stimulates peptidyl transfer by forming a helix that interacts with the ribosome to signal decoding in a novel manner.

How is the tmRNA open reading frame positioned for the ribosome to resume translating? Mutation of the tmRNA nucleotide A86 alters reading frame selection. Using a genetic selection, we identified SmpB mutants that restore normal frame selection to A86C tmRNA without altering frame selection on wild-type tmRNA. Through rational mutation of the SmpB tail we identified an SmpB mutant that supports peptidyl transfer but prevents translation of the tmRNA open reading frame. We conclude that SmpB plays a functional role in selecting the tmRNA open reading frame.

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I would like to thank both my immediate and extended family for their love and encouragement. My parents Michael and Jana Jones have taken especial care to guide and support me throughout my life. And also my dear husband Johnathan. I love him very much. Finally I acknowledge God's hand and express my deep gratitude for His mercy and goodness in all things.

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LIST OF ABBREVIATIONS

A site	Aminoacyl-tRNA binding site
E site	Ribosomal exit site
E. coli	Escherichia coli
EF	Elongation factor
fMet	formylated methionine
GAC	GTPase-associated center of ribosome
GDP	Guanosine diphosphate
GST	Glutathione S transferase
GTP	Guanosine triphosphate
IF	Initiation factor
KanR	Kanamycin resistance protein
mRNA	messenger RNA
ORF	Open reading frame
P site	Peptidyl tRNA binding site
RF	Release factor
RRF	Ribosome recylcling factor
S	Svedberg unit
SmpB	Small protein B
TLC	Thin layer chromatography
TLD	tRNA-like domain
tmRNA	transfer-messenger RNA
tRNA	transfer RNA
WT	Wild-type

CHAPTER 1: INTRODUCTION

The Ribosome and Translation

Ribosomes convert genetic information (encoded in nucleic acids) into a functional form (proteins). Cells cannot survive without functioning ribosomes. Many antibiotics kill bacteria by binding to the ribosome and preventing protein synthesis. Depleting or sequestering ribosomes results in cell death.

Bacterial ribosomes are composed of a large (50S) subunit and a small (30S) subunit. In *E. coli*, the large subunit contains thirty-six proteins and the 23S and 5S ribosomal RNAs (rRNAs). The small subunit is composed of twenty-one proteins and the 16S rRNA. The proteins in both subunits are found mostly on the outer surfaces while the rRNAs occupy the interior.[1, 2]

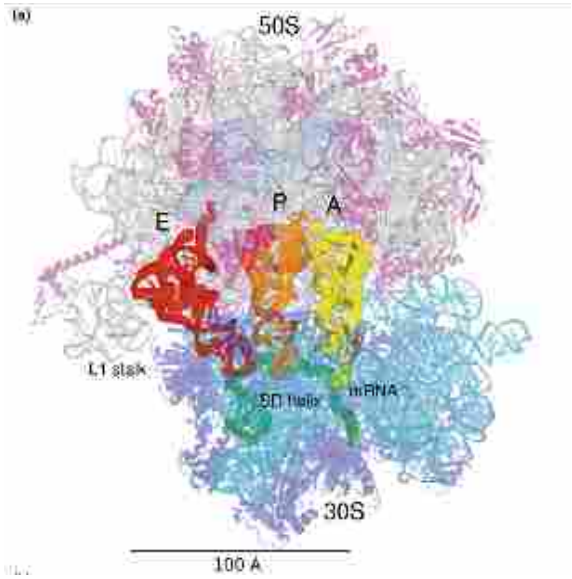


Figure 1-1: Crystal structure of a *Thermus thermophilus* 70S ribosome with tRNAs in the A site, P site and E site bound to an mRNA (green). From Korostelev, A.; Ermolenko, D. N.; Noller, H. F., Structural dynamics of the ribosome. *Curr Opin Chem Biol* **2008,** *12* (6), 674-83. Reprinted with permission from Elsevier.

Each subunit plays a different role in translation. The small subunit has a decoding center that selects tRNAs by monitoring the pairing of an mRNA codon with a tRNA anticodon. The large subunit contains a peptidyl-transferase center where peptide bonds are formed and a GTPase associated center (GAC) where proteins bind to facilitate various steps in translation. The two subunits come together on an mRNA template to form a functional 70S ribosome

(Figure 1-1).

There are three tRNA binding sites in a 70S ribosome: the aminoacyl or A site, the peptidyl or P site, and the exit or E site. During its transit of the ribosome, a tRNA will occupy each of these binding sites. The A site selects cognate or complimentary tRNAs to enter the ribosome. The P site holds the tRNA while it is bound to the nascent polypeptide chain. The E site ejects deacylated tRNAs from the ribosome to be recycled.

Translation by the ribosome is separated into three steps: initiation, elongation and termination. Prokaryotic initiation begins by positioning an mRNA start codon and its cognate tRNA in the P site of the 30S subunit. Three initiation factors bind the 30S subunit to aid in the initiation process. IF-3 binds the E site and prevents premature association of the 50S subunit. IF-1 binds near the decoding center in the A site to prevent tRNAs from interacting. IF-2 brings the initiator fMet-tRNA to the small subunit and positions it in the P site where it will pair with the mRNA start codon. An mRNA template binds directly to the 30S via its ribosome binding site (RBS) or Shine-Dalgarno sequence. Complimentary pairing between the RBS and the 16S rRNA positions the start codon in the 30S P site. The start codon is further situated by binding to the initiator tRNA. Once all of these factors are properly positioned on the small subunit, IF-3 dissociates and the 50S subunit binds. Binding of the 50S triggers GTP hydrolysis by IF-2, which releases both IF-1 and IF-2 from the small subunit. The 70S initiation complex is now fully assembled on an mRNA template with a tRNA in the P site.[3]

Once the 70S initiation complex is formed, the ribosome begins elongating the peptide chain one amino acid at a time. Elongation is a repetitive cycle of tRNA selection, peptidyl transfer and translocation. During tRNA selection, many different aminoacylated

tRNAs diffuse in and out of the A site. Each tRNA is bound near its 3′ terminus by elongation factor-Tu (EF-Tu) and GTP. The decoding center checks the pairing of the anticodon of each entering tRNA with the mRNA codon in the A site. Cognate tRNAs are selected to remain in the A site and noncognate tRNAs are rejected. Selection of a tRNA involves conformational changes in both the ribosome and the tRNA that activate EF-Tu. Activated EF-Tu hydrolyzes GTP and dissociates from the ribosome. This allows the tRNA to rotate and become fully accommodated in the A site.[4]

Following accommodation, the peptidyl-transferase center catalyzes the formation of a peptide bond between the amino acid on the A-site tRNA and the nascent polypeptide chain attached to the P-site tRNA. The ribosome catalyzes the amide bond formation through positioning of the two tRNAs. The ribosome reduces the entropic cost by positioning the A-site and P-site tRNAs such that their attached amino acids are in close proximity.[5] Additionally, the P-site tRNA is oriented with the 2′-OH on A_{76} to act as a proton shuttle in the reaction.[6] With the tRNAs in place, the peptide chain on the P-site tRNA is transferred onto the A-site amino acid, leaving an uncharged tRNA in the P site.

Translocation moves the peptide chain back into the P site and brings the next mRNA codon into the A site. This rearrangement is driven by elongation factor-G (EF-G), a GTPase that binds the ribosome following peptidyl transfer. Hydrolysis of GTP results in an EF-G conformational change that pushes the A-site peptidyl-tRNA and the mRNA codon to which it is bound into the P site. EF-G•GDP dissociates from the ribosome, leaving only an mRNA codon in the A site.[7] The cycle of tRNA selection, peptidyl transfer, and translocation is repeated until an mRNA stop codon enters the A site.

A stop codon signals translational termination by recruiting a class I release factor, RF1 or RF2. RF1 binds the stop codons UAG and UAA, while RF-2 binds UGA and UAA. Class I release factors stimulate transfer of the nascent peptide chain onto a water molecule, liberating it from the ribosome. Once the polypeptide has been released, the class II release factor RF3 binds the class I release factor and both dissociate from the ribosome. Ribosome recycling factor (RRF) and EF-G then separate the ribosome back into two separate subunits. IF-3 binds the small subunit to prevent reassociation with the large subunit until a new mRNA template has been bound and positioned for translation.[8]

Ribosome Stalling

Ribosomes become stalled if they are unable to complete the steps of elongation or termination. The use of the term "stall" in the literature is ambigious. It may refer to a temporary pause in translation that can be reversed, or it may refer to arrested ribosomes that are unable to resume translating or be released from their mRNA template by canonical means. We will use the term to mean irreversibly arrested. Ribosomes can be stalled indirectly through a lack of resources or directly by the binding of inhibitory molecules.

Some mRNAs cause ribosome stalling because they do not have a stop codon. Without a stop codon, the ribosome translates to the 3′ end of the mRNA where it cannot be released by release factors. Since transcription and translation occur simultaneously in bacteria, an mRNA can lose its stop codon before or after a ribosome begins translating. Some mRNAs are made without stop codons due to transcriptional errors. Premature termination of RNA polymerase results in nonstop mRNAs.[9] Additionally, bacterial exonucleases degrade RNA in a 3′-5′ direction, creating nonstop mRNAs by destroying the

mRNA stop codon on actively translating templates. Whatever their source, mRNAs that lack a stop codon stall ribosomes.

An mRNA that contains consecutive rare codons can also lead to ribosome stalling. Not all tRNAs are equally abundant in the cell. Rare mRNA codons call for tRNAs expressed at low levels. When the ribosome encounters a rare codon it pauses until the cognate tRNA is found. Most often the tRNA binds after a brief pause and the ribosome continues normal translation. However, a string of several rare codons in a row pauses the ribosome for so long that the downstream mRNA can become cleaved or degraded back to the stalled ribosome, creating a nonstop mRNA.[10]

Interestingly, a few nascent peptide sequences stall ribosomes even when the mRNA template has a stop codon and tRNAs are abundant. Peptide stalling sequences directly inhibit function of the ribosome by binding the peptidyl-transferase center or the peptide exit tunnel. SecM and TnaC are two bacterial leader peptides that stall ribosomes and regulate the expression of downstream genes.[11, 12] The genes downstream of SecM and TnaC are only expressed when the ribosome is paused on the leader peptide sequence. The downstream gene product releases the stalled ribosome, thereby downregulating its own synthesis. Because ribosomes stalled on such leader peptides are released, regulatory stalling events are reversible and are therefore better thought of as pausing events rather than truly arrested ribosomes that require rescuing.

Non-regulatory nascent peptide stalling sequences have also been discovered. Glu-Pro-Stop at the C-terminus of any protein causes the ribosome to stall irreversibly.[13] The P-site proline appears to be the greatest contributor to stalling in this sequence. Replacing it with the structurally similar molecule azetidine-2-carboxylic acid decreases stalling, while

exchanging the proline for 3,4 dehydroproline increases stalling. The glutamate also contributes to ribosome inhibition, and mutation to an amino acid other than aspartate or proline drastically reduces stalling. Since Glu-Pro-Stop is too short to make contacts with the exit tunnel, the dipeptide most likely inhibits the peptidyl-transferase center directly or interferes with termination machinery binding to the A-site stop codon.[13]

Irreversibly stalled ribosomes are a problem to the cell for several reasons. Multiple ribosomes will accumulate on a single mRNA, decreasing the pool of ribosomes available to translate needed proteins. In *E. coli* ribosomes stall so frequently that if there were no way to rescue them, all ribosomes would be stalled in a single generation and the cell would die.[14] On the other hand if stalled ribosomes are rescued, the released abnormal peptide could be toxic to the cell. Also, the mRNA that caused stalling can continue to stall other ribosomes until it is fully degraded. In order to survive, cells need a mechanism to rescue and recycle stalled ribosomes.

Trans-translation Overview

Rather than abandoning stalled ribosomes, bacteria have evolved a way to reactivate them. The two molecules responsible for this, transfer-messenger RNA (tmRNA) and small protein B (SmpB), are conserved among all eubacteria. Together SmpB and tmRNA facilitate template swapping on stalled ribosomes. The old nonfunctional mRNA is ejected from the ribosome and a portion of tmRNA is positioned in the ribosome as a new template. The tmRNA open reading frame ends in a stop codon, enabling the ribosome to be released normally. This process is known as trans-translation.[14]

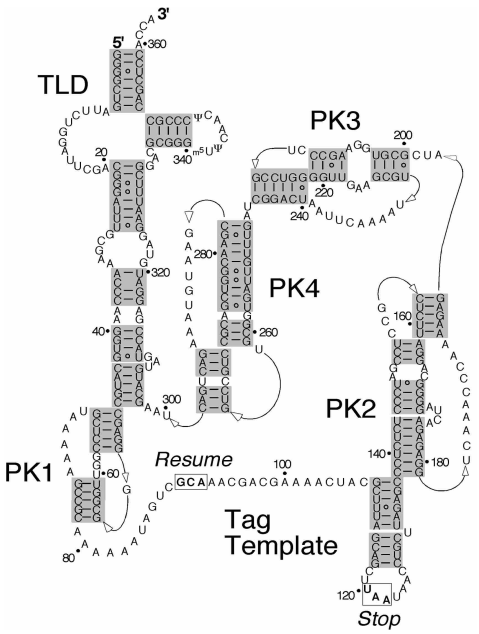


Figure 1-2: Secondary structure of tmRNA. The first and last codons of the open reading frame are boxed. PK stands for pseudoknot.

tmRNA has two distinct domains which enable it to act as both a tRNA and an mRNA during trans-translation (Figure 1-2).[15] The tRNA-like domain (TLD) is aminoacylated with alanine and enters the A site to accept transfer of the stalled peptide. Following translocation of the TLD to the P site, the first codon in the mRNA like portion of tmRNA is positioned in the A site. This enables the ribosome to resume translating on tmRNA using the normal elongation cycle. The short mRNA-like domain encodes a protease recognition sequence (ANDENYALAA in *E. coli*). A stop codon at the end of this sequence allows the ribosome to release the tagged peptide and dissociate from tmRNA. Using both the tRNA and mRNA-like domains of tmRNA, the ribosome makes one protein from two templates.[14]

SmpB is essential for all known functions of tmRNA.[16] The beta barrel core of SmpB binds the TLD of tmRNA with high affinity and specificity.[17, 18] Binding of SmpB to tmRNA protects both molecules from degradation and enhances tmRNA aminoacylation with alanine.[19-21] SmpB facilitates tmRNA entry into stalled ribosomes.[20, 22] SmpB has a C-terminal tail of 30 amino acids in *E. coli*. This tail is unstructured in solution but is required for transfer of the stalled peptide onto tmRNA.[23]

Together tmRNA and SmpB resolve all three problems associated with stalled ribosomes. Most importantly, tmRNA and SmpB release stalled ribosomes from their template mRNAs and return them to the pool of active ribosomes. Secondly, the aberrant

peptide has a protease recognition sequence added to its carboxy terminus. This targets the potentially toxic peptide for degradation once it is released from the ribosome. Finally, the troublesome mRNA is ejected from the ribosome and degraded in a tmRNA dependent manner.[24]

In the A site

How do tmRNA and SmpB mimic a tRNA to enter the ribosomal A site? In normal translation, cognate tRNAs are selected by the ribosome using induced-fit and kinetic proofreading mechanisms involving the decoding center of the 30S A site. These two mechanisms are incorporated into a kinetic model of decoding which includes both the initial selection of the tRNA and its accommodation into the A site (Figure 1-3).[25] tRNA selection and accommodation are irreversibly separated by GTP hydrolysis. Induced fit accelerates both the forward rate of EF-Tu activation before GTP hydrolysis (k_3), and accommodation of the tRNA following GTP hydrolysis (k_5). Noncognate tRNAs have faster rates of dissociation from the ribosome before and after GTP hydrolysis (k_{-2} and k_7) than cognate tRNAs, which proceed more rapidly towards peptidyl transfer (k_3 and k_5).

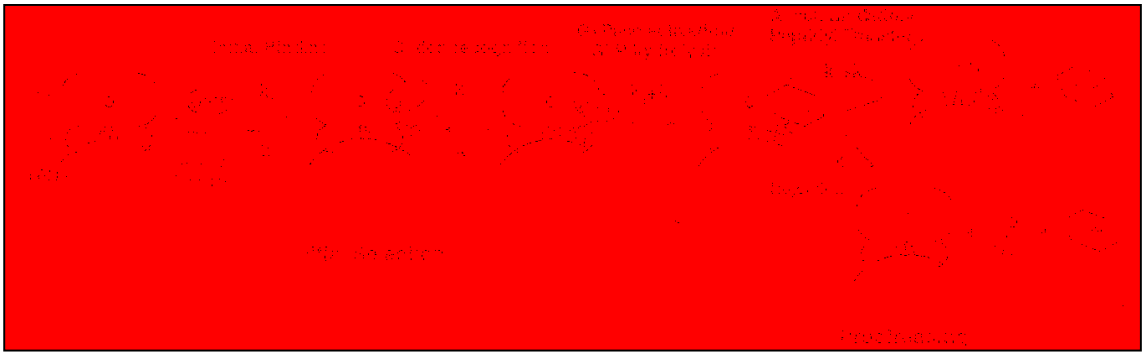


Figure 1-3: Kinetic model of decoding. From Cochella, L.; Green, R., An active role for tRNA in decoding beyond codon:anticodon pairing. *Science* **2005,** *308* (5725), 1178-80. Reprinted with permission from AAAS.

Initial tRNA selection depends on pairing of the codon and anticodon. As different tRNAs diffuse in and out of the A site, tRNAs that form noncognate codon-anticodon interactions dissociate quickly (k_{-1}) while a tRNA bound to its cognate remains in the A site longer. If the tRNA remains in the ribosome long enough, both the ribosome and the tRNA will undergo conformational changes to check pairing of the codon-anticodon.

A tRNA has to be deformed to bind both the codon in the small ribosomal subunit and EF-Tu in the large ribosomal subunit. A tRNA binding its cognate codon is stabilized by the ribosome in this strained state, whereas a noncognate interaction is not. Stabilization leads to a decreased dissociation of the tRNA from the ribosome (k_{-2}). This is the tRNA role in induced fit.[25]

The ribosome also undergoes conformational changes to check for correct pairing of the codon-anticodon. Ribosomal nucleotides A1492, A1493 and G530 check the accuracy of pairing between the codon and anticodon in the small subunit.[26] A1492 and A1493 flip out of ribosomal helix 44 and make minor groove interactions with the first two base pairs of the codon-anticodon. A1492 and A1493 act as calipers that only interact in the minor groove if the codon-anticodon bases are Watson-Crick paired.[26] G530 rotates to interact with the third base pair. This interaction is not as rigid and wobble pairing at the third position is allowed.[26] If the ribosomal nucleotides cannot make the proper interactions with the codon-anticodon helix, the noncognate tRNA more readily dissociates (k_{-2}). For cognate interactions, these seemingly small movements in ribosomal nucleotides lead to a rotation of the head and shoulder of the 30S subunit, bringing the ribosome into a more closed conformation around the codon-anticodon. This closed conformation holds the tRNA more tightly in the A site.

Following initial tRNA selection, GTP hydrolysis occurs. Deformation of the tRNA and domain closure of the ribosome both contribute to activation of EF-Tu (k_3) which hydrolyzes GTP (k_{GTP}).[25] GTP hydrolysis separates tRNA selection from accommodation. During accommodation, the 3'-aminoacylated end of tRNA moves almost 70 Å from EF-Tu to the peptidyl-transferase center. In this step noncognate tRNAs dissociate from the ribosome at a much higher rate (k_7) than cognate tRNAs (k_5). This is the final check for correct pairing of the codon-anticodon before the peptide bond is formed. The actual chemistry of peptide bond formation occurs very rapidly (k_{pep}), with accommodation being the rate limiting step in the whole process.

Since there is no codon-anticodon interaction when SmpB and tmRNA first enter stalled ribosomes, how does this complex trigger transfer of the stalled polypeptide onto the tmRNA alanine? Although tmRNA has a tRNA like domain (TLD), it is missing the anticodon stem. An x-ray crystal structure of the TLD bound to SmpB shows the body of SmpB replacing the missing stem (Figure 1-4).[27] Although the C-terminal SmpB tail is

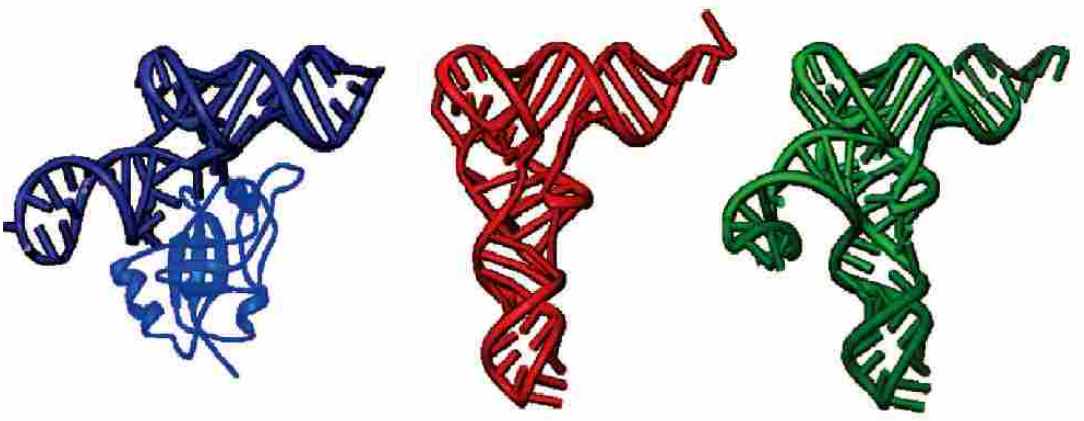


Figure 1-4: Molecular mimicry of tmRNA and SmpB. The TLD of tmRNA is shown in dark blue, and SmpB with the C terminal tail truncated is in light blue. In red is the structure of a $tRNA^{Phe}$ from yeast and in green is a $tRNA^{Ser}$ from *T. Thermophilus*. From Bessho, Y.; Shibata, R.; Sekine, S.; Murayama, K.; Higashijima, K.; Hori-Takemoto, C.; Shirouzu, M.; Kuramitsu, S.; Yokoyama, S., Structural basis for functional mimicry of long-variable-arm tRNA by transfer-messenger RNA. *Proc Natl Acad Sci U S A* **2007,** *104* (20), 8293-8. Reprinted with permission from PNAS.

truncated in the crystal structure, the beginning of the tail is positioned in such a way that the missing amino acids could interact with the ribosomal decoding center. Cryo-EM data of an EF-Tu-tmRNA-SmpB entry complex in the A site of a 70S ribosome also places the C-terminal tail of SmpB in the decoding center.[28]

Where does SmpB contact the ribosome? When added to empty 70S ribosomes, SmpB protects nucleotides in the small subunit P site and E site from chemical modification. The location of protected nucleotides corresponds to where the anticodon stem loop of a tRNA interacts during normal transit of the ribosome.[29] Directed hydroxyl radical probing studies of SmpB in stalled ribosomes (vacant A site only) reveal additional SmpB-ribosome interactions. Fe(II)-BABE produces hydroxyl radicals when Fe(II) is oxidized to Fe(III). By tethering Fe(II)-BABE to specific loci on SmpB, hydroxyl radicals can be produced at discrete locations in the presence of Fe(II) oxidizing agents. Fe(II)-BABE tethered to SmpB inside a stalled ribosome produces hydroxyl radicals that cleave ribosomal RNA immediately surrounding the Fe-BABE moiety. The position of rRNA cleavages maps the SmpB tail in the A site near the decoding center and along the mRNA downstream path.[30] SmpB can occupy each of the three tRNA binding sites in the ribosome.

Recently, Felden and co-workers suggested that the SmpB tail interacts directly with the A site nucleotides critical for normal translation: A1492, A1493 and G530.[31] To study a possible interaction between SmpB and these nucleotides, authors used nuclear magnetic resonance (NMR) techniques and a ribosomal A site mimic. An A site mimic is a short RNA stem loop corresponding to the 16S ribosomal helix 44. Helix 44 is part of the 30S decoding center and contains A1492 and A1493. The authors showed a change in resonance for nucleotides A1492 and A1493 when placed in solution with an SmpB-TLD complex.

Similarly, specific SmpB residues had altered NMR signals when placed in solution with the A site mimic. From these results, authors conclude that SmpB interacts with A1492 and A1493 following SmpB entry into the A site.[31]

Combining the structural information, it appears that SmpB interacts with the ribosome to replace a missing codon-anticodon interaction. Are interactions between SmpB and the ribosome sufficient to signal for peptidyl transfer or is the body of tmRNA functionally required? Shimizu and co-workers tested the involvement of tmRNA in peptidyl transfer by making a truncated tmRNA with only the aminoacylated TLD. When bound by SmpB, the TLD accepts transfer of the stalled peptide. Stalled ribosomes incubated with TLD-SmpB complexes cycle through multiple TLD-SmpBs and have polyalanine added to the stalled peptide.[32] The body of tmRNA does not contribute to decoding center trickery.

How does SmpB signal for peptidyl transfer to occur? Karzai and colleagues truncated the *E. coli* SmpB tail (Ala130-Arg160) at various lengths and tested the proteins ability to support peptidyl transfer.[23] They discovered that deleting residues 154-160 (Δ153) eliminates transfer of alanine onto stalled peptides. They also found that mutation of the highly conserved tail sequence D_{137}KR to A_{137}AA abolished peptidyl transfer. Neither of the inactive SmpB mutants was defective in binding to tmRNA or promoting its association with stalled 70S ribosomes. The authors concluded that D_{137}KR and I_{154}M functionally interact with the ribosome to signal for peptidyl transfer.[23] They further suggest that the SmpB tail could acquire structure in the A site to position these two regions. Others have also proposed that the SmpB tail forms a structure inside the ribosome. Based on the regular occurrence of basic amino acids in the SmpB tail[33] and the periodical cleavage pattern of Fe-BABE-generated hydroxyl radicals,[30] the SmpB tail may form a helix in the A site.

Although much has been discovered about how tmRNA and SmpB trigger the decoding center into allowing peptidyl transfer of the stalled peptide onto tmRNA, there are still unanswered questions. Does the deformability of tmRNA influence peptidyl transfer? Do other amino acids in the SmpB tail functionally interact with the ribosome? Does the SmpB tail acquire a helical structure in the A site that is important for peptidyl transfer? Are ribosomal nucleotides A1492 and A1493 functionally involved in the first decoding event in trans-translation? In Chapter 2 we offer insight into the last two questions.

Frame Selection in the P site

Following peptidyl transfer and translocation, the ribosome resumes translating on the tmRNA open reading frame (ORF). Amazingly, the ribosome proceeds with translation at the tmRNA resume codon as if there had been no break between templates. If the ribosome began translating even one nucleotide away from the resume codon, the proteolysis tag and the stop codon would be missed. With hundreds of nucleotides in tmRNA, how do stalled ribosomes consistently resume translation at the same spot?

Four pseudoknots dominate the structure of tmRNA. Since the pseudoknots clearly position the tmRNA ORF globally, could they also determine precisely where translation resumes on tmRNA? All four psuedoknots have been altered or deleted singly and in combination without disrupting tmRNA function.[34-36] This suggests that the tmRNA pseudoknots do not directly position the resume codon in the ribosomal A site.

A likely candidate for positioning the resume codon correctly in the ribosome is the resume codon itself. Williams *et. al* tested this theory by mutating the resume codon to encode a variety of different amino acids. Neither the resume codon sequence nor the amino

acid residue encoded affected frame selection.[37] This indicates that the resume codon does not bind the ribosome directly and a specific tRNA is not binding the resume codon sequence to position it in the A site. It appears that something other than the resume codon is responsible for its positioning in the A site.

The nucleotides upstream of the resume codon (Figure 1-5) were also implicated in precisely determining the tmRNA translation frame.[37, 38] Deletion of any one nucleotide from U85 to C89 results in translation of a mixture of frames.[39] Several nucleotide mutations in this region cause the ribosome to resume translating in the –1 frame. U85A causes the greatest –1 shift in frame selection, with the ribosome resuming at position 89 instead of 90 approximately 45% of the time.[39] Four nucleotides upstream of the resume codon is a universally conserved adenine.[40] Mutation of A86 to either a U or a C results in resuming translation exclusively in the +1 frame.[38, 39] The five nucleotides upstream of the resume codon play a significant role in the ribosomal selection of frame on tmRNA.

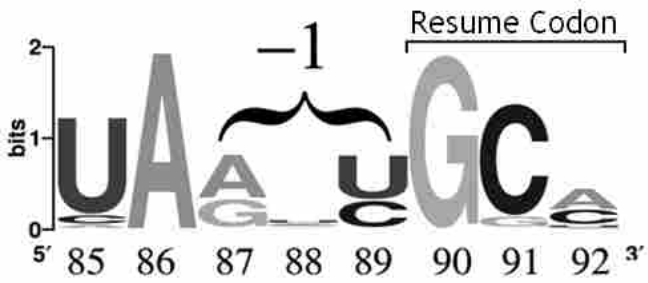


Figure 1-5: Sequence logo displaying the tmRNA upstream consensus of all 555 known tmRNA sequences. Created by Weblogo. Crooks, G. E.; Hon, G.; Chandonia, J. M.; Brenner, S. E., *WebLogo: a sequence logo generator.* Genome Res ***2004,*** 14 *(6), 1188-90.*

How do these nucleotides position the resume codon? Lim and Garber proposed that the three nucleotides immediately upstream of the resume codon, called the –1 triplet, bind the ribosome directly.[41] From computational analysis they determined that the –1 triplet could attain an A-form conformation and bind the ribosome prior to transfer of the stalled peptide onto tmRNA. Then when tmRNA translocates to the P site, the –1 triplet would go with it, leaving the

resume codon in the A site. Based on this hypothesis they developed a set of rules defining the allowed and forbidden –1 triplet sequences. All of the natural tmRNA –1 triplets fit their rules.[41] However, functional assays on all 64 possible –1 triplets gave results which did not support these rules and pointed instead to the nucleotides further upstream, specifically U85 and A86, as important in setting the frame.[38] Although these results discredited the –1 triplet theory, there was still no direct evidence of how U85 and A86 would position the resume codon in the A site after translocation.

One possibility is that a trans-acting factor binds the tmRNA upstream region to position the resume codon. Two factors have been shown to bind the upstream region of tmRNA *in vitro*: one of them is the ribosomal protein S1.[42, 43] The functional requirement for S1 in trans-translation has been debated *in vitro.*[44-46] Thus far *in vivo* it appears that trans-translation has little or no functional requirement for S1.[47]

SmpB has been shown to protect the upstream region of tmRNA from modification in some studies,[48, 49] but not in others.[19, 50] One recent study based on filter binding assays claims that SmpB binds the upstream region of tmRNA more tightly than it binds the TLD.[51] Perhaps the interaction between SmpB and the upstream region of tmRNA is transient or salt dependent, making it hard to detect. Despite the differences in detecting this interaction, Konno and colleagues show a shift in protection by SmpB on tmRNA mutants that are known to cause frameshifting.[48] Under their conditions, SmpB protects nucleotide U85 from modification. The mutation A86U, which causes translation to resume in the +1 frame, moves SmpB protection from position 85 to 86. Similarly, A84U/U85G resumes translation in the –1 frame and is protected at position 84 instead of 85.[48] SmpB remains a likely candidate for interacting with the upstream region of tmRNA to position the resume codon.

Using a genetic screen to rescue an A86C frameshifting tmRNA mutant, we provide the first *in vivo* evidence that SmpB plays a functional role in setting the frame on tmRNA. We also give an example of wild-type tmRNA paired with an SmpB mutant that can undergo peptidyl transfer but not resume translating any part of the tmRNA ORF. This previously uncharacterized role for SmpB is further evidence that SmpB is involved in positioning tmRNA in such a way that the ORF can be translated.

CHAPTER 2: THE ROLE OF SMPB IN THE A SITE

Unpublished work done in collaboration with Dr. Zhu Liu and the Rachel Green laboratory at John Hopkins University School of Medicine.

Introduction

Bacteria possess a conserved translational quality control system with two main components, small protein B (SmpB) and transfer-messenger RNA (tmRNA). tmRNA has both a tRNA like domain that is aminoacylated with alanine and an mRNA like domain that encodes a proteolysis tag. tmRNA and SmpB recognize stalled ribosomes on truncated or damaged mRNAs and enter the A site, where the stalled peptide is transferred onto the tmRNA alanine. Following translocation to the P site, the open reading frame of tmRNA is positioned in the A site such that the ribosome can resume translating. At the end of the short open reading frame, a stop codon signals for the ribosome to terminate translation normally. The ribosome is released to translate other mRNAs and the tmRNA-tagged protein is recognized by proteases and degraded.[14, 52]

How tmRNA and SmpB trick the ribosome into allowing peptidyl transfer onto tmRNA is unclear. Canonical peptidyl transfer is preceded by the correct pairing of a tRNA anticodon with an mRNA codon. Although tmRNA partially mimics a tRNA, it has no anticodon loop. Furthermore the body of tmRNA does not compensate for the missing anticodon; tmRNA can be truncated to contain only the tRNA-like domain and still undergo peptidyl transfer.[32] Structural data show SmpB poised to replace the missing tRNA anticodon and interact with the decoding center.[27, 28] Hydroxyl radical probing and footprinting experiments have detected interactions of SmpB with ribosomal RNA in the A site.[30, 31]

There is some speculation about how the SmpB tail triggers peptidyl transfer. Although unstructured in solution, several authors have proposed that the SmpB tail (residues 130-160 in *E. coli*) forms a structure in the A site to position amino acid residues that would stimulate peptidyl transfer.[23, 30, 33] *E. coli* tail residues D_{137}KR and I_{154}M are required for peptidyl transfer. Mutating these sequences to alanine or truncating the tail before Ile154 eliminates peptidyl transfer.[23] How and where these amino acid sequences interact with the ribosome has not been determined.

Other amino acids at the beginning of the SmpB tail have been implicated in making direct interactions with ribosomal decoding center nucleotides A1492 and A1493. These nucleotides are crucial to the canonical decoding process.[53] However, the SmpB-decoding center interaction was detected using an A site mimic and not a 70S ribosome. The functional relevance of the interaction has not been tested.

In this chapter we present several new findings that expand our understanding of the mechanism of trans-translation. First, the SmpB tail forms a helix inside the ribosome that is essential for peptidyl transfer. Second, the beginning of the SmpB tail plays an essential role in trans-translation after peptidyl transfer but before translation of the tmRNA open reading frame begins. Third, ribosomal nucleotides A1492 and A1493 are not significantly involved in the first peptidyl-transfer event.

Results

Functional relevance of conserved residues in the C-terminal tail

The SmpB tail has several highly conserved amino acid residues (Figure 2-1). Based on sequence alignment, we tested the functional relevance of the conserved residues

Figure 2-1: Conservation of amino acid residues in the SmpB C-terminal tail. Generated by Weblogo.[40] The height of each symbol indicates the conservation of the amino acid at that position. The *E. coli* residue number and sequence are printed below. The last two *E. coli* residues, $H_{159}R$, are not shown.

$K_{131}GKK$, $D_{137}KR$, K143, R145, and R153 in trans-translation. We used an *in vivo* tmRNA tagging assay to monitor the effects of SmpB mutations on trans-translation. Full length GST with a stalling sequence at the C terminus (Glu-Pro-Stop) is overexpressed as a substrate for tmRNA-mediated tagging. By mutating the tmRNA open reading frame to encode a His_6 epitope, tagging of GST can be visualized on an immunoblot. GST expression is also visualized and shown as a loading control. Expressing these modified constructs in combination with wild-type or mutant SmpB allows us to determine the relative activity of trans-translation.

The residues at the beginning of the SmpB tail, $K_{131}GKK$, were recently implicated in directly interacting with the ribosome decoding center.[31] To test the functional importance of these amino acid residues, we individually mutated each amino acid to alanine and monitored tagging levels. No effect was seen except for an approximately two-fold decrease in tagging with Gly132Ala. Mutating two lysines to alanine together reduced tagging significantly, as in $K_{131}GAA$ and $A_{131}GAK$. Mutating all three lysines to alanine at once, $A_{131}GAA$, abolished tagging. Interestingly, mutating glycine and only one lysine to alanine, $K_{131}AAK$,

also eliminated tagging (Figure 2-2). We refer to this mutant as $G_{132}K$:AA throughout the rest of the paper.

Figure 2-2: The effect of mutations in the SmpB sequence K_{131}GKK on tmRNA-mediated tagging.

Another highly conserved region of the SmpB tail, D_{137}KR, was previously shown to be essential for tmRNA mediated tagging.[23] While some mutation of the amino acid sequence is tolerated, changing all three residues to alanine, D_{137}KR:AAA, abolishes tagging. As expected, no detectable level of tagging is supported by the D_{137}KR:AAA mutant in our immunoblot assay (Figure 2-3).

The SmpB tail also has several conserved basic residues further downstream of K_{131}GKK and D_{137}KR. Residues 143, 145, 149 and 153 are conserved as amino acids with positively charged side chains in many SmpB proteins. In *E. coli*, only three of these basic residues are preserved as Lys143, Arg145, and Arg153. We tested the functional relevance of these positive charges by again mutating them to alanine individually or in combination and monitoring tagging levels in immunoblots. When all three charges were removed, no tmRNA mediated tagging was detected. When only two charges were removed, Lys143Ala

Figure 2-3: Immunoblot of tmRNA-mediated tagging on a GST substrate. SmpB mutants are labeled in each lane. Nonfunctional SmpB mutants do not support tagging of GST.

and Arg145Ala, there was no effect on tagging. Arg153Ala by itself also produced no defect in tagging (Figure 2-3).

The C-terminal tail functions as a helix

The periodicity of basic residues in the SmpB tail led to the hypothesis that once inside the ribosome, the tail could form a helix.[33] This hypothesis is supported by others who see a periodical footprinting pattern of the SmpB tail in the ribosomal A site.[30] We used a software analysis program (JPred) to predict whether or not the tail of *E. coli* SmpB forms a helix. According to JPred, a helix is likely to span approximately residues 142-157.

To test the functional relevance of a helical tail in trans-translation, we did an *in vivo* proline scanning experiment. Various SmpB residues from 135-154 were mutated to proline individually and tmRNA tagging activity monitored by immunoblot. Each amino acid mutated to proline was also mutated to alanine as a control to show that the specific amino acid lost was not important, just the ability of the tail to form a helix. We started proline scanning upstream of the predicted helix but did not go past residue 154, since residues 155 and beyond can be deleted without negatively affecting tagging.[23]

While some proline mutations had little effect on tagging, introducing a proline into the tail at or after Lys143 greatly decreases trans-translation efficiency (Figure 2-4). SmpB mutants Gln135Pro and Asp141Pro support tagging levels comparable to wild-type SmpB. However, Lys143Pro, Arg145Pro, Lys151Pro, and

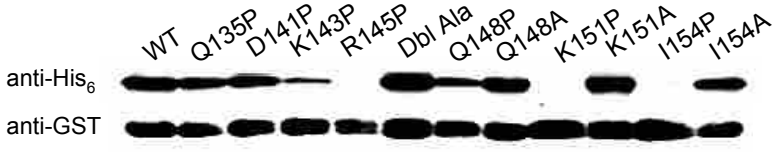


Figure 2-4: Proline mutations in the SmpB tail and their effect on tmRNA mediated tagging visualized by immunoblot. Dbl Ala refers to the K143A/R145A control. This mutant is also shown in Figure 2-3.

Ile154Pro yielded only negligible amounts of tagging and Gln148Pro was noticeably reduced. The alanine controls for these mutations all support tagging without any defect. These data indicate that the SmpB tail does form a helix inside the ribosome, and the structure is functionally important during some step in trans-translation.

Tail residues required for accommodation

Next we set out to determine at which step in trans-translation these various SmpB mutants have a defect. Two major roles for SmpB are binding to tmRNA and the ribosome. It is possible that our mutants are defective in binding to one or both of these. However, almost the entire SmpB tail can be truncated (up to Gly132) without decreasing the binding affinity of SmpB for tmRNA or the ribosome.[23] Although the tail may form functional interactions with the ribosome and/or tmRNA to signal for peptidyl transfer, these interactions do not contribute much to overall binding affinity. Since the mutations we found all fall in the area of the SmpB tail which can be truncated and still bind, we think it unlikely that mutation of one or two of these amino acids would significantly decrease binding.

Another known role for SmpB is supporting peptidyl transfer of the stalled peptide onto Ala-tmRNA. We used an *in vitro* peptidyl-transfer assay to determine the efficiency of various SmpB mutants in supporting dipeptide formation. Stalled ribosome initiation complexes were prepared with a ^{35}S labeled fMet-tRNA in the P site. Initiation complexes were incubated with either wild-type or mutant SmpB in Ala-tmRNA-SmpB-EF-Tu•GTP complexes and quenched at various timepoints by addition of potassium hydroxide. Products were separated on an electrophoretic TLC and visualized by audioradiography. The fMet-Ala signal compared to total signal gives the percent yield of dipeptide formation.

Although we used a 10-fold excess of tmRNA complexes in our peptidyl transfer reactions, Ala-tmRNA was not sufficiently saturating to determine the catalytic rate, k_{cat}, of peptidyl transfer. This is due to the difficulty of aminoacylating tmRNA *in vitro*. However, since reactant concentrations were the same between various tmRNA-SmpB complexes, we can compare the observed peptidyl transfer rate (k_{obs}). The rates reported here are preliminary. The experiments are being repeated in duplicate using a lower concentration of ribosomes and a higher concentration of tmRNA-SmpB complexes. This will increase the overall yield of the reaction and provide a more reliable k_{obs}.

We measured the ability of four SmpB mutants to support dipeptide formation: D_{137}KR:AAA, Δ153, Lys151Pro and G_{132}K:AA. D_{137}KR:AAA and Δ153 were previously shown to have peptidyl transfer defects using mass spectrometry analysis.[23] In our assay, both of these mutant complexes had an observed petidyl transfer rate of less than 0.01 s^{-1} (Figure 2-5). This is significantly lower than the observed rate for wild-type SmpB complexes (1.97 ± 0.15 s^{-1}). Lys151Pro complexes formed a dipeptide at an observed rate near 0.01 s^{-1}, similar to D_{137}KR:AAA and Δ153 (Figure 2-5). The slow observed rates for these three mutants indicate that they do not support peptidyl transfer at sufficient rates to see

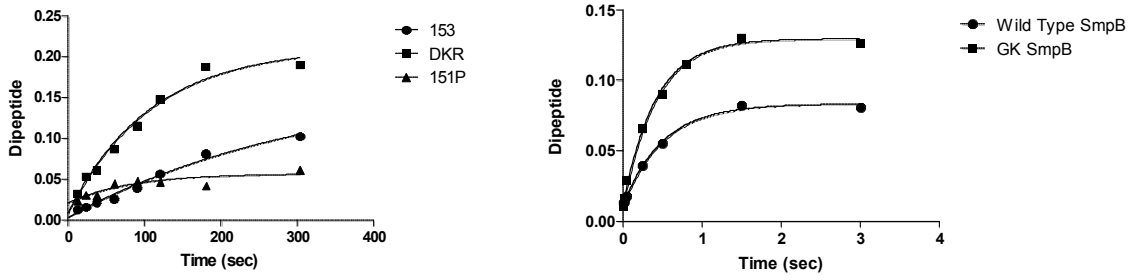


Figure 2-5: Plots of dipeptide formation in stalled ribosome complexes incubated with either wild-type SmpB-tmRNA or the indicated mutant SmpB-tmRNA. The observed peptidyl transfer rate of each reaction was determined from the initial slope of the curve. Made with GraphPad Prism 5.

tmRNA tagging *in vivo*. $G_{132}K$:AA complexes, however, support dipeptide formation at an observed rate of 2.40 ± 0.21 s^{-1}, comparable to wild-type complexes (Figure 2-5). Unlike the other SmpB mutants tested, the $G_{132}K$:AA defect in supporting tmRNA mediated tagging is not in peptidyl transfer, but a step further downstream.

To further characterize the $G_{132}K$:AA defect in trans-translation, we tested the ability of this mutant complex to make a tripeptide. In order to make fMet-Ala-Ala, the tmRNA-SmpB complex must translocate to the P site and position the tmRNA open reading frame in the A site. While we were able to make a tripeptide with wild-type SmpB-tmRNA complexes, no tripeptide formed using a $G_{132}K$:AA-tmRNA complex (data not shown).

This isolates the $G_{132}K$:AA defect to either translocation to the P site or some event in the P site that allows translation of the tmRNA open reading frame to begin. We tested for misreading of the tmRNA open reading frame in the immunoblot assay. By moving the His_6 epitope into the +1 and –1 frames, we are able to detect tagging that begins one nucleotide away from the correct resume codon. No tagging was detected in the +1 or –1 frames (Figure 2-6).

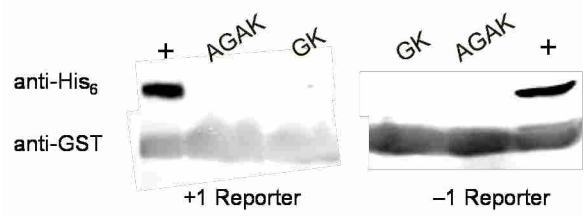


Figure 2-6: Immunoblot analysis of frame choice. tmRNA-mediated tagging in the –1 and +1 frames with $G_{132}K$:AA or $A_{131}GAK$ SmpB. Wild-type SmpB is coupled with U85A –1 tmRNA or A86C +1 tmRNA as a control for visualizing tagging (+).

rRNA nucleotides in the decoding center are not required for accommodation

Lysines 131 and 133 in the SmpB tail were recently implicated in interacting with the decoding center nucleotides A1492 and A1493.[31] SmpB is able to protect these and other nucleotides in the A site from chemical modification *in vitro*.[30, 31] We set up peptidyl transfer

experiments to determine if any interaction of the SmpB tail with A1492 and A1493 is functionally relevant to the first peptidyl-transfer step of trans-translation.

The mutations A1492G and A1493G reduce EF-Tu activation at least 40-fold and tRNA accommodation 5 to 20-fold.[53] In collaboration with Rachel Green's lab, we were able to purify ribosomes with these individual mutations and test them in the dipeptide assay. Stalled ribosome complexes were prepared with fMet in the P site and a phenylalanine mRNA codon in the A site. This short three-nucleotide extension does not interfere with SmpB-tmRNA entry into the ribosome.[54] The control Phe-tRNA has drastically reduced yields of dipeptide formed in both 1492G and 1493G ribosomes compared to wild-type ribosomes (Figure 2-7). If these nucleotides are critical to peptidyl transfer in trans-translation, we would expect to see a similar decrease in dipeptide yield when these ribosomal mutants are paired with SmpB-tmRNA complexes. However, the dipeptide yield with SmpB-tmRNA on mutant ribosomes was not significantly decreased compared to the yield on wild-type ribosomes (Figure 2-7). The observed rates of these reactions are currently being measured.

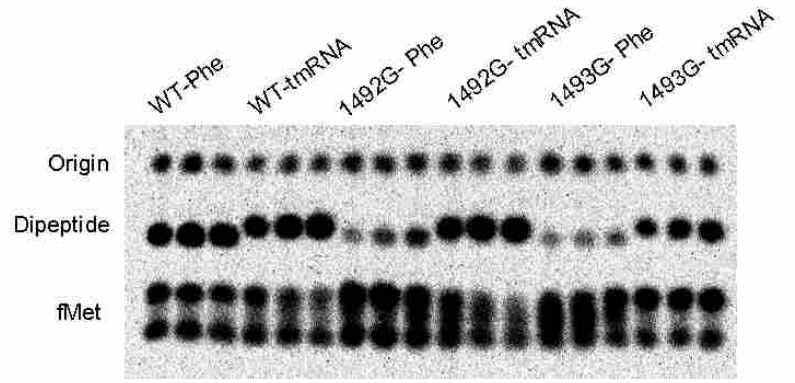


Figure 2-7: Mutant ribosomes and peptidyl transfer. Autoradiograph image of electrophoresed TLC. Wild-type or mutant ribosome complexes were incubated with either a Phe-tRNA or tmRNA-SmpB. Each reaction was incubated at 37˚C for 1.5 min., 5min., or 10 min. (left to right) before quench with KOH. fMet-Phe runs slightly farther than fMet-Ala.

Discussion

In this chapter we present new information about the SmpB tail and its functional role in trans-translation. We characterize the previously hypothesized helical structure of the SmpB tail within the ribosome and conclude that a helix is required to support peptidyl transfer. Lys151Pro would prevent the tail from forming a helix, and it destroys trans-translation activity *in vivo* as well as SmpB-mediated peptidyl transfer *in vitro*. Loss of the lysine residue itself is not the problem, as Lys151Ala supports tmRNA mediated tagging as well as wild-type SmpB. Some proline mutants do support tmRNA mediated tagging, indicating the helical boundaries. The helix begins around Lys143 and continues to at least Ile154. Interestingly, there appears to be some flexibility in the middle of the helix, as tmRNA tagging with Gln148Pro is only partially reduced. Although each proline mutant was not tested for its ability to support peptidyl transfer *in vitro*, we propose they all behave similarly based on their inability to support tmRNA tagging *in vivo*. SmpB tail mutations previously shown to be nonfunctional in peptidyl transfer (D_{137}KR:AAA and Δ153) were used throughout our work as controls. D_{137}KR is just upstream of the helix and I_{154}M is near the C-terminus of the helix. Perhaps the functional purpose of the helix is positioning these crucial residues within the A site. We note that the basicity of the tail also appears to be important, though we do not characterize at which step in trans-translation the positive charges are required.

We also examined the functional significance of another conserved region at the start of the SmpB tail, K_{131}GKK. Single mutation of each amino acid in this sequence to alanine had no effect on tagging except for Gly132Ala, which decreased tagging two-fold. The absence of a sidechain on glycine allows it more flexibility than other amino acids, while the

methyl group on alanine partially constrains the allowed angles. This part of the tail could be required to form a tight kink inside the ribosome, easily allowed by glycine and slightly hindered by alanine. Surprisingly, combining the slightly decreased Gly132Ala mutation with Lys133Ala eliminated all tagging. This GK mutant, $K_{131}AAK$, retains two positive charges in the area. Mutants with only one positive charge, $A_{131}GAK$ or $K_{131}GAA$, were able to support tagging, though at a significantly decreased levels. Removing all three positive charges, $A_{131}GAA$, eliminates tagging just as the $G_{132}K$:AA mutant.

The $G_{132}K$:AA mutant is different from all other SmpB mutants studied in that it supports peptidyl transfer as well as wild-type SmpB. $G_{132}K$:AA is nonfunctional due to a defect downstream of decoding, after transfer of the stalled peptide onto tmRNA. This is a novel function for SmpB, whose previously characterized functions were binding to tmRNA and improving its aminoacylation, as well as associating with the ribosome and promoting peptidyl transfer.

The $G_{132}K$:AA mutant does not allow the ribosome to begin translation of the tmRNA open reading frame. While wild-type SmpB supports formation of fMet-Ala-Ala *in vitro*, $G_{132}K$:AA does not. This is not due to a slight slip in selection of the resume codon: the $G_{132}K$:AA complex does not support tmRNA tagging in the 0, +1 or –1 frames. It is possible that the open reading frame is not positioned in the mRNA tunnel at all, so the ribosome has no recognizable template on which to resume translation. Another possibility is that $G_{132}K$:AA complexes cannot translocate from the A site to the P site. Work in our lab continues to isolate which if either of these failings describe the $G_{132}K$:AA mutant.

Specific amino acids in SmpB were recently proposed to directly interact with ribosomal nucleotides A1492 and A1493, possibly mimicking canonical decoding to signal

for peptidyl transfer. However, we show that an interaction between SmpB and A1492 or A1493 is not absolutely required for peptidyl transfer. While both A1492G and A1493G mutant ribosomes decode a Phe-tRNA with diminished efficiency, neither mutation greatly affects SmpB-tmRNA decoding. The observed rates of these reactions are currently being measured so that we can determine if A1492 and A1493 play a slight role in the first peptidyl transfer event of trans-translation or if they do not contribute. This is one instance where the trans-translation system may not mimic normal translation.

SmpB makes other contacts with the ribosomal A site which could be required for peptidyl transfer. We propose crosslinking amino acid residues in or near DKR and IM to the ribosome. Once ribosomal nucleotides which are near these amino acids in the A site have been identified, they can be functionally analyzed to determine their relevance to SmpB stimulated peptidyl transfer.

We conclude that SmpB is even more critical to the trans-translation process than previously noted. Not only does SmpB protect tmRNA from degradation, enhance its aminoacylation, promote its stable association with the ribosome and signal for peptidyl transfer to occur, but it also functions after peptidyl transfer to allow translation of the tmRNA open reading frame to begin. SmpB is involved in transitioning the ribosome from its old mRNA template to the new tmRNA template.

Materials and Methods

Materials: Enzymes for cloning were purchased from New England Biolabs. The mouse anti-His_6 antibody was purchased from Cell Signaling Technology and the rabbit anti-

GST antibody from Sigma. Both secondary antibodies (anti-mouse IRDye 800 and anti-rabbit IRDye 680) were from LI-COR Biosciences.

Immunoblot analysis of tagging – The pDH210 plasmid expresses the GST protein with the stall-inducing sequence Glu-Pro-Stop added to the C-terminus. It also expresses tmRNA altered to encode an ANDH_6D tag. Variants of pDH210 were made that encode this His_6-containing tag only if the ribosome resumes on tmRNA in either the –1 or +1 frame, by deleting C98 or by inserting a G immediately before G90, respectively. Δ*ssrA-smpB* cells carrying pDH210 and an SmpB expression plasmid (pDH113) were grown in ampicillin and tetracycline to an OD_{600} of 0.5. The expression of GST was induced with 1 mM IPTG. After 2.5 h, the cells were pelleted and lysed with SDS. Protein in the crude lysate was quantified via Lowry assay and each sample was resolved by 10% SDS-PAGE. The protein was transferred to PVDF membrane and His_6-tagged GST was bound by a mouse anti-His_6 antibody. Binding of a rabbit anti-GST antibody was used to control for protein expression and loading. Fluorescent secondary antibodies (anti-mouse IRDye 800 and anti-rabbit IRDye 680) were added and the blot was visualized with an Odyssey Infrared Imaging System (LI-COR Biosciences).

In vitro translation components – Tightly coupled 70S ribosomes were purified from MRE600 as described previously.[55] Mutant ribosomes were purified as described.[53] Initiation factors and EF-Tu were purified as described previously.[56, 57] tmRNA was prepared from double-stranded DNA template using run-off transcription by T7 RNA polymerase,[58] then purified and aminoacylated as described previously for tRNAs.[59] Aminoacylation and formylation of the initiator $tRNA^{fMet}$ with radiolabelled [^{35}S]-methionine using S100 extract was done as previously described.[60] The aminoacylated tRNA was

purified by phenol and chloroform extraction followed by ethanol precipitation and resuspended in 20 mM potassium acetate buffer, pH 5.1, with 1 mM DTT. Wild-type and mutant SmpB were overexpressed with an N-terminal His_6 tag in BL21 using a pET15b vector. The protein was purified on a Ni-NTA column.

Initiation complexes – Stalled ribosome initiation complexes were prepared by incubating 2 μM 70S ribosomes with 6 μM mRNA (GGG AAT TCG GGC CCT TGT TAA CAA TTA AGG AGG TAT ACT ATG TTC), 2 mM GTP, and 3 μM each IF1, IF2, IF3, fMet-$tRNA^{35SfMet}$ in 1×219 buffer (50 mM Tris HCl, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$, 1 mM DTT) at 37 °C for 30 min. Following incubation a minimal amount of $MgCl_2$ was added to bring the final Mg^{2+} concentration to 10 mM. Complexes were purified by spinning through a 1.3 mL sucrose cushion (1.1 M sucrose, 20 mM Tris-HCl, pH 7.5, 500 mM NH_4Cl, 10 mM $MgCl_2$, 0.5 mM EDTA) at 69 krpm in a TLA100.3 rotor for 2 h. The complexes were resuspended in 1×219 buffer, aliquoted and frozen at –80°C until used.

Ternary complexes – Ternary complexes were prepared in 1×219 buffer by incubating 2 mM GTP with either 2.5 μM tmRNA and 5 μM SmpB, or 2.5 μM Phe-$tRNA^{Phe}$ at 37 °C for 2 min. Then EF-Tu (previously incubated with 2 mM GTP for 10 min at 37 °C) was added to a final concentration of 5 μM and the complex was incubated at 37 °C for another 5 min.

Dipeptide Assay – Immediately following incubation of the ternary complexes, ribosome initiation complexes were added to a final concentration of 100 nM. Samples of each reaction were quenched with 100 mM potassium hydroxide at various time points. For faster reactions 250 mM ribosomes were added to the ternary complex in a quench-flow instrument (RQF-3 quench-flow, KinTek Corporation). Samples were electrophoresed on

cellulose TLC plates in pyridine-acetate pH 2.8. The fraction of dipeptide formed was measured using ImageQuant v5.2 (Molecular Dynamics) and plotted against time. The observed rate of dipeptide formation was determined from the initial slope of a one phase association curve fitted to each plot in GraphPad Prism 5.

CHAPTER 3: SMPB CONTRIBUTES TO READING FRAME SELECTION IN THE TRANSLATION OF TMRNA

This chapter is modified from a manuscript *in press*: Watts, T.; Cazier, D.; Healey, D.; Buskirk, A., SmpB Contributes to Reading Frame Selection in the Translation of Transfer-Messenger RNA. *J Mol Biol* **2009**.

Introduction

Stalled ribosomes in eubacteria are rescued and recycled by a highly conserved quality control mechanism. Ribosomes stall upon reaching the 3'-end of mRNAs that lack a stop codon. With empty A sites, these ribosomes are trapped on the defective mRNA because they cannot efficiently bind release factors. Instead, stalled ribosomes recruit transfer-messenger RNA (tmRNA) and its binding partner, small protein B (SmpB) to their empty A sites. SmpB and aminoacylated tmRNA function first as a tRNA, transferring alanine to the nascent polypeptide. The stalled ribosome then resumes translation using tmRNA as a template, translating a short open reading frame that encodes a protease-recognition sequence. Through the action of tmRNA and SmpB, known as trans-translation, the aborted polypeptides are tagged for degradation by cellular proteases and the ribosome is released at a stop codon and recycled.[14, 16, 61]

As the ribosome switches templates from the defective mRNA to tmRNA, how is the appropriate codon in tmRNA selected for translation to resume? The global structure of tmRNA plays little role in the selection of the correct frame. The four pseudoknots that dominate the tmRNA structure can be replaced with unrelated sequences with little or no loss of tmRNA activity.[34-36] Instead, the reading frame is chosen locally, by five bases immediately upstream of the resume codon.[37-39] Mutations in this upstream sequence

(U_{85}AGUC) lead to reduced tmRNA function and errors in frame selection *in vitro* and *in vivo*. Mutation of the first two of these nucleotides is particularly deleterious: the U85A mutation, for example, partially shifts translation to the –1 frame.[39] Mutation of the universally conserved A86 leads to severe loss of function:[37, 39] the A86C mutation shifts translation entirely to the +1 frame *in vivo.*[38] From these data, we proposed that the resume codon is chosen by its placement in the ribosome as determined by the binding of an unidentified ligand to A86.[38]

A variety of candidates have been reported to bind the sequence upstream of the resume codon. One suggestion is that the last of these upstream nucleotides, the so called –1 triplet (G_{87}UC), is recognized directly by rRNA in the ribosomal decoding center,[41] although this hypothesis does not withstand analysis of tmRNA activity *in vivo.*[38] Another candidate is ribosomal protein S1, previously shown to crosslink to U85.[42] Cryo-electron microscopy structures of tmRNA bound inside 70S ribosomes reveal that S1 affects the structure of the tmRNA template sequence.[43] Though S1 cannot interact directly with tmRNA on the ribosome, it has been proposed that free S1 binds tmRNA and stabilizes a functional, open complex that is then passed to stalled ribosomes.[43] In support of this model, one study presents evidence that S1 is required for tmRNA to serve as a template *in vitro.*[46] There are also reports that refute this proposed role for S1, however, using reconstituted translation systems[44, 45] as well as *in vivo* functional analysis.[47]

Another promising candidate is SmpB, a protein that plays a role in the stability and aminoacylation of tmRNA and is required for its entry into the ribosome. SmpB binds the tRNA-like domain (TLD) of tmRNA in a well-characterized interaction.[27] Interest in SmpB has focused on its ability to license tmRNA entry into the ribosomal A site through

interacting with the decoding center.[23, 32] Recent reports suggest that more than one binding site for SmpB exists in tmRNA, raising the possibility of additional functions for this protein. Felden and co-workers showed that SmpB binding reduces the accessibility of the upstream sequence to nucleases in probing assays and proposed that SmpB plays a role in resume codon selection.[49] After further characterization with surface plasmon resonance and filter binding assays, they report that this interaction is higher in affinity than SmpB binding to the TLD.[51] An interaction between SmpB and the upstream region was likewise reported by Himeno and co-workers using chemical probing assays. Intriguingly, the site of SmpB binding shifted in tmRNA mutants known to alter the frame in which translation resumes.[48] On the other hand, several crosslinking and chemical probing assays have failed to detect an interaction between SmpB and the tmRNA upstream sequence.[19, 50, 62]

The role of S1 and SmpB in frame selection remains controversial because binding has only been detected in some assays *in vitro* and these RNA-protein binding events have not been shown to affect tmRNA activity either *in vitro* or *in vivo*. To test for a functional interaction between SmpB and the upstream region of tmRNA, we identified mutations in SmpB that restore the function of an A86C mutant tmRNA. This mutation strongly reduces tmRNA activity in several assays and causes tmRNA to be translated exclusively in the +1 frame.[38] Several SmpB mutations were identified which rescue tmRNA function and alter frame selection on A86C tmRNA. These results demonstrate definitively that SmpB plays a biologically relevant role in setting the frame on tmRNA.

Results

SmpB mutations restore A86C tmRNA function

We used a genetic selection to identify mutations in SmpB that suppress the defect (improper frame selection) in A86C tmRNA. The selection relies on the tmRNA tagging process to complete the synthesis of the kanamycin resistance protein (KanR).[35, 38] Ribosomes are programmed to stall at the end of a truncated KanR protein that lacks the C-terminal 15 amino acids. tmRNA rescues these stalled ribosomes and tags the nascent polypeptide with the missing 15 amino acids; these are encoded by an altered tmRNA template sequence. In this way, tmRNA function completes the KanR protein and makes the cells kanamycin resistant (Figure 3-1). While cells with wild-type (A86) tmRNA survive equally well with or without kanamycin, only about 1 in 10^6 cells expressing the A86C tmRNA mutant form colonies on selective media, even at the lowest stringency conditions (15 μg/mL kanamycin at 25°C). This low background survival rate allowed us to select for

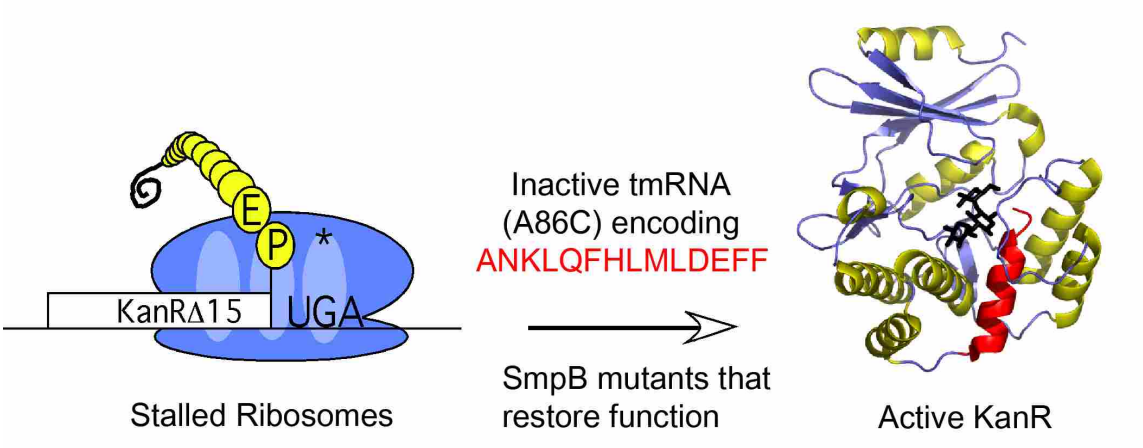


Figure 3-1: Genetic selection for SmpB mutants that restore A86C tmRNA activity. Translation of a truncated KanR gene is stalled during termination at the sequence Glu-Pro-Stop. The resulting KanR protein lacks the C-terminal 15 amino acids (red) and is inactive unless these stalled ribosomes are rescued by tmRNA that has been altered to encode the last 14 amino acids, ANKLQFHLMLDEFF. Roughly 10^8 SmpB mutants were screened to identify those that restore tagging levels sufficient to synthesize KanR and confer cellular survival on kanamycin plates.

SmpB mutants that suppress the A86C defect and restore high levels of tmRNA function and kanamycin resistance.

We generated a library of ~10^8 SmpB mutants using error-prone PCR and subjected it to the KanR selection with A86C tmRNA, obtaining survival levels 100-fold higher than background. The SmpB genes were recloned from this enriched pool and reselected, with nearly all the cells surviving on selective plates. Sequencing revealed three related but distinct SmpB clones that were designated A1, A2 and A5 (Table 3-1). The A1 clone has two changes, Tyr24Cys and Val129Ala. A2 has these and the additional Glu107Val mutation. A5 shares the same Tyr24Cys mutation as A1 and A2 but coupled instead with Ala130Gly. The tagging activity of these three SmpB clones was measured by plating cells on media containing 15 μg/mL kanamycin at 25 °C. A1 conveyed 50% survival while A2 and A5 both conveyed ~90% survival, several orders of magnitude higher than the background level of 1 in 10^6. These findings show that mutations in SmpB can compensate for deficiencies in the function of the upstream region of tmRNA and the critical nucleotide A86 in particular.

Clone	SmpB mutations			
A1	Tyr24Cys		Val129Ala	
A2	Tyr24Cys	Glu107Val	Val129Ala	
A5	Tyr24Cys			Ala130Gly

Table 3-1: SmpB clones that restore A86C tmRNA activity. Changes in the SmpB sequence are shown for three clones that survived the KanR selection for tmRNA activity. The codons for the Tyr24Cys and Val129Ala mutations are the same at the DNA level in each clone that they appear.

SmpB mutations affect frame choice on A86C tmRNA

To verify that the selected SmpB mutants restore tmRNA activity, we directly measured the tmRNA tagging levels with immunoblots. In the immunoblot assay, tmRNA directs the addition of a His_6 tag to the end of the full-length GST protein. The sequence

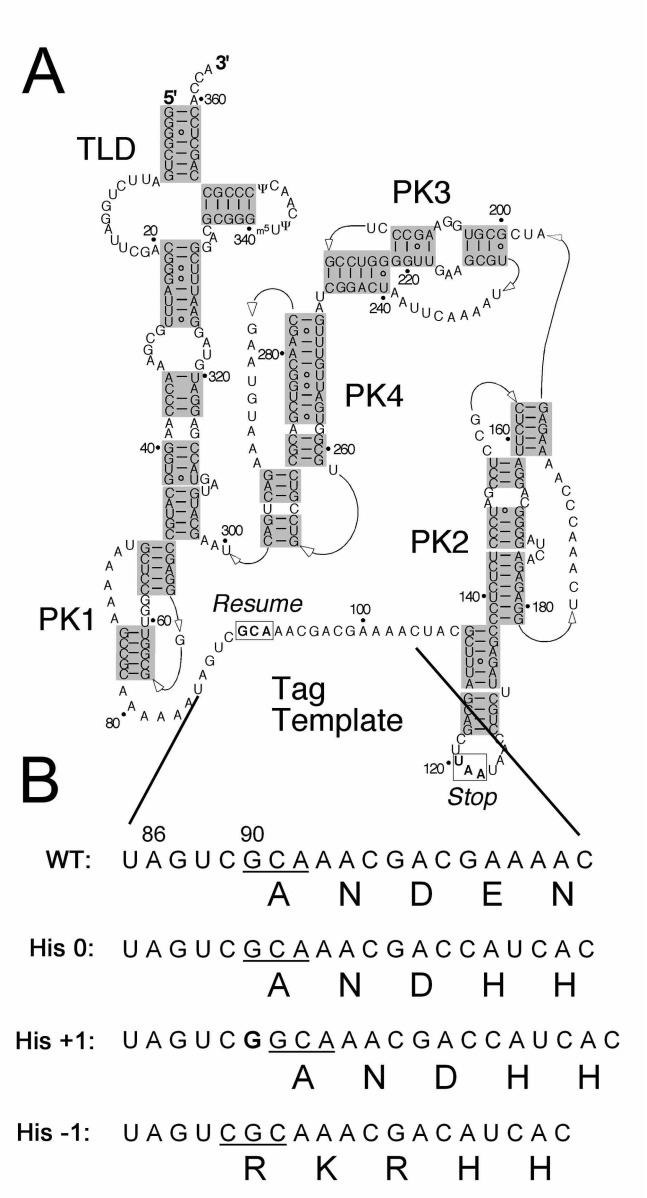


Figure 3-2: Sequence and structure of tmRNA. A) Secondary structure of *E. coli* tmRNA, including the tRNA-like domain (TLD), four pseudoknots (PK1-4), and the resume and stop codons boxed at either end of the tag template sequence. B) Fragment of the tmRNA sequence including the first five codons in the tag template as well as five nucleotides of the upstream sequence known to play a role in frame selection. The resume codon is underlined. The His 0 template encodes ANDH_6D in the natural frame; addition of a G before G90 leads to the synthesis of this tag only when the +1 frame is read in the His +1 reporter. Likewise, deletion of C98 leads to synthesis of RKRH_6D only upon translation in the –1 frame in the His –1 reporter.

Glu-Pro-Opal was used at the C-terminus of GST to induce stalling during termination.[13] The tmRNA template sequence was altered to encode ANDH_6D. Tagging in the natural (or 0) frame was detected by immunoblot using an anti-His6 antibody. We also developed additional tools to detect translation of the tmRNA template in other frames.[38] We created a +1 frame construct by inserting a single G before G90, the first nucleotide in the resume codon. This tmRNA encodes a His_6-tag only if tmRNA is translated in the +1 frame. Likewise, tmRNA in which C98 is deleted only encodes the His_6-tag when read in the –1 frame (Figure 3-2). These three tmRNA constructs allow tagging in all three frames (–1, 0, +1) to be visualized on an immunoblot for any given tmRNA and SmpB mutant pair.

Wild-type SmpB yields no detectable activity in the 0 frame and high levels of +1 frame tagging with A86C tmRNA (Figure 3-3 A). In contrast, the selected A1, A2, and A5 SmpB clones all restore significant levels of tagging in the 0 frame; the A2 mutant is the most active. The A2 mutant also reduces the +1 frame tagging seen with wild-type SmpB (t-test $P < 0.01$). It appears that the total level of tagging remains constant in the A2 mutant—tagging in the +1 frame decreases at about the same level as tagging in the 0 frame increases (Figure 3-3B). No significant reduction of +1 frame tagging was detected with A1 or A5, but this is expected since these mutants restore 0 frame tagging at lower levels. No tagging in the –1 frame was detected with the wild-type or mutant SmpB clones (data not shown). U85A tmRNA was used as a control to verify that tagging in the –1 frame could be visualized. The restoration of tagging in the natural frame and reduction in the +1 frame

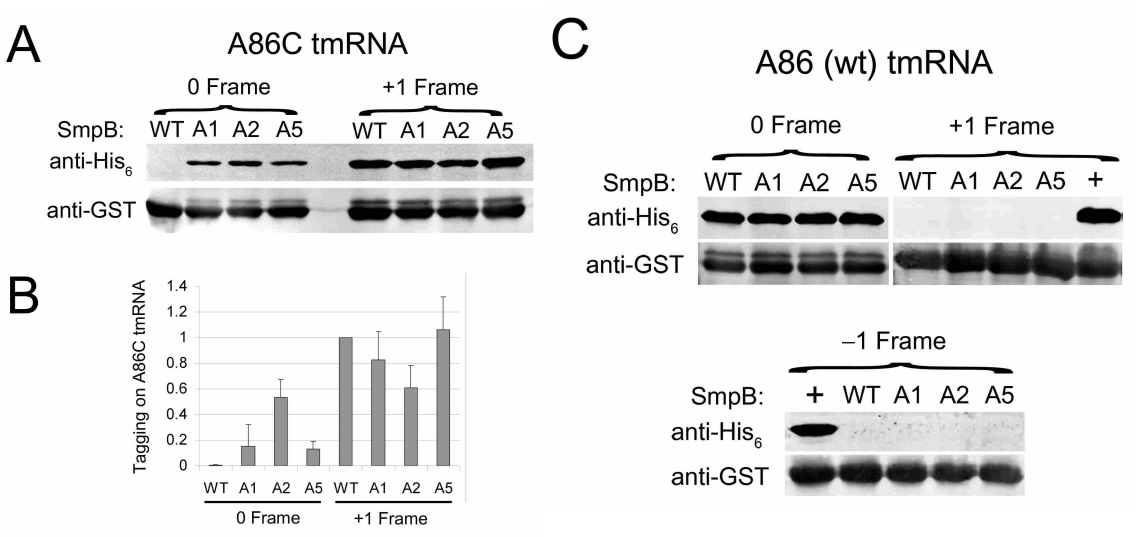


Figure 3-3: SmpB mutants alter frame selection on A86C tmRNA but not wild-type tmRNA. A) Tagging by A86C tmRNA with the selected SmpB clones. B) Quantification of the level of A86C tmRNA tagging divided by the level of GST expression, normalized to the level of +1 tagging with wild-type SmpB. Error bars report the standard deviation of three independent experiments. C) Tagging by wild-type tmRNA (A86) in all three frames in combination with various SmpB alleles. A86C and U85A were used as positive controls for the detection of +1 and –1 frame tagging, respectively, with wild-type SmpB (the + lanes).

suggests that these SmpB mutants rescue tmRNA function in the KanR selection by restoring the proper reading frame on A86C tmRNA. These data corroborate the genetic findings that SmpB is functionally tied to the upstream region of tmRNA. They also provide evidence that SmpB plays a role in selection of the reading frame.

Analysis of the role of the selected mutations in restoring tagging

How do these SmpB mutants alter frame selection and restore function? Our model of frame selection relies on two interactions: the known binding of SmpB to the ribosomal P site[30, 50] and the controversial binding of SmpB to the upstream sequence of tmRNA. Altering either of these could potentially restore function to A86C tmRNA. If the selected mutants act by repositioning SmpB in the ribosome, then they should act in a dominant manner, altering frame selection on any tmRNA, not just A86C tmRNA. To test this hypothesis, we analyzed reading frame selection with SmpB clones A1, A2, and A5 with wild-type (A86) tmRNA. Immunoblot analysis revealed no evidence of increased translation in either the +1 or –1 frame (Figure 3-3 C). This result shows that our SmpB mutants are not sufficient to alter frame recognition alone—they only do so in the context of the A86C mutation. This suggests that they act by restoring the interaction of SmpB with the tmRNA upstream sequence.

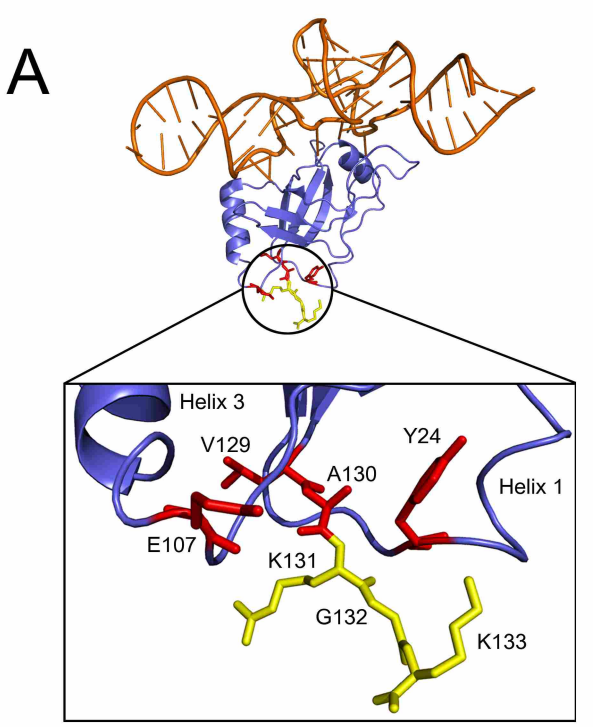
How is the binding to the upstream sequence on tmRNA restored? Perhaps the interaction with A86 that was lost was compensated for by a new interaction with C86. Are the selected SmpB clones specific for C86 or do they support tmRNA activity with the wild-type tmRNA? Immunoblot analysis with A86 tmRNA revealed that the mutants show no loss of function in the normal frame (Figure 3-3 C). We also tested the A1, A2, and A5 clones in the KanR assay against A86 tmRNA encoding the KanR tag; the SmpB mutants did

not lower the level of survival. These findings indicate that specificity for the tmRNA upstream sequence was relaxed to allow either adenosine or cytidine at position 86, rather than altered to be specific for the cytidine nucleotide. Relaxed specificity is the simpler solution in most cases; in directed evolution experiments a negative selection against the wild-type sequence must be used to obtain truly altered specificity.[63]

What is the role of each amino acid change in the selected SmpB clones? The Tyr24Cys mutation appears in all three (Table 3-1). To test if the cysteine side chain was functionally important, we replaced the Tyr24Cys mutation with Tyr24Ala in A2. The resulting SmpB protein supported survival levels similar to the original A2 mutant in the KanR assay, suggesting that Tyr24Cys is a loss-of-function mutation. Replacing the large tyrosine side chain with a smaller amino acid is sufficient. In contrast, the Glu107Val mutation found in A2 appears to be a gain of function mutation. The A2 clone is the most active of the three selected mutants, with the greatest increase in 0 frame tagging. The only difference between A1 and A2 is that A2 also has the Glu107Val mutation. Perhaps the large Val side chain provides a site for a hydrophobic interaction.

Which mutations are necessary for altered function and how do they work together? We tested all four mutations found in the three selected clones both individually and in pairs. The following mutants were assayed by immunoblot with A86C tmRNA: the single mutations Y24C, E107V, V129A, A130G, and double mutations Y24C/E107V, E107V/V129A, E107V/A130G, and V129A/A130G. None of these yielded a detectable amount of tagging in the 0 frame (data not shown), in contrast to the substantial signals seen with the original pairings. We conclude that at least two mutations are required to rescue A86C tmRNA: Tyr24Cys and either Val129Ala or Ala130Gly.

When mapped onto the atomic structure of *Thermus thermophilus* SmpB,[27] these three residues cluster in a junction at the bottom of the protein, at the opposite end from the well-characterized tmRNA binding site (Figure 3-4 A). This junction is formed from the beginning of the C-terminal tail and loops linked to helices 1 and 3. The fourth selected mutation, Glu107Val, is found nearby in the loop following helix 3. The clustering of these smutations in this single site implicates this junction at the bottom of SmpB in the frame selection process.



The Tyr24 side chain makes hydrophobic interactions with several other residues in this junction region. We introduced mutations to test the importance of these hydrophobic interactions in the natural function of the SmpB protein (i.e. paired with wild-type A86 tmRNA). Replacement of Tyr24 with charged residues Glu or Arg results in a complete loss of

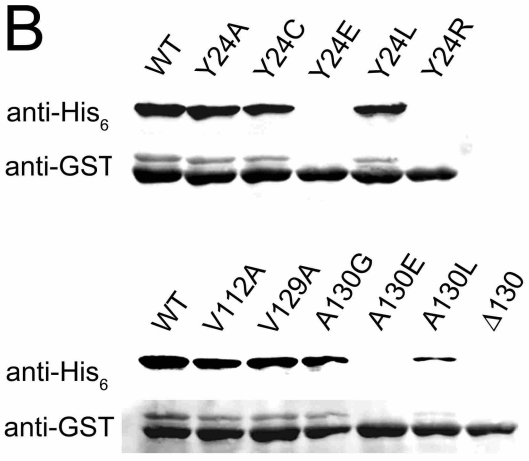


Figure 3-4: Structure and mutagenesis of SmpB. A) The structure of the SmpB-tmRNA complex from *T. thermophilus* is shown with the tRNA-like domain of tmRNA in orange and SmpB in blue(Bessho, 2007). Residues mutated in the selected SmpB clones are shown in red. The first three amino acids in the C-terminal tail ($K_{131}GK$) are shown in yellow. Residue labels depict the *E. coli* numbering and sequence. Created with Pymol. B) Tagging of stalled GST was measured by immunoblot as in Fig. 3-2 but with wild-type (A86) tmRNA encoding $ANDH_6D$ in the natural (0) frame. This series of SmpB mutants was designed to test the structural role of residues at the junction where the C-terminal tail begins.

SmpB function (Figure 3-3B), while mutation to Leu is well tolerated. Mutation to the smaller side chains Cys or Ala also has little or no effect on SmpB activity. Analysis of the atomic structure likewise suggests that the Ala130 side chain interacts with Tyr24. Mutation of Ala130 to the charged Glu or the bulky Leu dramatically reduce SmpB function (Figure 3-3B). Deletion of Ala130, effectively replacing it with Lys131, likewise destroys SmpB function. These mutagenesis results support the conclusion that Tyr24 and Ala130 form hydrophobic interactions that are essential for SmpB activity. This junction region has to be fine-tuned carefully to avoid disrupting these key interactions. Immunoblot analysis reveals that none of the selected mutations alone inhibit SmpB activity on A86 tmRNA (Figure 3-3B). We propose that the Tyr24Cys, Val129Ala, and Ala130Gly mutations identified in our selection introduce structural plasticity into this junction region, and suggest that this is what allows these SmpB mutants to interact productively with both A86 and A86C tmRNA.

Discussion

Our findings demonstrate that SmpB and the region upstream of the resume codon on tmRNA are functionally linked; both play a role in establishing the reading frame on tmRNA. The A86C mutation in tmRNA leads to the total loss of 0 frame tagging and high levels of +1 frame tagging. We identified several SmpB mutants that restore the function of A86C tmRNA both in a genetic selection and in a direct assay for the addition of the tmRNA tag. The A2 mutant restores 0 frame tagging at about the same level that it reduces +1 frame tagging (Figure 3-3 B). This suggests that the overall tagging efficiency is the same and that A2 increases tmRNA activity by simply restoring the proper frame in tmRNA translation. Interestingly, the mutant SmpB clones do not alter frame selection on wild-type (A86)

tmRNA. As the SmpB mutants act with specificity (only on A86C tmRNA) in reorienting frame choice, we conclude that they restore the interaction between SmpB and the upstream sequence.

How might SmpB set the frame during trans-translation? SmpB binds to the tRNA-like domain of tmRNA to form a structure that mimics normal tRNAs.[27] SmpB serves as the anticodon stem of this structure and licenses entry of the SmpB-tmRNA complex into the ribosomal A site. Following accommodation and peptidyl transfer, this complex moves to the P site.[50, 64] SmpB binds the 30S P site with high affinity even without tmRNA.[29, 30] We envision a model in which interactions with the P site position SmpB precisely such that its draws tmRNA into the A site (Figure 3-5). With the first codon (GCA) lying in the mRNA channel in the decoding center, translation begins with tmRNA as a template. Intriguingly, the SmpB residues identified in our selection as affecting frame selection (Tyr24, Val129, and Ala130) cluster together in a hydrophobic pocket located on what would be the A-site face of SmpB. We propose that these mutations act together to alter the structure of this pocket, allowing SmpB to bind the upstream region of either A86 or A86C tmRNA.

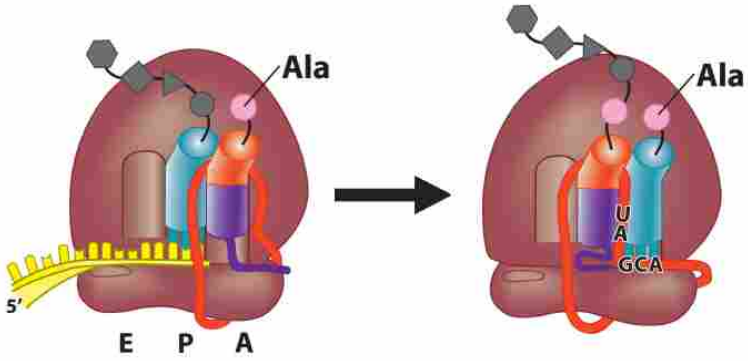


Figure 3-5: Model of tmRNA frame positioning. Following translocation, SmpB (purple) interactions with the upstream region of tmRNA (orange) position the resume codon, GCA, in the ribosomal A site. Figure made by Hilary Johnson.

The SmpB tail, beginning with K_{131}GKK, emerges from this hydrophobic pocket. Mutating K_{131}GKK to K_{131}AAK abolishes tmRNA-mediated tagging due to an SmpB

supported event following peptidyl transfer but before translation of the tmRNA open reading frame begins. Positioning the tmRNA resume codon in the A site is a major event during this window. Although there is no evidence that GK directly binds the upstream tmRNA region, the G_{132}K:AA defect is further evidence that the junction at the bottom of SmpB is involved in allowing translation of the tmRNA open reading frame to begin following the first peptidyl transfer event. Future investigations into the GK region will involve crosslinking these amino acids to tmRNA.

Materials and Methods

Materials – Enzymes for cloning were purchased from New England Biolabs. MegaX DH10B electrocompetent cells were purchased from Invitrogen. The mouse anti-His6 antibody was purchased from Cell Signaling Technology and the rabbit anti-GST antibody from Sigma. Both secondary antibodies (anti-mouse IRDye 800 and anti-rabbit IRDye 680) were from LI-COR Biosciences.

SmpB Library Construction – The selection plasmid expresses the ssrA, smpB, and truncated kanR genes[35] and conveys resistance to chloramphenicol and ampicillin. From an arabinose-inducible promoter, this plasmid expresses a truncated KanR protein lacking the C-terminal 15 amino acids, with the sequence Glu-Pro-Stop added to the C-terminus to induce ribosome stalling. The ssrA tag template sequence was altered to encode the last 14 amino acids of the kanamycin resistance protein (KanR), ANKLQFHLMLDEFF, instead of the normal degradation tag, ANDENYALAA. The ssrA gene also contains the A86C mutation. EagI and EcoRV cloning sites in the selection plasmid were used to insert the SmpB gene mutagenized by error-prone PCR[65] with the following primers: 392,

GGTATCAACAGGGACACCAGG and 470, CCAGTCACGTAGCGAAGATC. The SmpB library was introduced into MegaX DH10B competent cells by electroporation and was amplified, purified, and then introduced into the ΔssrA-smpB strain (a gift from Brice Felden[66]) for selection in the KanR assay.

KanR assay for tmRNA activity – ΔssrA-smpB cells expressing A86C tmRNA and SmpB from the selection plasmid were grown overnight in 2xYT with ampicillin. Saturated cultures were diluted to an OD600 of approximately 0.3 in fresh media containing 2% arabinose and grown for 4 hours to induce expression of the KanR protein. The cells were plated onto selective media: 2xYT, ampicillin, chloramphenicol, 2% arabinose, and 15 μg/mL kanamycin. Growth comparisons (selective vs. non-selective plates) were made after incubation for 48 h at 25 °C. Mutant smpB genes from selected clones were amplified by PCR and cloned into fresh selection vector and re-introduced into the selection strain to verify their phenotype.

Immunoblot analysis of tagging – The pDH210 plasmid expresses the GST protein with the stall-inducing sequence Glu-Pro-Stop added to the C-terminus. It also expresses tmRNA altered to encode an ANDH_6D tag. Variants of pDH210 were made that encode this His6-containing tag only if the ribosome resumes on tmRNA in either the –1 or +1 frame, by deleting C98 or by inserting a G immediately before G90, respectively. ΔssrA-smpB cells carrying pDH210 and an SmpB expression plasmid (pDH113) were grown in ampicillin and tetracycline to an OD_{600} of 0.5. The expression of GST was induced with 1 mM IPTG. After 2.5 h, the cells were pelleted and lysed with SDS. Protein in the crude lysate was quantified via Lowry assay and each sample was resolved by 10% SDS-PAGE. The protein was transferred to PVDF membrane and His_6-tagged GST was bound by a mouse anti-His6

antibody. Binding of a rabbit anti-GST antibody was used to control for protein expression and loading. Fluorescent secondary antibodies (anti-mouse IRDye 800 and anti-rabbit IRDye 680) were added and the blot was visualized with an Odyssey Infrared Imaging System (LI-COR Biosciences).

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