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The Role of SmpB Protein in *Trans*-translation

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Abstract of the Dissertation

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Trans-translation is a quality control mechanism utilized by bacteria to cope with the consequences of translation of incomplete or damaged mRNAs. The two unique components of this system are tmRNA (transfer messenger RNA) and its protein partner SmpB (Small protein B). In the early stages of the *trans*-translation mechanism, SmpB and tmRNA must recognize stalled ribosomes and bind in the ribosomal A-site. I present results that argue for binding of a pre-formed SmpB•tmRNA•EF-Tu(GTP) quaternary complex to stalled ribosomes to initiate the *trans*-translation process. SmpB protein possesses a C-terminal tail that lacks structure in solution. Through site directed mutagenesis and *in vivo* based functional assays, I've shown that deletion of the tail, or mutation of specific residues near the C-terminus abolishes SmpB's activity in supporting *trans*-translation. Interestingly, C-terminal tail mutants still retain their ability to support the two known functions of the protein, binding to tmRNA and promoting association of the SmpB•tmRNA complex with stalled ribosomes. Hence, I've identified a novel function of SmpB protein in *trans*-translation, a function that requires an intact C-terminal tail. I've assembled an *in vitro trans*-translation system in order to pinpoint the specific function of the SmpB C-terminal tail in the molecular mechanism of *trans*-translation. I've shown that the C-terminal tail is required to support transpeptidation onto tmRNA both *in vivo* and *in vitro*. Therefore, the novel SmpB function performed by the tail occurs during the tRNA-like function of tmRNA. Utilizing the *in vitro trans*-translation system, I was able to demonstrate that, like canonical tRNAs, tmRNA requires EF-Tu-mediated GTP hydrolysis for peptide bond formation. However, the SmpB C-terminal tail does not appear to be involved in eliciting activation of the EF-Tu GTPase, as mutant SmpBs lacking the C-terminal tail were fully functional in supporting GTP hydrolysis activity *in vitro*. Overall, results presented in this thesis provide unique insight into the mechanism of the elegant translational quality control mechanism known as *trans*-translation.

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Chapter 1: Introduction

Trans-translation is a bacteria-specific translational quality control mechanism that rescues ribosomes stalled on defective mRNAs, directs the degradation of aberrant protein products, and facilitates the decay of incomplete or damaged mRNAs [1-14]. The two unique components of this system are tmRNA (transfer messenger RNA) and its protein partner SmpB (Small protein B). tmRNA (also known as SsrA RNA, or 10Sa RNA) is a bi-functional stable RNA molecule with both tRNA- and mRNA-like features and activities. SmpB is a small, basic RNA binding protein that is essential for all known physiological activities of tmRNA [4, 15-17]. Briefly, *trans*-translation consists of stalled ribosome recognition and assembly of an SmpB•tmRNA•EF-Tu(GTP)•stalled ribosome complex. The SmpB•tmRNA•EF-Tu(GTP) quaternary complex then behaves in a manner analogous to a tRNA•EF-Tu(GTP) ternary complex. The tRNA-like domain (TLD) of tmRNA enters the ribosomal A-site where the growing peptide is transferred onto it. The ribosomal reading frame then shifts to translate the mRNA sequence contained in the mRNA-like domain of tmRNA. This leads to the addition of a C-terminal proteolysis tag to the new peptide, which facilitates its degradation by cellular proteases [2, 5, 18, 19]. In addition, the SmpB•tmRNA system facilitates the decay of the causative non-stop mRNA [6, 14, 20]. The aim of my investigations has been to gain further insight into the mechanism of this elegant translational control process.

1.1 Prokaryotic Protein Synthesis

The synthesis of cellular proteins from template mRNAs is termed translation. Translation is catalyzed by a large ribonucleoprotein complex, the ribosome, with tranfer RNAs (tRNAs) serving as the adaptor molecules, linking the mRNA sequence to the amino acid sequence of nascent polypeptides. The mechanism of translation may be divided into three distinct phases: initiation, elongation, and termination. These phases are represented schematically in figure 1.1. In bacteria, initiation is the loading of ribosomes onto the mRNA, through a specialized tRNA molecule ($tRNA^{fMet}$) and the activities of three protein initiation factors (IF-1, IF-2, and IF-3). Elongation is the polypeptide synthesis step where mRNA sequence is translated into protein amino acid sequence. Elongation requires tRNAs, aminoacyl tRNA synthetases, and elongation factors EF-Tu, EF-Ts, and EF-G. Termination involves the release of the nascent protein and

mRNA as well as recycling of ribosomes for translation of other mRNAs. Protein release factors RF-1, RF-2, and RF-3 as well as ribosome recycling factor (RRF) are involved in this process.

1.1.1 The Ribosome

The ribosome is the macromolecular machine that catalyzes peptide bond formation and facilitates the translation of the genetic code carried on mRNA. Ribosomes are large ribonucleoprotein complexes made up of approximately 2/3 RNA and 1/3 protein by mass. Due to its high density, the components of the ribosome are named according to their rates of sedimentation. The ribosome consists of a small (30S) subunit and a large (50S) subunit. The two subunits associate to form the 70S ribosomal complex. The 30S subunit contains 21 proteins and one RNA (16S rRNA) while the large subunit contains 34 proteins and two RNAs (23SrRNA and 5SrRNA). 70S ribosomes possess three tRNA binding sites: the aminoacyl (A) site, the peptidyl (P) site, and the exit (E) site. Elements of the A, P, and E-sites are present in both the 30S and 50S subunits.

1.1.2 Translation Initiation

Translation initiation involves the assembly of 70S ribosomal complexes primed to translate a given mRNA. The product of translation initiation is the 70S initiation complex with a specialized tRNA (initiator tRNA or $tRNA^{fMet}$) bound to an AUG mRNA initiation codon in the ribosomal P-site (figure 1.1). This is accomplished through the activities of several protein and RNA elements and begins with the assembly of a 30S pre-initiation complex containing the 30S ribosomal subunit bound to the mRNA and initiator tRNA.

Elements in the mRNA 5' untranslated region (5' UTR) are important for directing proper translation initiation. Most mRNAs contain a Shine Delgarno (SD) sequence in their 5' untranslated region (UTR). This sequence base pairs with the anti-Shine Delgarno sequence near the 3' end of 16S rRNA and this interaction favors association of the 30S subunit with the mRNA [21-23]. The mRNA generally also possesses an AUG initiation codon, which base pairs with the anticodon sequence of $tRNA^{fMet}$ to determine the translation start site. Assembly of the 30S pre-initiation complex is aided by the activities of several proteins. Methionyl-tRNA synthetase (MetRS) is responsible for charging $tRNA^{fMet}$ with methionine, and methionyl-tRNA transformylase (MTF) formylates Met-$tRNA^{fMet}$ (converting it to fMet-$tRNA^{fMet}$). This formylation event is thought to prevent initiator tRNA from being utilized in translation elongation [21, 23-25]. Formylation, as well as sequence determinants on the initiator tRNA drive its interaction with initiation factor 2 (IF-2) [21, 26-28] (see below). During translation initiation, initiation factor 1 (IF-1) binds in the A-site of the 30S subunit performing at least 2 functions: 1) directing intitiator tRNA to the ribosomal P-site by blocking access to the A-site and 2) preventing binding of elongator tRNAs to the A-site during initiation [21, 23, 29-36]. Initiation factor 3 binds the 30S subunit, although its specific

binding site is unclear at this time. It is involved in preventing premature association of the 50S subunit and in directing the selection of initiator tRNA [35, 37-44]. Association of the 50S ribosomal subunit with the 30S pre-initiation complex is catalyzed mainly by IF-2 and is coupled to release of IF-1 and IF-3 [35, 45, 46]. IF-2 is a GTP-binding protein and its activity is regulated through cycles of GTP binding and hydrolysis. The precise functional significance of GTP hydrolysis remains to be firmly elucidated, but it may be involved in promoting the release of IF-2 from the initiation complex [47-50]. IF-2 release leaves a 70S ribosome initiation complex with initiator tRNA in the P-site and the next mRNA codon to be translated in the A-site, primed for translation elongation (figure 1.1).

1.1.3 Translation Elongation

Elongation is the stage of protein synthesis where the genetic code, carried as nucleotide sequence on mRNA, is translated into the amino acid sequence of proteins. A schematic representation of the mechanism of translation elongation is depicted in figure 1.2. Elongator tRNAs possess an anticodon sequence which base pairs with cognate mRNA triplets and binds a specific amino acid via an esther linkage. As such tRNAs serve as adaptor molecules for transmission of the genetic code. Protein elongation is catalyzed by 70S ribosomes as well as numerous protein factors. Aminoacyl-tRNA synthetases catalyze the attachment of specific amino acids to the appropriate tRNA molecule, and are thus, essential for the fidelity of protein synthesis. Following initiation, and after each successive round of elongation, the mRNA codon that specifies the next amino acid to be added to the growing polypeptide resides in the ribosomal A-site. tRNAs, in complex with EF-Tu and GTP, then sample the A-site. When the correct (cognate) tRNA enters the A-site, its anticodon base pairs with the mRNA codon. Proper codon-anticodon interactions lead to conformational changes in the ribosome that stabilize binding of the correct tRNA and also stimulate GTP hydrolysis by EF-Tu. The GDP form of EF-Tu has reduced affinity for tRNA and, thus, dissociates from the complex. This allows the amino acid acceptor arm of the tRNA to fully enter the ribosomal A-site in a process termed accommodation. Accommodation results in entry of the amino acid acceptor arm of the tRNA molecule into a region in the 50S subunit known as the peptidyl transferase center. Here the growing peptide is transferred onto the A-site tRNA in a process known as transpeptidation. Transpeptidation is a ribosome catalyzed event, and the active site is composed entirely of RNA [51-59]. Another protein factor, EF-Ts, serves as the guanyl-nucleotide exchange factor for EF-Tu, promoting conversion of EF-Tu(GDP) to the active EF-Tu(GTP) form.

After transpeptidation, the ribosome is left with a deacylated tRNA in the P-site and a peptidyl tRNA in the A-site. The ribosome then moves three bases down the length of the mRNA, in a process termed translocation, in order to facilitate another round of translation elongation. Translocation is catalyzed by another protein translation elongation factor known as EF-G. EF-G binds in the ribosomal A-site and promotes movement of the A-site peptidyl tRNA to the P-site and the deacylated P-site tRNA to the E-site. This movement leaves the

next mRNA codon to be translated in the ribosomal A-site, ready for the next round of translation elongation. EF-G is a GTP binding protein and hydrolysis of GTP is required for its activity in translocation [60-62].

1.1.4 Translation termination, peptide release, and ribosome recycling

Translation elongation continues until the ribosome encounters an in-frame termination codon. There are three termination codons (or stop codons) in bacteria (UAA, UAG, and UGA). Two protein release factors recognize these stop codons (RF-1 recognizes UAA and UAG, and RF-2 recognizes UAA and UGA). These release factors bind in the ribosomal A-site and promote hydrolysis of the P-site peptide-tRNA linkage, releasing the new polypeptide (figure 1.1). RF-3, another GTP binding protein, then binds the A-site promoting release of RF-1 or RF-2 [63, 64]. Finally, GTP hydrolysis on RF-3 promotes the dissociation of RF-3 from the ribosome [63, 64]. Peptide release leaves a 70S ribosome complex with mRNA and a deacylated P-site tRNA. Disassembly of this complex into free 50S and 30S ribosomal subunits is required to initiate a new round of protein synthesis. This process is catalyzed by another protein translation factor known as ribosome recycling factor (RRF) along with the aforementioned factors EF-G, IF-1, and IF-3 [21, 65-67] (figure 1.1). IF-3 is thought to promote the dissociation of the deacylated P-site tRNA, and IF-1 enhances this activity [21, 66-68]. It is important to note that the presence of an in-frame termination codon is required to elicit rapid peptide release and ribosome recycling via this efficient mechanism. The next section will describe some of the causes and consequences of translation of mRNAs that lack an in-frame stop codon.

1.2 *trans*-translation

Gene mutation, DNA Damage, mRNA damage, and translational errors may all lead to the ribosome reaching the 3' end of an mRNA without encountering an in-frame termination codon. This event could have two potentially hazardous consequences for the bacterium. First, since an in-frame stop codon is required to recruit the translation termination apparatus, mRNAs lacking in-frame stop codons lead to ribosome stalling and significant loss of translational efficiency. Secondly, aberrant protein products translated from incomplete mRNAs may be harmful to cells. These consequences are summarized schematically in figure 1.3. The SmpB•tmRNA quality control system solves both problems by recognizing and rescuing stalled ribosomes, and directing the addition of a C-terminal proteolysis tag to incomplete protein products [3-5, 10, 16, 69-74]. This process is known as *trans*-translation.

1.2.1 tmRNA and SmpB

tmRNA is a unique bi-functional RNA molecule that exhibits features and activities similar to both a tRNA and an mRNA. Figure 1.4 is a secondary structural model of tmRNA alongside $tRNA^{Ala}$. The 5' and 3' ends (of tmRNA) together form a tRNA-like domain (TLD) with sequence and structural similarity to $tRNA^{Ala}$ [75-79]. The tmRNA TLD possesses an amino acid acceptor stem, a TΦC loop and a D-arm, but lacks an anticodon arm. Like $tRNA^{Ala}$, tmRNA can be charged with alanine through the action of alanyl-tRNA synthetase (Ala-RS) [79, 80]. The structure of the tmRNA TLD has been solved crystallographically in complex with SmpB [70, 81] (see below for details). tmRNA exhibits additional secondary structural elements in the form of four RNA pseudoknots (pk1-pk4). The specific functions of the tmRNA pseudoknots remain unclear, however several studies suggest that pk1 is important for tmRNA function [82-86]. Mutations that disrupt the structure of pk1 also effect the efficiency of tmRNA charging as well as *trans*-translation activity [82, 83]. However, a more recent genetic analysis suggests that pk1 may be replaced by engineered RNA hairpin structures with minimal loss in tmRNA activity, suggesting that pk1 might serve a largely stuctural role [86]. Less is known about the roles of pseudoknots pk2, pk3, and pk4. Substitution of these pseudoknots with single stranded species yielded minimal loss of tmRNA activity in *trans*-translation [84]. However, results of a more recent study suggest that these pseudoknots may be important for the structure and/or stability of tmRNA [87]. Between pseudoknots 1 and 2 in the tmRNA sequence lies an mRNA-like open reading frame (ORF) which codes for a proteolytic degradation tag (ANDENYALAA in *E. coli*) followed by tandem UAA termination codons. The mRNA-like activity of tmRNA was initially suggested by the observation of a discreet set of smaller products present in a purified interleukin 6 (IL-6) protein preparation [9]. These smaller species turned out to be incomplete IL-6 protein products appended with the tmRNA-derived tag sequence. Later the tRNA-like and mRNA-like features were combined by Keiler and colleagues into the initial model of *trans*-translation [5] (see below).

Small protein B (SmpB) is a small basic RNA binding protein. It's structure has been solved both by NMR and X-ray crystallography [70, 81, 88, 89]. SmpB has an antiparallel β-barrel type structure with an embedded oligonucleotide binding (OB) fold [70, 81, 88, 89]. SmpB also possesses a C-terminal tail which is unstructured in solution [70, 81, 88, 89].

1.2.2 The mechanism of *trans*-translation

Figure 1.5 is a model of the mechanism of *trans*-translation. It begins with recognition of stalled ribosomes by SmpB•tmRNA. The TLD of tmRNA is charged with alanine by Ala-RS and a quarternary complex of SmpB•tmRNA•EF-Tu(GTP) (analogous to the tRNA•EF-Tu(GTP) ternary complex involved in translation elongation) binds in the A-site of stalled ribosomes. GTP is hydrolyzed, EF-Tu(GDP) is released, and the tmRNA TLD is accommodated into the peptidyl tranferase center in the 50S subunit. The growing peptide is then tranferred onto the tmRNA TLD. Next, the ribosomal reading frame shifts to

translate the tmRNA encoded mRNA sequence. This mRNA sequence is translated normally until the ribosome reaches a termination codon at the end of the tmRNA ORF. This allows rapid translation termination, protein release, and ribosome recycling. The nascent protein, now carrying the tmRNA encoded degradation tag is efficiently degraded by cellular proteases. Several cellular proteases, including ClpXP, ClpAP, lon, ftzH, and Tsp have been shown to be involved in degradation of tmRNA tagged proteins [2, 5, 19, 90, 91]. The SmpB•tmRNA system also promotes the degradation of the aberrant mRNA by RNase R via an unknown mechanism that requires translation of the defective transcript [6, 8, 20, 92].

1.2.3 SmpB•tmRNA substrates

What differentiates a stalled ribosome from a normally translating ribosome, making it a substrate for SmpB•tmRNA mediated tagging, remains an open question. A variety of situations which delay the progress of protein synthesis have been shown to elicit tagging by SmpB•tmRNA. Tagging can occur at the end of a non-stop mRNA, but also at internal positions on the mRNA [5, 93-96]. However, it is likely that in cases of internal tagging, a co-translational mRNA cleavage event leads to the generation of a non-stop mRNA [92, 97-100]. As such, one possible mechanism for the differentiation between normal and stalled ribosomes is the presence or absence of mRNA sequence either within or 3' to the ribosomal A-site. Tagging has been reported at positions corresponding to termination codons, presumably due to a delay in binding of release factors to the A-site, as compared to the rate of cognate tRNA A-site entry [92, 96, 101, 102]. Indeed, depletion of the cognate release factor has been shown to enhance the rate of tagging at a specific termination codon [103, 104]. In addition, tRNA scarcity can lead to SmpB•tmRNA-mediated tagging [6, 8, 95, 99]. This observation is highlighted by the observation that overexpression of an mRNA containing tandem rare arginine codons leads to tagging, an effect that is rescued by driving increased expression of the cognate tRNA. Tagging has also been observed in response to programmed ribosome stalling events such as translation of the secM arrest sequence [93, 105]. In summary, various ribosome stalling events can drive recruitment of SmpB•tmRNA and elicit *trans*-translation.

1.2.4 Physiological Significance

The *smpB* and *ssrA* (the gene coding for tmRNA) genes are represented in all sequenced bacterial genomes. The SmpB•tmRNA translational quality control system has been shown to be important for both survival and pathogenesis in different bacterial species. Genome wide mutagenesis results suggest that the *ssrA* and *smpB* genes are essential in both *Mycoplasma genitalium* and *Mycoplasma pneumoniae* [106]. It was also established directly that the *ssrA* gene is essential in *Neisseria gonorrhoeae* [107]. Although non-essential, *ssrA* has been shown to be important for virulence in *Salmonella enterica* [108, 109], as well as

Yersinia pseudotuberculosis [109]. Hence, the SmpB•tmRNA system is a bacteria-specific quality control mechanism that is important for survival and virulence in bacterial pathogens. In *E. coli*, *ssrA* is not essential. *ssrA* deficient strains, however, exhibit slower growth at high temperature, slow recovery from carbon starvation, and reduced motility [79, 110]. Similarly, *ssrA* is not required for normal growth of *Bacillus subtilis*, however *smpB* and *ssrA* deletions lead to reduced growth rate at both low and high temperatures [111]. *ssrA* mutants also fail to support lytic development of two bacteriophages, λimmP22 hybrid phage and temperature sensitive variants of phage Mu [4, 112, 113].

SmpB•tmRNA mediated tagging is also thought to serve as a mechanism for translational control of gene expression under normal growth conditions. A number of regulatory proteins have been shown to be tagged by the SmpB•tmRNA system, for example YbeL, GalE, RbsK, and the LacI repressor are all substrates for SmpB•tmRNA[96, 114]. In the case of LacI, it is thought that tagging of the repressor serves as a negative feedback mechanism to maintain optimal repressor concentrations. Consistent with this hypothesis, *ssrA*$^-$ cells exhibit a delay in induction of the lac operon [96, 114]. Interestingly, in *Yersinia pseudotuberculosis*, loss of SmpB and tmRNA leads to a severe defect in expression of virulence effector proteins, apparently at the level of transcription [109]. This effect contributes to the loss of virulence observed in these mutants. More recently, tmRNA has been implicated in the maintenance of cellular concentration of the *E. coli* stress sigma factor RpoS [115]. RpoS is upregulated in response to various cellular stresses. The stationary phase upregulation of RpoS is substantially reduced in the absence of tmRNA, and tmRNA's effect appears to be at the level of RpoS translation [115].

1.2.5 Comparison of prokaryotic and eukaryotic translation surveillance mechanisms

Unproductive ribosome stalling is a very basic cellular event with universal causes and consequences. However, prokaryotic and eukaryotic cells deal with the phenomenon in distinct ways. Bacterial *trans*-translation, in comparison to eukaryotic mechanisms, is a more robust response to ribosome stalling, as non-stop mRNAs, rare codon containing mRNAs, as well as mRNAs with specific stall sequences are all targets for SmpB•tmRNA [5, 93-95]. In contrast, eukaryotic cells exhibit at least two distinct mechanisms for preventing ribosome stalling. The nonstop decay pathway (NSD) targets mRNAs lacking in-frame termination codons [116-121], while the no-go decay (NGD) pathway deals with mRNAs containing internal stall sites (i.e. mRNAs with secondary structural elements like stem loops and pseudoknots) [122]. Our current knowledge of the NSD and NGD pathways is limited as study of these two mechanisms is in its infancy. NSD involves exosome mediated 3'-5' mRNA decay (although a 5'-3' non-stop mRNA decay activity has been reported [117]), mediated by the C-terminal domain of ski7p [116, 120]. No-go decay is initiated by an mRNA cleavage event performed by an as yet unidentified nuclease and involves two proteins, Dom34p and Hbs1p, which

exhibit similarity to the eukaryotic release factor eRF-1 [122]. Translation inhibition as well as nascent protein destabilization are thought to limit the accumulation of protein products translated from nonstop mRNAs [117-119, 121], but the fate of nascent polypeptides is unclear in no-go decay. It has been convincingly demonstrated that non-stop mRNAs lead to translational repression as non-stop mRNA reporters accumulate in polysome fractions [117], however the mechanism of ribosome rescue and restoration of translational efficiency is unclear. Similarly, secondary structure elements present in mRNAs that elicit no-go decay must result in sequestration of ribosomes and it is, as yet, unclear how this unproductive stalling is alleviated.

Trans-translation is an elegant, simple and comprehensive mechanism for dealing with unproductive ribosome stalling. It can efficiently deal with different types of stalling events, including non-stop mRNAs, rare codons, and engineered stall sequences. In doing this it promotes ribosome rescue, directs the degradation of aberrant protein products, and facilitates the decay of the causative non-stop mRNA. As such, it seems surprising that such a complete mechanism for dealing with a host of universal cellular problems has been lost in eukaryotes. One possible explanation is that eukaryotes have evolved more efficient mechanisms for the individual activities performed by SmpB•tmRNA. Another possibility is that a *trans*-translation-like mechanism would interfere with productive ribosome stalling events. For instance, SmpB•tmRNA might interfere with translational control processes like programmed ribosomal frameshifting or with delayed translation to facilitate proper co-translational folding of nascent proteins. More details about the way eukaryotic cells deal with unproductive ribosome stalling events may help shed light on this issue.

1.3 The roles of SmpB in *trans*-translation

1.3.1 SmpB RNA binding activity

SmpB protein is required for all known physiological activities of tmRNA. The tmRNA model suggests that SmpB performs two distinct functions in *trans*-translation. SmpB binds tmRNA specifically and is required for promoting association of tmRNA with stalled ribosomes [4, 15, 16, 123-128]. SmpB may also promote charging of the tmRNA TLD with alanine by alanine-tRNA synthetase (Ala-RS) [15]. Two major unanswered questions with regard to the role of SmpB are: 1) what are its specific RNA binding partners and 2) what is the stoichiometry of these protein-RNA complexes. SmpB has been shown on numerous occasions to bind specifically to tmRNA, however the stoichiometry of this complex is unclear [123-126]. Optical biosensor and melting curve analysis combined with mutational studies suggests that one copy of SmpB binds to a single binding site on the D-arm of tmRNA in *Thermus thermophilus* [129]. These results are mirrored by two co-crystal structure models of SmpB in complex with the TLD of tmRNA from *Thermus thermophilus* and *Aquifex aeolicus* [70, 81]. These models depict a single SmpB binding site on the D-arm of the tmRNA TLD. In

contrast, two footprinting studies predict multiple SmpB binding sites on tmRNA. Wower *et. al.* found that three SmpB molecules can bind per tmRNA tRNA-like domain, one on the acceptor stem and T-arm (which overlaps the EF-Tu binding sites) and the other two on the anticodon stem [130]. An additional footprinting study reported SmpB footprints in both the D-loop and T-arm of the TLD, along with an additional set of footprints near the resume codon of the full-length tmRNA molecule [131].

Another source of uncertainty lies in the nature of SmpB•ribosome binding activity. It is clear from a number of sources that SmpB is required for association of tmRNA with stalled ribosomes [4, 124, 128, 132]. However, the location and number of SmpB ribosome binding sites, as well as the order of events in the assembly of the SmpB•tmRNA•stalled ribosome complex remains uncertain. Footprinting of rRNA by SmpB interactions with 70S ribosomes or isolated ribosomal subunits has revealed two SmpB binding sites on ribosomal RNA, one near the 30S P-site, the other close to the factor binding site in the 50S A-site [133]. *In vitro* SmpB-ribosome binding studies in another report suggest that SmpB binds to both ribosomal subunits [128]. Predicting the stoichiometry of the SmpB•tmRNA•stalled ribosome complex is complicated by two contradictory cryo-EM maps of the pre-accomodated complex. The first model depicts a single SmpB molecule bound to the D-loop region of the tmRNA TLD [134]. A second model depicts two SmpB molecules in the very same pre-accomodated complex [135]. The low resolution of these maps, along with the small size of SmpB and the disparity between reconstructions calls into question the reliability of these assignments. Hence, the stoichiometry of this complex remains an open question.

An additional point of uncertainty lies in the order of events in the formation of the SmpB•tmRNA•stalled ribosome complex. One model predicts that a pre-formed SmpB•tmRNA complex recognizes and binds stalled ribosomes to initiate *trans*-translation. This model is supported by numerous studies that suggest that tmRNA is the specific high affinity RNA binding partner of SmpB [4, 15, 123-128, 136]. However, based on *in vitro* SmpB•ribosome binding studies and on *in vivo* interactions in the absence of tmRNA, it has been proposed that free SmpB may pre-bind the ribosome to recruit tmRNA to stalled ribosomes [137].

1.3.2 Putative role of SmpB in the tRNA-like function of tmRNA

The tmRNA TLD shares structural and functional characteristics with canonical tRNAs. However, tmRNA lacks an anticodon arm. As such, the mechanism of initial A-site binding and accommodation of the tmRNA TLD is necessarily different from that of tRNAs. It has been proposed, that SmpB may function as an anticodon arm mimic [4, 16, 70, 81, 134, 138]. That is, SmpB may make contacts in the ribosomal A-site that promote GTP hydrolysis by EF-Tu and elicit the ribosomal conformation changes required for accommodation of the tmRNA TLD. This notion is supported by a recent co-crystal structure model of SmpB in complex with the tmRNA TLD [81]. This reconstruction demonstrates that the

SmpB•tmRNA TLD complex structurally mimics a tRNA, forming the traditional 90° L-shape. However, SmpB occupies the positions corresponding to the D-stem and anticodon arm in the complex. SmpB is required to support association of tmRNA with stalled ribosomes, an event upstream of GTP hydrolysis and peptide bond formation in the *trans*-translation mechanism [4, 15, 16, 123-128]. As such it is difficult to directly test a putative anticodon-like role for SmpB. Such analysis would require the isolation of SmpB mutants that are able to bring tmRNA to the ribosome, but fail to support the tRNA-like function of tmRNA.

The kinetic sheme and elemental rate constants for each distinct kinetic step in the process of codon recognition for tRNA ternary complexes have recently been determined. Rapid quench and stop flow kinetics techniques have been utilized to measure the rates of initial ribosome binding, codon recognition, GTPase activation, accommodation, and peptide bond formation [61, 139-150]. However, very little kinetic information about these processes is available for SmpB•tmRNA A-site interactions. Ivanova *et. al.* measured the rate of trans-transfer (i.e. peptide bond formation) that occurs upon SmpB•tmRNA interactions with stalled ribosomes *in vitro* [97]. The observed rate is similar to that of transpeptidation onto a cognate tRNA. However, no information regarding the rates of GTPase activation, GTP hydrolysis or accommodation is currently available. Analysis of the kinetics of the tRNA-like activities of SmpB•tmRNA would be informative both in elucidating the mechanistic details of the process, as well as testing a putative anticodon-like function of SmpB.

1.3.3 The SmpB C-terminal tail

SmpB protein exhibits a C-terminal extension whose structure is not well defined in four separate structural models of the protein [70, 81, 88, 89]. It is, hence, likely that this region is unstructured in solution. However, it is possible that this region is still functionally relevant in *trans*-translation. There is extensive precedent in the literature to support the idea that a ribosome-associated protein may contain extensions that are unstructured in solution but gain structure within the context of the ribosome. For example both ribosomal proteins S4 and S19 contain extended termini which are disordered in NMR solution structures [151]. In the crystal structure of the 30S subunit, however, these extensions exhibit defined structures and make contacts with 16S rRNA [152]. In a recent report, Hoang and colleagues demonstrated that the C-terminal tails of ribosomal proteins S9 and S13 contribute to the binding of P-site tRNA to the 30S ribosomal subunit [153]. In addition, functional roles for both the N- and C-terminal extensions of eukaryotic translation initiation factor eIF-1A have recently been demonstrated [154]. Hence, the extended termini of ribosomal proteins have been shown to play a functional role in translation. Therefore, it would not be surprising to discover that the SmpB C-terminus gains structure upon ribosome binding to make critical contacts required for tmRNA mediated tagging.

1.4 Figures

Figure 1.1

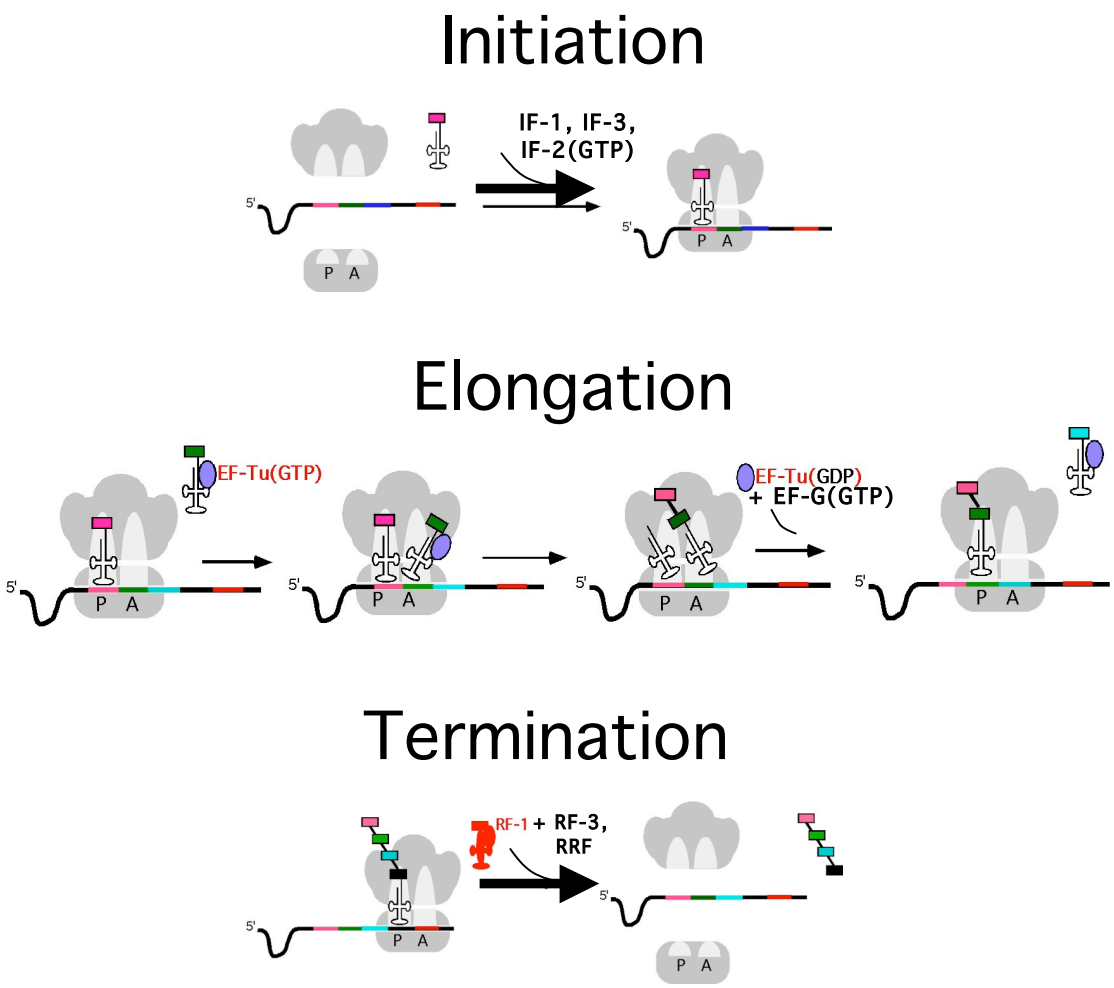

Figure 1.1: *Outline of the mechanism of protein synthesis.* Protein synthesis may be divided into translation initiation, elongation and termination phases. See text for details.

Figure 1.2

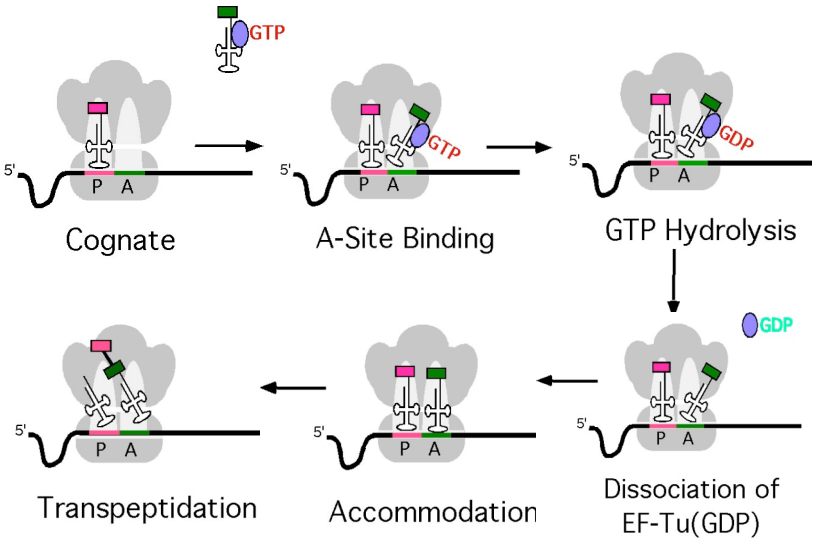

Figure 1.2: *Schematic representation of the codon recognition process*. Cognate tRNAs enter the ribosomal A-site in a ternary complex with EF-Tu(GTP). Codon anticodon interactions elicit conformational changes leading to GTP hydrolysis by EF-Tu. EF-Tu(GDP) dissociates, allowing accommodation of the tRNA. This is followed by transpeptidation onto the A-site tRNA.

Figure 1.3

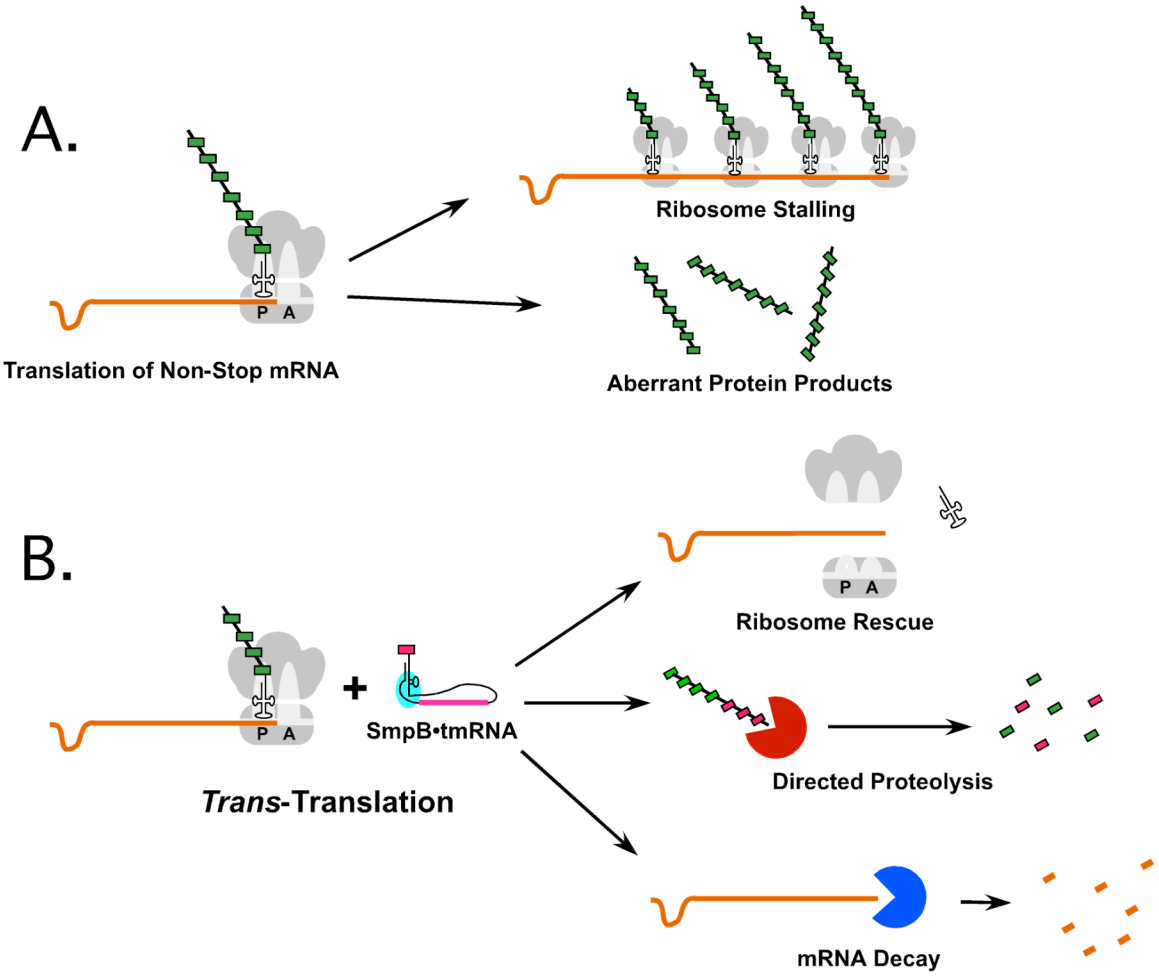

Figure 1.3: *Consequences of non-stop mRNAs*. **A.** As mRNAs are translated on polysomes, a single non-stop mRNA may lead to the hijacking of multiple ribosomes, causing a substantial net loss in translational efficiency. A second consequence of non-stop mRNAs is the production of incomplete proteins that may be toxic. **B.** *Trans*-translation solves both problems by facilitating ribosome rescue, and directing the degradation of aberant protein products. SmpB•tmRNA also directs the decay of the causative mRNA, preventing future ribosome stalling events

Figure 1.4

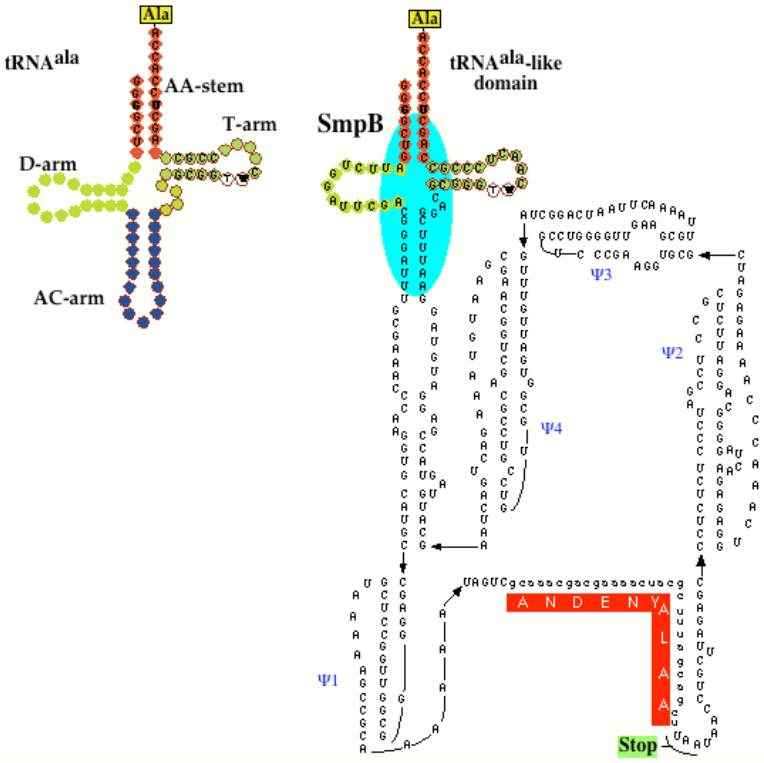

Figure 1.4: *Secondary structure model of tmRNA*. A model of the secondary structure of tmRNA (on the right) is shown along side that of $tRNA^{Ala}$. Pseudoknots 1-4 are labelled Ψ1-Ψ4. The mRNA-like ORF is in red. SmpB protein is depicted in cyan.

Figure 1.5

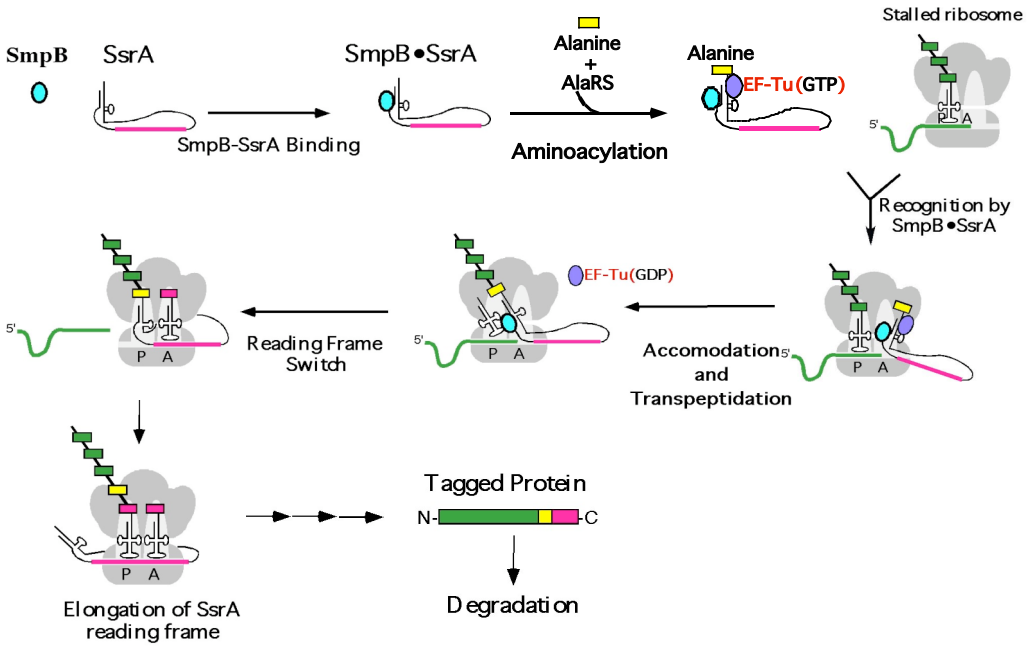

Figure 1.5: *Model of the mechanism of trans-translation*

Chapter 2: Functional SmpB•Ribosome Interactions Require tmRNA

2.1 Summary

Small protein B (SmpB) is a requisite component of the transfer messenger RNA (tmRNA)-mediated bacterial translational quality control system known as *trans*-translation. The initial binding of tmRNA and its subsequent accommodation into the ribosomal A-site are activities intimately linked to SmpB protein function. From a mechanistic perspective, two key unanswered questions that require further investigation are: 1) what constitutes a stalled ribosome recognition complex and 2) does SmpB pre-bind ribosomes to recruit tmRNA. I have assessed, both *in vivo* and *in vitro*, the nature and stability of free SmpB interactions with stalled ribosomes and examined whether these interactions are functionally relevant. I present evidence to demonstrate that interaction of free SmpB with ribosomes is salt sensitive and significantly more labile than interaction of the SmpB-tmRNA complex with ribosomes. Upon dissociation of 70S ribosomes, SmpB partitions primarily with tmRNA rather than ribosomal subunits. This finding is consistent with biochemical and structural data demonstrating that tmRNA is the high-affinity binding partner of SmpB. Moreover, I show that under normal physiological conditions, roughly similar numbers of SmpB and tmRNA molecules are present in cells. My investigations also reveal that upon induction of a nonstop mRNA, SmpB is enriched in stalled ribosome fractions only in the presence of tmRNA. Based on these findings, I conclude that SmpB does not pre-bind stalled ribosome and that functional SmpB-stalled ribosome interactions require tmRNA. I propose that a 1:1:1 complex of SmpB•tmRNA•EF-Tu (GTP) recognizes and binds a stalled ribosome to initiate *trans*-translation.

2.2 INTRODUCTION

The specific roles of SmpB protein in *trans*-translation remain a matter of debate. It is clear from a number of studies that SmpB binds tmRNA specifically and is required for association of tmRNA with stalled ribosomes [12, 123, 126-128, 136, 155, 156]. However, the location and number of SmpB ribosome binding sites, as well as the order of events in the assembly of the SmpB•tmRNA•stalled ribosome complex remain uncertain. To gain deeper insight into SmpB-ribosome interactions, I set out to study the assembly of the SmpB•tmRNA•stalled

ribosome complex *in vivo*, under normal log-phase growth conditions, and with canonical SmpB and tmRNA expression levels. I found that under certain non-physiological conditions free SmpB can interact with ribosomes, however this interaction is labile and salt sensitive. I showed that ribosome-bound SmpB co-localizes with tmRNA upon dissociation of ribosomal subunits. In addition, I report that SmpB is enriched in stalled ribosomes only in the presence of tmRNA. Taken together, my investigations lead me to conclude that stalled ribosomes are recognized and bound by a pre-formed SmpB•tmRNA complex (along with EF-Tu and GTP) to initiate *trans*-translation.

2.3 Experimental Procedures

2.3.1 Buffers, Strains, and Plasmids

Buffer A contained 50mM Tris (pH 7.5), 70mM NH_4Cl, 30mM KCl, 10mM $MgCl_2$, and 20mM DTT. Buffer B contained 50mM Tris (pH 7.5), 10mM $MgCl_2$, 20mM DTT, and either 100, 200, or 300mM NH_4Cl. Buffer C contained 40mM Tris (pH 7.5), 10mM $MgCl_2$, 2mM β-mercaptoethanol, 10% sucrose, and either 100, 200 or 300mM NH_4Cl. Buffer D contained 40mM Tris (pH 7.5), 1mM $MgCl_2$, 2mM β-mercaptoethanol, and either 100 or 300mM NH_4Cl. Buffer E contained 25mM Tris (pH 7.5), 100mM NH_4Cl, 30mM KCl, 8mM $MgCl_2$ and 1mM DTT. Buffer F contained 50mM Tris (pH 7.5), 20mM $MgCl_2$, 2mM β-mercaptoethanol, 10mM Imidazol, and either 100 or 300mM NH_4Cl. Buffer G contained 50mM Tris (pH 7.5), 10mM $MgCl_2$, and 2mM β-mercaptoethanol, and either 100, 200, or 300mM NH_4Cl.

The strain W3110 *ssrA*::CAT has been described previously [156]. pλ-cI-N-4-AGG-Flag has been described previously and is referred to in the text as pλ- cI-N AGG [14]. pλ-cI-NS was prepared by sequential rounds of PCR mutagenesis to convert the rare arginine codons (AGG) to abundant codons (CGG) and to introduce a trpA transcriptional terminator near the 3' end. The plasmid pET28BA was described previously [156]. The plasmid ptrnfM for expression of initiator tRNA was a kind gift from Dr. Uttam RajBhandary (MIT). The plasmid pETrpS12 for expression of ribosomal protein S12 was a kind gift from Dr. Harry Noller (UC Santa Cruz).

2.3.2 Protein and RNA Purification

His-tagged protein translation factors IF-1, IF-2, IF-3, EF-Tu, EF-Ts, AlaRS, MetRS, and MTF as well as SmpB were purified using Ni^{2+}-NTA affinity chromatography followed by ion exchange. Briefly, proteins were purified by batch affinity chromatography using Ni^{2+}-NTA agarose (Qiagen, Valencia,CA) followed by FPLC ion exchange using either a MonoS (HR 10/10) column or a MonoQ (HR 10/10) column (GE Healthcare, Piscataway, NJ) eluted with a linear

salt gradient. Ribosomal protein S12 was purified according to Culver and Noller [157, 158].

tmRNA was purified from cells as follows. 6L of BL21(DE3)/pLysS/pET28BA cells were grown to OD_{600} around 0.5-0.7. SmpB and tmRNA were simultaneously induced for 2 hours with 10μM IPTG. The SmpB•tmRNA complex was purified by affinity chromatography using Ni^{2+}-NTA agarose resin (Qiagen, Valencia,CA). tmRNA was separated from SmpB via RNA extraction using TriReagent LS (MRC, Cincinnatti,OH). tmRNA was further purified via FPLC anion exchange using a MonoQ (HR 10/10) column and a linear salt gradient (GE Healthcare, Piscataway, NJ).

A $tRNA^{fMet}$ enriched tRNA pool was purified by isolating total tRNA from cells expressing $tRNA^{fMet}$ from the plasmid ptrnfM. tRNA purification was performed as described [159]. Preparative purification of charged fMet-$tRNA^{fMet}$ was performed as follows. Charging and formylation reactions were performed in buffer E. Reactions (10mL) included 20μM $tRNA^{fMet}$, 200μM L-methionine, 150μg N^{10}-formyl-tetrahydrofolate, 2μM Met-RS, 5μM MTF, and 3mM ATP, incubated at 37°C for 20min. RNA was precipitated with isopropanol, washed with ethanol, and extracted with TriReagent LS. The charged and formylated fraction was separated from uncharged tRNA via FPLC hydrophobic interactions chromatography using a Hi-Trap Phenyl Sepharose HP column (GE Healthcare, Piscataway, NJ). This product was further purified by FPLC anion exchange using a MonoQ (HR 10/10) column (GE Healthcare, Piscataway, NJ).

2.3.3 Ribosome Association Assays

Ribosomes were purified as previously described, with modifications [160]. 750mL cultures were grown to OD_{600} ~ 1.0. Cells were harvested and resuspended in 15mL of buffer G with the NH_4Cl concentrations given in the text. Cells were lysed by French press, and the lysate was centrifuged at 30,000Xg for 1hr. The S30 supernatant (12mL) was then layered onto 12mL of a 32% sucrose cushion in buffer G. The sucrose cushions were then centrifuged at 100,000XG for 16hr. The ribosome pellets were again washed with 5mL of buffer G and ribosomes were resuspended in 1mL of Buffer G.

For analysis of SmpB ribosome association, a fraction of this ribosome preparation was resolved on a 15% Tris-Tricine gel and western blots were developed using α-SmpB (a rabbit polyclonal antibody raised against *E.coli* SmpB) and IR800 dye conjugated α-rabbit secondary antibody (Rockland, Gilbertsville, PA). Blots were scanned using the Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, NE).

For separation of ribosomal subunits, 70S ribosomes were purified via pelletting through a sucrose cushion as above. Ribosomes were then resuspended in buffer D and loaded onto a 10-40% linear sucrose gradient in buffer D. Gradients were subjected to centrifugation at 25,000 RPM for 16hr in SW28 rotor (Beckman Coulter, Fullerton,CA). Fractions were subjected to

western blotting using αSmpB polyclonal antibody [123, 156]. For analysis of tmRNA ribosome association, the RNA from 250μL of each gradient fraction was extracted with 750 μL of Tri Reagent LS (MRC, Cincinnatti, OH). RNA samples were run on a 1.5% formamide-formaldehyde denaturing agarose gel and northern blots were developed using a biotinylated full-length tmRNA dsDNA probe and a Biotin detection kit (Roche Diagnostics, Indianapolis, IN).

For *in vitro* SmpB ribosome binding assays, I first assembled fM pre-*trans*-translation complexes in buffer A. The reaction mix contatained 5μM fMet-$tRNA^{fMet}$, 3μM 70S ribosomes, 3μM IF-1, 3μM IF-2, 3μM IF-3, 1mM GTP and 8μM fM mRNA. Reactions were incubated at 37°C for 45 minutes, and then used directly for ribosome binding assays. tmRNA charging and SmpB ribosome binding reactions were performed in one step. This reaction was performed in buffer B and contained 750nM pre-*trans*-translation complexes, 750nM SmpB, 750nM EF-Tu, 750nM EF-Ts, 1μM Ala-RS, 2mM ATP, 250μM GTP, 1mM L-alanine, 750nM tmRNA, and 75μM total *E. coli* tRNA. These reactions (200μL) were incubated at 37°C for 10 minutes. To separate ribosomes from free reaction components, ribosomes were pelleted through 500 μL of a 10% sucrose cushion in buffer C (41,000RPM for 16hr at 4°C in a TLA100.3 rotor (Beckman Coulter, Fullerton, CA)). Ribosomes were resuspended in buffer B, normalized based on A260, and run on 15% tris-tricine PAGE. Westerns were developed using a polyclonal antibody directed against SmpB.

2.3.4 SmpB cell concentration measurements

W3110 cells were grown to mid-log phase, harvested, resuspended in buffer G, and lysed by sonication. Insoluble material was removed by centrifugation. The supernatant fraction, along with a titration of either purified SmpB or ribosomal protein S12, was subjected to western blot analysis using a polyclonal antibody directed against either SmpB or S12. The relationship between Western Blot signal and protein concentration was fit to a linear regression using Microsoft Excel software, and the concentrations of SmpB and S12 were solved based on the equation of the linear fit.

2.3.5 Stalled Ribosome Enrichment

Reporter mRNAs were overexpressed in either W3110 or W3110 *ssrA*::CAT cells from either pλ-cI-N-AGG or pλ-cI-NS constructs by growing cells (1.5L) to OD_{600}=1.0 and inducing with 1mM IPTG for 45min. Cells were harvested and resuspended in 15mL buffer F. Cells were lysed by French Press and ribosomes were purified by pelletting through a 32% sucrose cushion in buffer F as described above. Ribosomes were resuspended in 5mL of buffer F, to which 200μL of Ni^{2+}-NTA agarose resin (Qiagen, Valencia, CA) was added and incubated for 2hr at 4°C. Unbound material was allowed to flow through by gravity flow and the resin was washed 4 times with 1mL of buffer F. Stalled

ribosomes were eluted with 250μL of buffer F plus 200mM imidazol. Ribosome concentrations from starting material and eluted fractions were normalized based on A_{260} and subjected to Western blotting using αSmpB antibody.

2.4 RESULTS

2.4.1 Interaction of free SmpB with ribosomes is salt sensitive *in vivo*

I was interested in gaining detailed mechanistic insight into interactions of SmpB protein with tmRNA and stalled ribosomes. It has been recently proposed that free SmpB, not associated with tmRNA, recognizes stalled ribosomes to recruit tmRNA [137]. However, the evidence for interaction of free SmpB with ribosomes is based mainly on low stringency *in vitro* experiments or *in vivo* studies involving SmpB and/or tmRNA overexpression. I, therefore, set out to determine whether free SmpB interacts with ribosomes *in vivo*, under normal physiological concentrations of all the interacting partners. To this end, I utilized a previously reported high stringency ribosome isolation protocol [156] followed by Western blot analysis (using an SmpB specific antibody) to determine the amount of SmpB protein that co-purifies with ribosomes. To my surprise, SmpB interaction with ribosomes was only observed in wild type cells (Fig. 2.1, lanes 2, 4). Interaction of SmpB with ribosomes under these conditions required the presence of tmRNA in cells, as this interaction was completely absent in *ssrA*$^-$ cells. This result disagreed with previous reports regarding SmpB•ribosome interactions [128, 131, 133, 137, 161]. I surmised that the discrepancy might be due to the different stringency conditions used for ribosome isolation in these studies as compared to mine. Thus, I repeated the experiment using lower stringency conditions (reducing the salt concentration from 300mM to 100mM NH_4Cl). Under these low stringency conditions, interaction of free SmpB with ribosomes was observed (Fig. 2.1, lanes 1, 3), albeit the number of SmpB molecules per ribosome was only around 40% of that value in the otherwise isogenic wild type cells. This reduction in SmpB levels may be due in part to decreased stability of SmpB in the absence of tmRNA. Reduced SmpB levels in *ssrA*$^-$ cells, however, cannot account for the differences in salt sensitivity observed in the presence or absence of SmpB. These results suggest that interaction of SmpB with ribosomes in the absence of tmRNA is incomplete, labile and salt sensitive, observed only under low stringency conditions.

Next, I further characterized the observed salt sensitivity of this interaction. I purified ribosomes from *ssrA*$^-$ and wild type cells using a range of salt concentrations (100, 150, 200, 250, and 300mM NH_4Cl). With ribosomes isolated from *ssrA*$^-$ cells, association of free SmpB with ribosomes began to decrease at 150mM NH_4Cl and was barely detectable at NH_4Cl concentrations of 200mM or higher (Fig. 2.2, lanes 3-7). In contrast, in the presence of tmRNA, I observed little difference in SmpB ribosome association between 100 and 300mM salt

concentrations (Fig. 2.2, lanes 1, 2). Therefore, the salt sensitivity of free SmpB-ribosome interaction is so severe that SmpB binding is disrupted at moderate ionic strength conditions (Less than 200mM salt), even below physiological ionic strength [162, 163]. These findings suggest that the observed SmpB•ribosome interaction under low salt is an artifact of low stringency purification conditions, arguing against the possibility that free SmpB could bind ribosomes *in vivo*.

2.4.2 Free SmpB•ribosome interactions are salt sensitive *in vitro*

The results of the *in vivo* ribosome binding assays described above suggest that interactions of SmpB with ribosomes in the absence of tmRNA might be non-specific and physiologically irrelevant. However, those data reflect the binding capacity of free SmpB for the total cellular ribosome pool, only a fraction of which is stalled, or a substrate for SmpB•tmRNA mediated tagging. Hence, I examined the salt sensitivity of free SmpB interactions with stalled ribosomes *in vitro*. To this end, I programmed ribosomes on a nonstop mRNA (fM-mRNA, containing a 5-UTR, a ribosome binding site, and an initiation codon). These ribosomes contain the AUG initiation codon bound to fMet-tRNA^{fMet} in the ribosomal P-site and an empty A-site, lacking both tRNA and mRNA. The absence of an A-site mRNA codon renders these *in vitro* stalled ribosome complexes an ideal substrate for *trans*-translation. I incubated these stalled ribosomes with SmpB, EF-Tu, GTP, ATP, and Alanyl-tRNA Synthetase (Ala-RS) with or without tmRNA, and added a 100 fold molar excess of total *E. coli* tRNA as a non-specific competitor. To evaluate the stability of these ribosome complexes, I performed the binding reactions in the presence of either 100, 200, or 300mM NH_4Cl. Ribosome complexes formed in these reactions were separated from free components by pelleting through a 10% sucrose cushion, and the amount of SmpB that co-purified with stalled ribosomes was measured by Western blot analysis using an SmpB specific antibody. The results are summarized in figure 2.3.

In the presence of tmRNA, I observed a small, stepwise decrease in SmpB ribosome binding activity from 100 to 300mM salt. 300mM NH_4Cl resulted in only a small (15-20%) reduction in SmpB binding to stalled ribosomes (Fig. 2.3), suggesting that the vast majority of SmpB-tmRNA-stalled-ribosome complexes generated under these conditions are stable and resistant to the effects of high salt. Increasing salt concentration had a much more dramatic effect on the ribosome binding capacity of free SmpB. 300mM salt reduced the amount of bound SmpB by 80-85% (Fig. 2.3). Thus, the results of *in vitro* SmpB-stalled ribosome binding measurements mirror those of *in vivo* SmpB-total ribosome studies. Both methods show the interaction of SmpB with ribosomes in the absence of tmRNA to be far more labile than the interaction in the presence of tmRNA. The salt sensitivity of the interaction of free SmpB with ribosomes is somewhat less pronounced *in vitro*, as compared to the observed *in vivo* salt sensitivity (Fig. 2.2 vs. Fig. 2.3). This is most likely due to the myriad of potential non-specific nucleic acid targets present *in vivo*, which are able to substantially

diminish any potential nonspecific interactions of SmpB protein with ribosomes. Therefore, one would expect that non-specific interactions would be less prevalent *in vivo* than *in vitro*

2.4.3 SmpB and tmRNA are present at a 1:1 ratio in cells

The results of both *in vivo* and *in vitro* ribosome binding studies suggested that interaction of free SmpB with ribosomes is non-specific. However, it remained formally possible that this interaction is functionally relevant, but too labile and short-lived to survive ribosome purification techniques. In order for SmpB to pre-bind the ribosome it must either: a) possess greater or comparable affinity for the ribosome than it does for tmRNA, or b) be present in cells in a molar excess over tmRNA. Moore and Sauer recently measured the ratio of the number of copies of tmRNA to the number of ribosomes, based on comparison to 5S ribosomal RNA [164]. They found a ratio of one tmRNA molecule per approximately 15 to 20 ribosomes. To gain deeper insights into the molecular mechanism of SmpB protein function, I performed quantitative western blotting to determine the ratio of SmpB to ribosomes, based on comparison to ribosomal protein S12. Figure 2.4 shows the results of one measurement. Briefly, I performed western blotting with an SmpB specific antibody from S30 extract (total cellular soluble material) of wild type cells along with a titration of purified SmpB. The same S30 extract was also blotted along with a titration of purified ribosomal protein S12. Analysis of the intensities of the SmpB and S12 bands in the S30 extract as compared to the intensities of the bands in the purified protein lanes gave the concentrations of SmpB and S12 in the S30 samples. I was then able to calculate the ratio of SmpB to S12. The results of six independent experiments gave an [SmpB]:[S12] ratio of 1:14.2+/-2.37. These data suggest a roughly 1:1 ratio of SmpB to tmRNA in *E. coli*. Similarly, a 1:1 ratio of SmpB to tmRNA has been reported in other bacterial species [13, 165].

2.4.4 SmpB partitions with tmRNA

I next set out to determine the relative affinity of SmpB for tmRNA and ribosomes. 70S ribosomes can be dissociated *in vitro* into 50S and 30S subunits by incubation in low (1 mM) Mg^{2+}. When SmpB•tmRNA•ribosome complexes are dissociated, tmRNA is released. If ribosomal subunits are then separated by density gradient centrifugation, free tmRNA fails to co-migrate with either subunit and remains in the topmost gradient fractions. I decided to utilize this phenomenon as an indicator of the relative affinities of SmpB for tmRNA and ribosomes. I purified ribosomes from wild type cells, dissociated the subunits *in vitro* and then separated the subunits using a 10-40% linear sucrose gradient. If SmpB prefers to bind to tmRNA rather than ribosomes, I would expect it to co-migrate with tmRNA in the top gradient fractions. If, in contrast, the ribosome is the preferred binding target of SmpB, then it should migrate with the ribosomal subunit on which its primary binding site resides. When I performed this separation under low stringency (100mM NH_4Cl) conditions, I found that the

majority of SmpB co-migrated with tmRNA in the early gradient fractions with a small amount of SmpB bound to either ribosomal subunit (Fig. 2.5, top panel). When higher stringency (300mM NH_4Cl) was used, the partitioning of SmpB protein with its preferred binding partner, tmRNA, was even more dramatic. In this case, nearly all of the SmpB co-migrated with tmRNA in the topmost fractions, with only traces of SmpB co-migrating with the 50S subunit (Fig. 2.5, bottom panel). These results demonstrate that tmRNA is the primary binding partner of SmpB. These results are also in agreement with previously published data regarding this phenomenon [128, 161]. Since similar numbers of tmRNA and SmpB molecules are present in cells and SmpB partitions primarily with tmRNA, it is unlikely that SmpB can pre-bind the ribosome to recruit tmRNA. I, therefore, propose that a preformed SmpB•tmRNA complex recognizes stalled ribosomes, and that the reported pre-binding of free SmpB to the ribosome and its subunits is the result of non-specific interactions of a basic RNA binding protein with a large cellular ribonucleoprotein complex under artificial conditions.

2.4.5 SmpB is enriched in stalled ribosomes *in vivo*, only in the presence of tmRNA

In order to test the hypothesis that SmpB binds stalled ribosomes only in complex with tmRNA, I set out to purify a pool of stalled ribosomes and assess whether SmpB is enriched in these ribosomes over the total ribosome pool. I expected to observe SmpB enrichment in stalled ribosomes purified from wild type cells, but not in stalled ribosomes purified from *ssrA*$^-$ cells. However, if SmpB pre-binds the ribosome in the absence of tmRNA, then SmpB should be enriched in stalled ribosomes purified from either wild type or *ssrA*$^-$ cells. To accomplish this, I expressed a reporter mRNA (λ-CI-N-4-AGG) which codes for a variant of the λ repressor N-terminal domain with an N-terminal 6His epitope and a string of 4 rare arginine codons (AGG), making it a substrate for tmRNA-mediated tagging [14]. Briefly, ribosomes stall on the rare codon-containing segment of this construct, while displaying the already translated N-terminal 6His epitope. These ribosomes may then be captured on Ni^{2+}-NTA beads. It should be noted that the product of this purification is not expected to contain only stalled ribosomes, as ribosomes might be captured at any point beyond translation of the N-terminal 6His epitope. However, as the stalled state is expected to be the most kinetically long-lived state between 6His epitope translation and peptide release, this pool of ribosomes should be greatly enriched for stalled ribosomes. Hereafter, I will refer to this stalled ribosome-enriched pool as stalled ribosomes.

I purified these stalled ribosomes from both wild type cells and *ssrA*$^-$ cells under low (100mM NH_4Cl) and high (300mM NH_4Cl) stringency conditions. Figure 2.6 depicts the results of this assay. As expected, in wild type cells, regardless of stringency, I observed around a 5-fold enrichment of SmpB in the stalled ribosome fraction versus total ribosomes. In contrast, no enrichment of SmpB was observed in *ssrA*$^-$ cells under low stringency conditions (Fig. 2.6A). In

ssrA$^-$ cells under high stringency conditions, no SmpB ribosome binding was observed in either the total or stalled ribosome fractions, consistent with the results of *in vivo* ribosome association assays described above (Fig. 2.6B). These results lend further support to the conclusion that functional SmpB•stalled ribosome interactions require the presence of tmRNA.

I next repeated this assay using a different *trans*-translation substrate, a non-stop mRNA reporter. I replaced the rare (AGG) arginine codons with abundant (CGG) codons and inserted a strong trpA transcriptional terminator at the 3'-end. This new reporter (λ-cI-NS) mRNA still codes for the N-terminal 6His epitope but lacks an in-frame termination codon. The results of stalled ribosome enrichment experiments with this construct were quite similar to those with the rare codon-containing construct. I observed an approximately 8-fold enrichment of SmpB in stalled ribosomes from wild type cells (Fig. 2.7). In *ssrA*$^-$ cells, I saw only an insignificant enrichment under low stringency, and no SmpB-ribosome association in either total ribosomes, or the enriched ribosome pool under high stringency conditions (Fig. 2.7).

I have, thus, demonstrated that interaction of free SmpB protein with ribosomes is salt sensitive. Further, I've shown that SmpB is present at a similar intracellular concentration relative to tmRNA, and that SmpB partitions with tmRNA upon ribosomal subunit dissociation. Finally, I have demonstrated that SmpB is enriched in stalled ribosome fractions only in the presence of tmRNA. Taken together, these results demonstrate that tmRNA is required for specific and functional interactions of SmpB with stalled ribosomes. Based on these findings, it is clear that SmpB does not pre-bind the ribosome *in vivo*, and that SmpB•tmRNA binding occurs prior to stalled ribosome recognition to initiate *trans*-translation.

2.5 DISCUSSION

SmpB is required for tmRNA-mediated recognition and rescue of stalled ribosomes. Despite extensive biochemical and structural studies, the mechanism by which SmpB facilitates the productive engagement of tmRNA with stalled ribosomes has remained a matter of debate. Two competing models have emerged in recent years. The first model suggests that a preformed complex of SmpB•tmRNA•EF-Tu(GTP) recognizes stalled ribosomes to initiate *trans*-translation. This model is supported by numerous genetic, biochemical and structural studies that demonstrate that tmRNA is the specific high-affinity binding partner of SmpB [1, 12, 16, 70, 81, 87, 123, 129, 156]. This SmpB•tmRNA interaction is illustrated in two recently solved co-crystal structure models [70, 81]. Amino acid residues required for high affinity tmRNA binding are clustered on a unique surface of the protein. Mutations that alter these amino acids disrupt SmpB-tmRNA interactions and prevent ribosome rescue and peptide tagging [123]. Moreover, it has been shown that SmpB can bind both the alanine-charged and

uncharged forms of tmRNA and that binding of SmpB enhances the aminoacylation of tmRNA by ala-RS [126, 136, 155]. SmpB and EF-Tu (GTP) can bind simultaneously to the tRNA-like domain of tmRNA and protect the labile ester linkage from hydrolysis [126].

Studies in *E. coli* and *C. crescentus* have shown that the intracellular levels of tmRNA and SmpB are reduced when one of the binding partners is missing [13, 137, 165, 166]. Most strikingly, in *C. crescentus*, the levels and stability of SmpB and tmRNA are under cell cycle control [165]. SmpB is reported to protect tmRNA from degradation by RNase R. This study also demonstrates that SmpB binds with high affinity to tmRNA and that this direct binding is responsible for its protection from degradation by RNase R. Taken together, these finding are consistent with a model wherein the complex of SmpB-tmRNA forms first, is aminoacylated by AlaRS, bound by EF-Tu(GTP) and subsequently recognizes and binds stalled ribosomes. Direct binding of SmpB to tmRNA and its subsequent aminoacylation and delivery to ribosomes would thus protect a large fraction of tmRNA from cellular nucleases, and a large fraction of SmpB protein from proteases. In contrast, if SmpB binds stalled ribosomes first, as proposed by the pre-binding model described below, then its intracellular concentration should not be affected by the presence or absence of tmRNA, as the bulk of SmpB would be bound by ribosomes and inaccessible to cellular proteases.

The second model suggests that SmpB might pre-bind stalled ribosome to recruit tmRNA and initiate *trans*-translation [131, 133, 137, 161]. Two lines of observations led to this conclusion. The first observation was that SmpB co-purifies with 70S ribosomes or the dissociated 50S and 30S subunits in an *ssrA*$^-$ strain [137]. Careful examination of the data presented in this report, however, reveals that the SmpB-ribosome preparations were generated under low salt conditions (60-100mM NH_4Cl), and as such agrees with my data depicted in figures 2.1 and 2.2. The question, then, is whether this free SmpB-ribosome interaction, observed only under low stringency conditions, is specific and functionally relevant. I reason that this interaction is likely due to the non-specific binding of a highly basic RNA binding protein, in the absence of its canonical RNA binding partner, to a highly abundant large ribonucleoprotein complex, the ribosome. The labile nature and salt sensitivity of this interaction (Figs. 2.1, 2.2, and 2.3) is consistent with the pre-binding interaction being of a nonspecific nature. In agreement with this notion, two related studies [128, 161], aimed at assessing the interactions of free SmpB with ribosomes, demonstrated that when ribosomal subunits are dissociated, the vast majority of SmpB co-localizes with tmRNA and not with ribosomal subunits, even under low stringency conditions. This result, although not interpreted as such, agrees with my finding that tmRNA, and not the ribosome, is the preferred binding partner of tmRNA. Furthermore, if SmpB can pre-bind stalled ribosomes then it should be enriched in stalled ribosome fractions, irrespective of the presence or absence of tmRNA. My experiments addressing this issue clearly show that this is not the case. SmpB is enriched in stalled ribosome fractions only when tmRNA is present, and not in its absence (Figs. 2.6 and 2.7).

The second observation that led to the SmpB-ribosome pre-binding hypothesis was that an *in vitro* generated SmpB ribosome complex could recruit tmRNA to drive peptide bond formation *in vitro* [137]. For this experiment, the authors measured the extent of peptide bond formation at 10 second, 5 minute, and 15 minute time points. Peptide bond formation occurs on a time scale of milliseconds. Therefore, it is entirely conceivable that SmpB could recycle from ribosome to tmRNA, in the time scale of this experiment, to generate the SmpB•tmRNA•EF-Tu(GTP) quaternary complex required for participation in the transpeptidation reaction. A further point of uncertainty associated with the pre-binding model is the suggestion that a pre-formed SmpB•tmRNA•EF-Tu (GTP) complex might still be needed to deliver tmRNA to the pre-bound SmpB-ribosome complex [135, 137]. This phenomenon is presented as evidence for the requirement of two SmpB molecules to elicit *trans*-translation. The fact that SmpB partitions primarily with tmRNA and that there are equal numbers of SmpB and tmRNA molecules present per cell is inconsistent with this proposal and can't be reconciled with the multiple SmpB requirement of this model (see below).

Finally, a cryo-EM reconstruction of a free SmpB-ribosome complex has recently been modeled [161]. Low stringency conditions and a large molar excess of SmpB over ribosomes were used to generate these complexes. The density assigned to SmpB was located near the decoding center of the 30S subunit. This positioning of SmpB is provocative, and may represent one of the binding sites of SmpB in the SmpB•tmRNA•EF-Tu(GTP) complex. Interestingly, this SmpB binding site on a stalled ribosome was identified by comparing the differences between cryo-EM maps generated from SmpB-ribosome complexes containing either a nonstop mRNA or a longer mRNA. Therefore, the 30S-decoding center is the only site on the ribosome where a difference in density would be expected.

The results presented in this chapter are consistent with the first model of stalled ribosome recognition and rule out SmpB ribosome pre-binding as a physiologically relevant phenomenon. I have shown that interaction of free SmpB with ribosomes occurs only under artificial low stringency conditions. In general, several previous studies have looked at interaction of free SmpB with ribosomes but failed to identify its labile and non-specific nature. This highlights the importance of choosing appropriate stringency conditions and the use of competitors to disrupt non-specific interactions when studying binding *in vitro*. This consideration is particularly critical when looking at interaction of a basic protein with RNA, where charge interactions with the RNA backbone can drive binding to essentially any nucleic acid.

The results of this study also shed new light on the related issue of stoichiometry of the SmpB•tmRNA•stalled-ribosome complex. I have shown that free SmpB does not specifically bind the ribosome. Further, I have demonstrated that the intracellular concentrations of SmpB and tmRNA are similar. This strongly suggests that the stoichiometry of the SmpB•tmRNA complex is 1:1. The primary SmpB binding site on tmRNA has been conclusively identified [1, 12, 16, 70, 81, 87, 123, 129, 156]. Since the intracellular concentrations of SmpB and tmRNA are

similar, the 1:1 complex, with SmpB bound to the primary high-affinity binding site, would be favored at equilibrium. This conclusion is consistent with recent analyses of the interaction between tmRNA and SmpB, from *Thermus thermophilus* and *Aquifex aeolicus* [70, 81, 129], which found only one SmpB contacting the D-loop equivalent of tmRNA. Furthermore two co-crystal structure models of SmpB in complex with the tmRNA TLD predict a 1:1 SmpB:tmRNA stoichiometry [70, 81]. Initial cryo-EM derived models of the complex formed by SmpB-tmRNA-EF-Tu-GDP-Kirromycin and 70S ribosomes suggested the presence of a single molecule of SmpB protein in the pre-accommodated complex [134]. Similarly, analysis of affinity purified tmRNA•ribosome complexes also suggested the presence of only one SmpB molecule at a late stage of the *trans*-translation process [167].

Another cryo-EM reconstruction of SmpB-tmRNA-EF-Tu-GDP-Kirromycin in complex with a stalled ribosome suggested the presence of two molecules of SmpB, one in the decoding center and the other in the A-site compartment of 50S subunit [135]. As a consequence of the inclusion of the second SmpB molecule, the tRNA-like domain of tmRNA is now oriented towards the tmRNA ORF [135]. It is difficult to visualize how tmRNA could participate in peptide bond formation with its tRNA-like domain facing the ORF rather than the peptidyl-transferase center. This second SmpB molecule will undoubtedly interfere with peptide bond formation. Equally puzzling is the suggestion that both molecules of SmpB interact with two dissimilar parts of the tRNA-like domain of tmRNA, presumably using the same RNA binding surface. It is not clear from these studies how the same SmpB RNA binding residues could specifically interact with two distinct tmRNA sequence and structure elements.

Under normal physiological conditions, stalled ribosomes constitute only a small fraction of the total cellular pool of ribosomes (15,000-20,000 total ribosomes per cell). Normal translating ribosomes are present in vast excess (10 to 20-fold) over SmpB and tmRNA [164]**.** I reason that pre-binding of SmpB to ribosomes is highly unlikely, as most SmpB would be sequestered and unavailable to support tmRNA recruitment. In contrast, if pre-formed quaternary complexes of SmpB•tmRNA•EF-Tu(GTP) were responsible for recognizing stalled ribosomes then this surveillance system would be ideally primed to engage and rescue stalled ribosomes. In conclusion, I have demonstrated that specific and functional SmpB-ribosome binding requires tmRNA. My results rule out the possibility of SmpB pre-binding of ribosomes to recruit tmRNA and initiate *trans*-translation. My results also suggest that the stoichiometry of the SmpB•tmRNA•stalled ribosome complex is 1:1:1. I, therefore, propose that ribosome rescue is initiated by a preformed SmpB•tmRNA•EF-Tu (GTP) complex that recognizes and binds the A-site of stalled ribosomes to commence *trans*-translation.

2.6 Figures

Figure 2.1

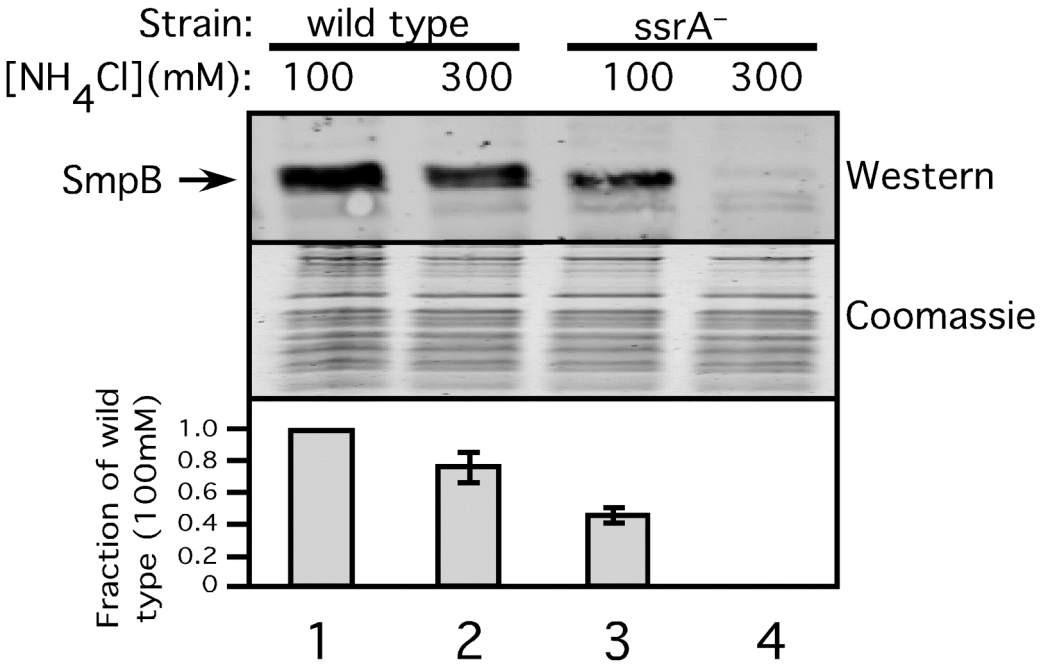

Figure 2.1: *SmpB ribosome binding in the presence and absence of tmRNA*. Ribosomes were purified using the indicated NH_4Cl concentrations in the purification buffer and subjected to Western blotting using an SmpB specific antibody. Coomassie staining of total ribosomal proteins present in each ribosome preparation is shown as a loading control. Graph represents mean +/- standard deviation (3 independent experiments) of the Western signal relative to lane 1.

Figure 2.2

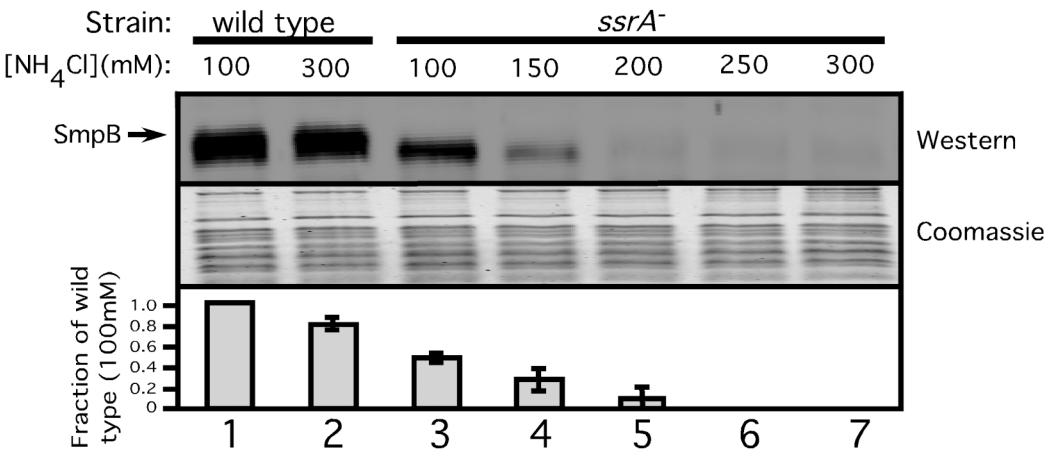

Figure 2.2 *SmpB ribosome binding in the presence and absence of tmRNA*. Ribosomes were purified using the indicated NH_4Cl concentrations in the purification buffer and subjected to Western blotting using an SmpB specific antibody. Coomassie staining of total ribosomal proteins present in each ribosome preparation is shown as a loading control. Graph represents mean +/- standard deviation (3 independent experiments) of the Western signal relative to lane 1.

Figure 2.3

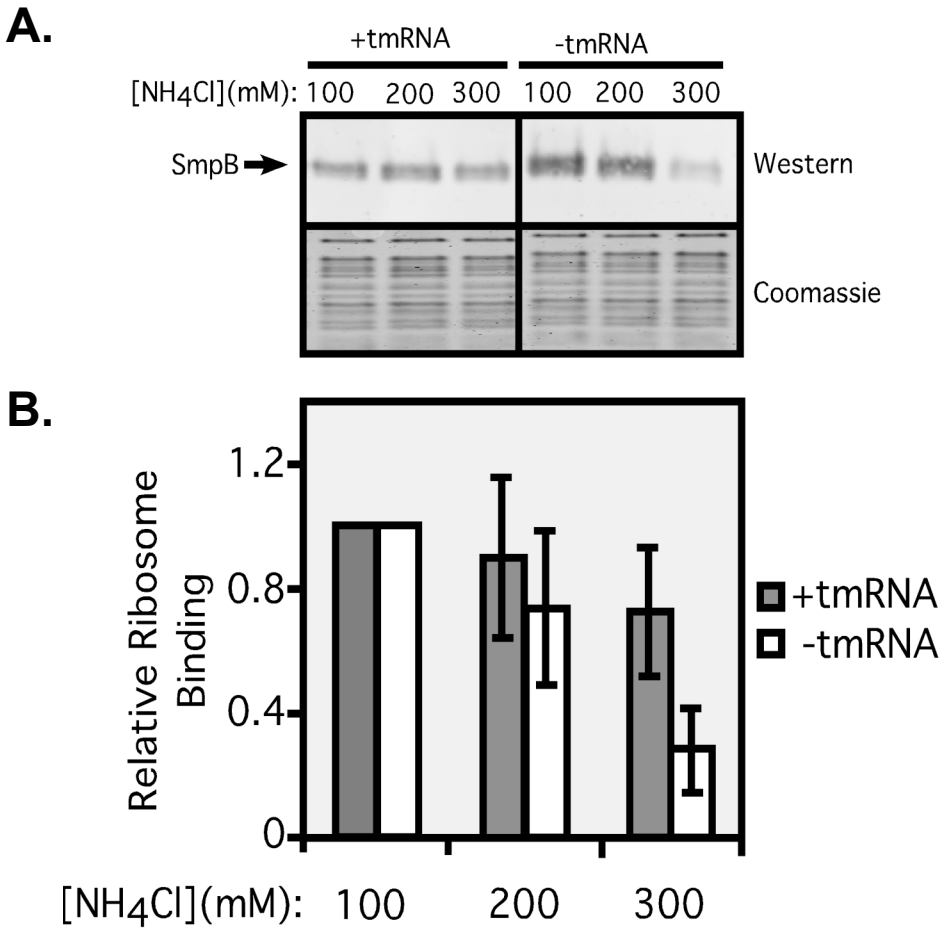

Figure 2.3 *In vitro analysis of the salt sensitivity of SmpB-ribosome binding.* **A.** SmpB-stalled ribosome complexes were generated *in vitro* and subjected to Western analysis using an SmpB specific antibody. Coomassie staining of total ribosomal proteins was used as a loading control. **B.** Graph represents mean +/- standard deviation (four independent experiments) of the Western signal relative to lane 1.

Figure 2.4

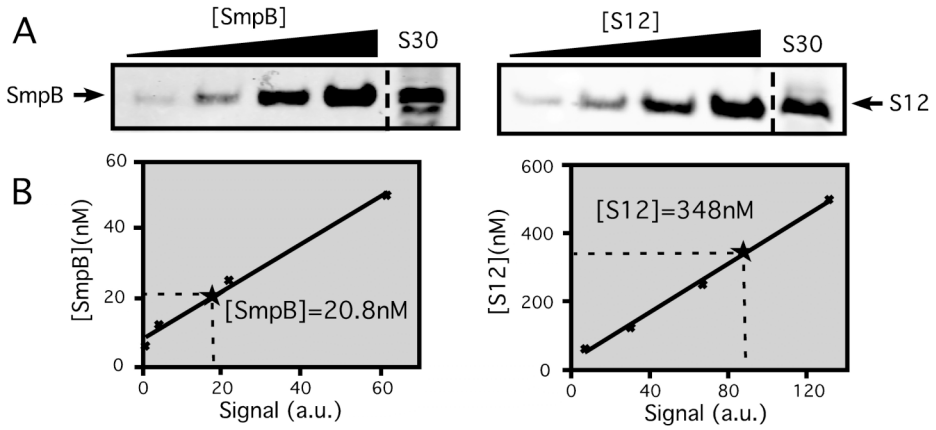

Figure 2.4: *SmpB:ribosome ratio measurements.* Panel A depicts quantitative western blots measuring the concentrations of SmpB and ribosomal protein S12 in S30 extracts from W3110 cells. S30 extracts, along with titrations of either purified SmpB or purified ribosomal protein S12 were subjected to Western blotting with antibodies directed against either SmpB or S12. Panel B shows curve fit of the Western blot signals (in arbitrary units) versus concentration of either SmpB or S12 standards. Asterisks indicate the concentrations of SmpB or S12 in S30. The summary of data from six independent experiments gave an SmpB:S12 ratio of 1:14.2+/-2.4.

Figure 2.5

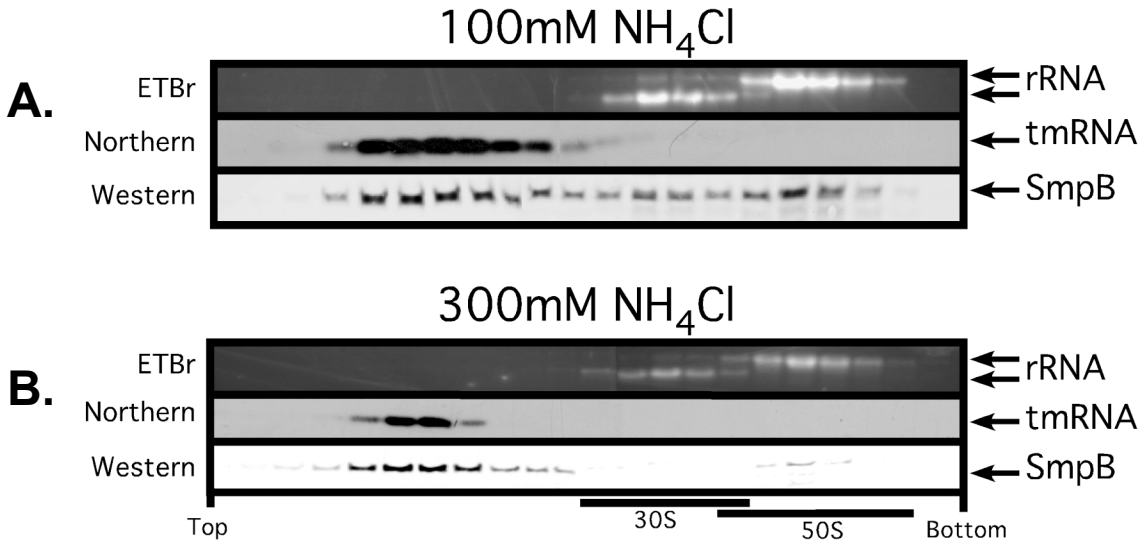

Figure 2.5 *Ribosomal subunit dissociation assay.* Ribosomal subunits were dissociated *in vitro* and subjected to density gradient centrifugation. The RNAs present in gradient fractions were separated electrophoretically and subjected to Ethidium bromide staining (top panels) and Northern blotting using a tmRNA specific probe (middle panels). The same gradient fractions were subjected to Western analysis using an SmpB specific antibody. Subunit dissociation assays were performed with either 100mM **(A)** or 300mM **(B)** NH_4Cl.

Figure 2.6

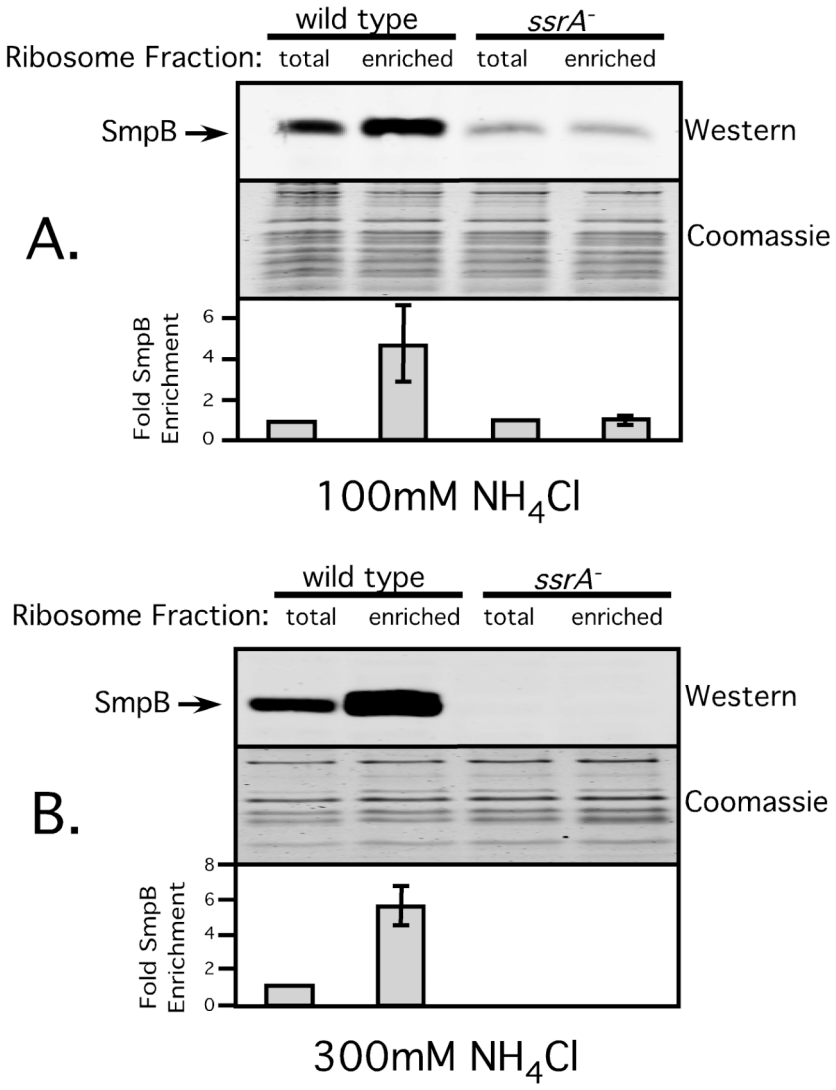

Figure 2.6 *Enrichment of SmpB in stalled ribosomes*. Stalled ribosome fractions were purified from wild type or *ssrA*$^-$ cells. Proteins in enriched and total ribosome fractions were resolved electrophoretically, stained with coomassie blue (middle panels), and subjected to western analysis using an SmpB specific antibody (top panels). Bar graphs (bottom panels) represent the mean +/- standard deviation (three independent experiments) of the fold enrichment of SmpB in stalled ribosomes over total ribosomes. Experiments were performed with either 100mM **(A.)** or 300mM **(B.)** NH_4Cl.

Figure 2.7

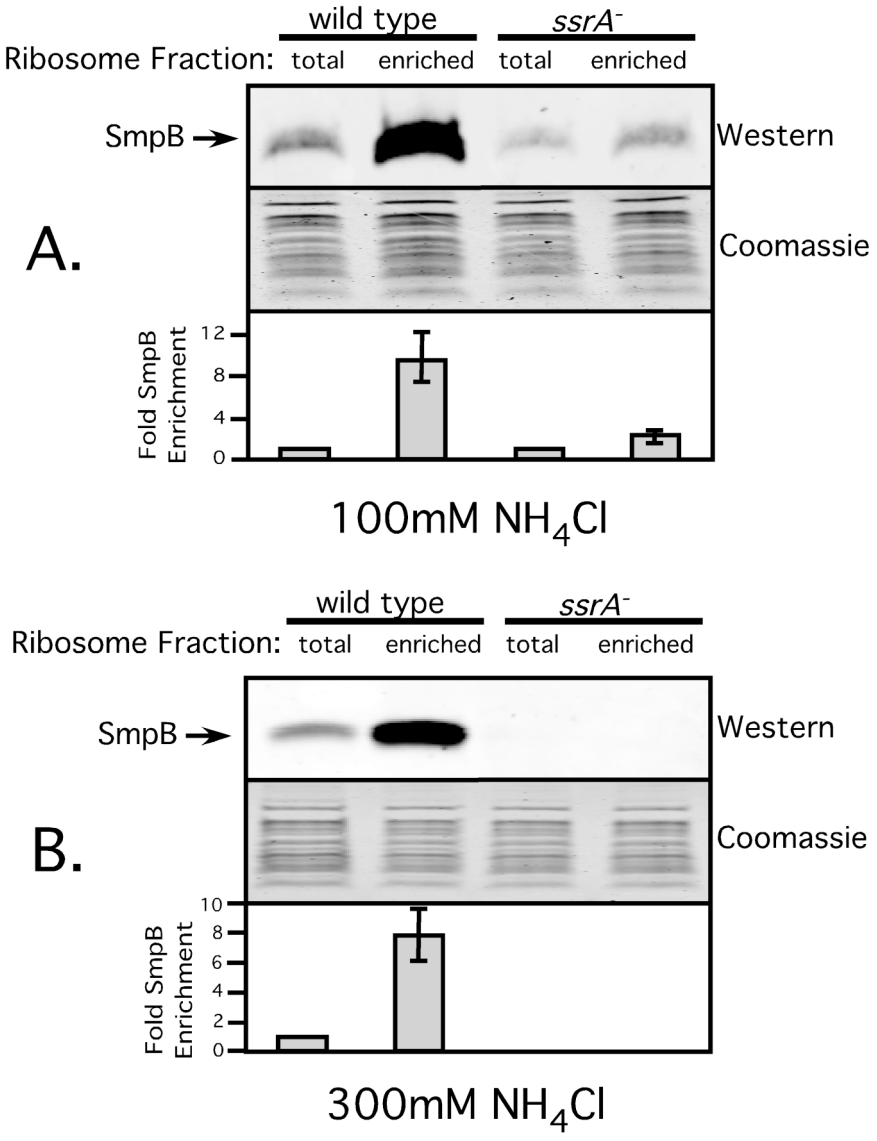

Figure 2.7 *Enrichment of SmpB in ribosomes stalled on a non-stop message*. Stalled ribosome fractions were purified from wild type or *ssrA*$^-$ cells. Proteins in enriched and total ribosome fractions were resolved electrophoretically, stained with coomassie blue (middle panels), and subjected to western analysis using an SmpB specific antibody (top panels). Bar graphs (bottom panels) represent the mean +/- standard deviation (three independent experiments) of the fold enrichment of SmpB in stalled ribosomes over total ribosomes. Experiments were performed with either 100mM **(A.)** or 300mM **(B.)** NH_4Cl.

Chapter 3: *In vivo* analysis of the role of the SmpB C-terminal tail in *trans*-translation

3.1 Summary

SmpB is required for all known activities of tmRNA. The two known functions of SmpB are binding tmRNA and promoting stable association of the SmpB•tmRNA complex with 70S ribosomes. Using mutational analysis and biochemical experiments, I discovered a novel SmpB function. This function is required for a step in the tagging process downstream of tmRNA binding and ribosome association, but prior to transpeptidation onto tmRNA and establishment of the tmRNA reading frame. My results demonstrate that residues in the C-terminal tail of SmpB confer a hitherto unrevealed function that is essential for *trans*-translation. Based on these results I propose that upon binding stalled ribosomes the unstructured C-terminal tail of SmpB acquires contacts that are critical for productive accommodation of tmRNA into the ribosomal A-site.

3.2 Introduction

Small protein B (SmpB) is essential for all known tmRNA functions; however, its exact mechanistic function in *trans*-translation is unclear. The two known functions of SmpB are specific binding to tmRNA and promoting its stable association with 70S ribosomes [4, 16, 127]. Although not strictly required, SmpB may also stabilize tmRNA and promote its efficient charging by alanine tRNA synthetase [15, 127]. Recently, a great deal of structural information regarding SmpB and tmRNA has become available. NMR solution structures of SmpB from *Aquifex aeolicus* [88] and *Thermus thermophilus* [89] along with two co-crystal structures of SmpB in complex with the tRNA-like domain of tmRNA [70, 81] have been solved. The core SmpB structure is quite similar in all four structural models; however, none of the models were able to discern the structure of the protein's C-terminal extension. Thus, the C-terminal tail of SmpB appears to be unstructured.

In this study, I directly tested the functional relevance of the C-terminal tail of SmpB protein. I identified residues in the C-terminal tail of SmpB that are critical in supporting tmRNA tagging activity. Interestingly, mutations in these residues do not affect the ability of SmpB to bind tmRNA *in vivo* or *in vitro*, nor do they

affect its ability to support stable association of tmRNA with stalled ribosomes. I propose that the SmpB C-terminal extension gains structure within the context of the ribosome and acquires contacts that are required to support a previously unidentified function of SmpB protein. This novel function is crucial for events that are downstream of ribosome association but prior to transpeptidation and establishment of the tmRNA-encoded reading frame.

3.3 Experimental Procedures

3.3.1 Nomenclature

SmpB truncation mutants are named using the number of the C-terminal residue of the protein. For example, $SmpB^{155}$ includes amino acids 1-155 of the wild type SmpB protein. $SmpB^{DE}$ is I154D/M155E, $SmpB^{RK}$ is I154R/M155K, $SmpB^{AA}$ is I154A/M155A, $SmpB^{LI}$ is I154L/M155I, and $SmpB^{QQ}$ is I154Q/M155Q.

3.3.2 Plasmids, Strains and Phage

The generation of strains W3110ΔsmpB1, W3110ΔsmpB1(DE3), and W3110 *ssrA*::CAT, and the plasmids pKW24, pPW500 and pETBA have been described previously [4]. The plasmid pTC9, for overexpression of terminal tRNA nucleotidyl transferase, was a kind gift from Dr. Charles McHenry (UCHSC). The $ssrA^{H6}$ gene was amplified by PCR from pKW24. This PCR product was used as mutagenic primer to convert pETBA to $pETBA^{H6}$. The λ-N trpAT construct was amplified by PCR from pPW500 with a 5' primer including an NcoI restriction site and 3' primer containing a PstI site. This PCR product was cut by both enzymes and ligated into NcoI/PstI digested pACYCDuet-1 plasmid (Novagen) to generate pACYCDuet-λ. SmpB mutations were generated using the QuickChange method (Stratagene) to alter the SmpB coding sequences of pETBA and $pETBA^{H6}$. All mutants were confirmed by sequencing of the smpB gene in each one of the corresponding plasmids. λ-immP22 dis c2–5 hybrid phage induction assays were performed as described [4]. The number of plaques formed when $SmpB^{WT}$ was expressed was taken as efficiency of plating (EOP) of 1.

3.3.3 Protein Purification

$SmpB^{WT}$, $SmpB^{DE}$, and $SmpB^{153}$ were overexpressed from ΔB (DE3)/pLysS/$pETBA^{H6}$ cells using 1mM IPTG induction. Cells were collected and resuspended in lysis buffer I [50mM Tris (pH7.5), 1M NH_4Cl, 10mM $MgCl_2$, 10mM Imidazole, and 2mM β-mercaptoethanol]. Cells were lysed by sonication and cell debris was removed by centrifugation. SmpB was purified from the supernatant using affinity chromatography over Ni-NTA beads (Qiagen). The Ni-NTA eluted protein was further purified by ion exchange chromatography on a MonoS (HR 10/10) FPLC column (Amersham) using a linear salt gradient. Buffer IA

contained 50mM Tris (pH8), 50mM NH_4Cl, 5mM $MgCl_2$, and 2mM β-mercaptoethanol, and Buffer IB contained 50mM Tris (pH8), 1.0 M NH_4Cl, 5mM $MgCl_2$, and 2mM β-mercaptoethanol. Purification products were analyzed by electrophoresis, flash frozen in liquid nitrogen and stored at -80°C.

Terminal tRNA nucleotidyl transferase was overexpressed from DH5α/pTC9 cells using 1mM IPTG induction for 3hrs. Cells were harvested and resuspended in 30mL of lysis buffer II containing 50mM Tris (pH 7.5), 150mM KCl, 10 mM $MgCl_2$, and 2mM βMercaptoethanol. Cells were lysed by sonication and cell debris was removed by centrifugation. Solid Ammonium Sulfate was added to the lysate to a final concentration of 30% (w/v) and the solution was stirred at 4°C for 1hr. The sample was then centrifuged at 30,000xG for 30 min. The Ammonium Sulfate concentration in the Supernatant was then raised to 45% and processed as before. The 45% pellet was resuspended in Buffer IIA [20mM Potassium Phosphate (pH7.4), 1mM $MgCl_2$, 1mM DTT, 0.1mM PMSF, and 5% glycerol (v/v)]. The resuspended protein sample was then loaded onto an FPLC monoQ (HR 10/10) column (Amersham), washed with buffer IIA and eluted with a linear pH gradient of 100% Buffer IIA to 100% Buffer IIB [200mM Potassium Phosphate (pH 4.5), 1mM $MgCl_2$, 1mM DTT, 0.1mM PMSF, and 5% glycerol (v/v)].

3.3.4 In Vitro Transcription and RNA Labeling

tmRNA113 was transcribed *in vitro* from PCR products. Two primers representing the 5' and 3' ends of the tmRNA113 coding sequence (with overlapping complimentary sequence) were extended by PCR using pfu Turbo DNA polymerase (Stratagene). This PCR extension product was used as template in a second PCR reaction with 5' primer designed to add a T7 promoter sequence immediately 5' of the first transcribed base. This PCR product was gel purified and used as template for in vitro transcription using T7 RNA Polymerase (USB). Transcription reactions were treated with DNaseI (Amersham) to digest template DNA, and RNA was purified on a 12% denaturing polyacrylamide gel (SequaGel, National Diagnostics). tmRNA113 RNA was labeled at its 3'end using purified terminal tRNA nucleotidyl transferase. The labeling reaction (100uL) contained 50mM Glycine (pH9), 10mM $MgCl_2$, 1mM $NaPP_i$, approximately 100pmoles of tmRNA113, 10mM α^{32}P ATP (3000 Ci/mmol), and 1μg of purified terminal tRNA nucleotidyl transferase. Reactions were incubated at 37°C for 15min, then 5 units of yeast pyrophosphatase was added and reactions were incubated at 37°C for an additional 1min, before reactions were quenched using TriReagent LS (MRC).

The probe used for Northern Blots was biotinylated full-length tmRNA dsDNA. The tmRNA gene was amplified from pETBA by PCR and tmRNA dsDNA was gel purified and biotinylated using a psoralen biotin labeling kit (Ambion).

3.3.5 Endogenous tmRNA^{H6} Tagging Assays

The endogenous tagging assay protocol was modified from Karzai et al. [4]. 50mL cultures of either *ssrA*::CAT, or Δ*smpB*1(DE3) with pETBA^{H6} were grown to late log phase (when Δ*smpB*1(DE3) cells were used, they were continuously induced with 10μM IPTG). Cells were harvested and resuspended in 1mL of lysis buffer III [8M Urea, 100mM Potassium Phosphate (pH8), 10mM Tris (pH8), and 5mM β-Mercaptoethanol], and lysed by rocking at room temperature for 1 hr. Cell debris was collected by centrifugation and the supernatant was added to 100μL of Ni-NTA agarose resin (Qiagen). Resin binding was allowed to proceed for 1 hour at RT. Resin-sample slurries were then applied to a miniature chromatography column and allowed to flow through by gravity flow. The resin was washed 4 times with 1mL of lysis buffer III and his-tagged proteins were eluted in 200mL of an elution buffer containing: 8M urea, 100mM Acetic acid, and 20mM β-Mercaptoethanol. Samples were resolved on a 15% Tris-Tricine gel and western blots were developed using either αHis6-HRP (Santa Cruz) or IR800-conjugated αHis6 (Rockland) antibodies. Total level of endogenous tagging activity was quantified from western blots using the IR dye conjugated antibody and the Odyssey Infrared Imaging System and data analysis software (LI-COR).

3.3.6 Gel Mobility Shift Assays

3'-end Labeled tmRNA[113] (~100pM) was incubated with varying concentrations of purified SmpB variants in a gel shift buffer containing 50mM Tris (pH7.5), 10 mM $MgCl_2$, 300mM KCl, 2mM β-Mercaptoethanol, 100mg/mL BSA, 0.01% NP-40 (v/v), 5% Glycerol (v/v), and 200nM total *E. coli* tRNA. Reactions were incubated for 60 min. at 4ºC then loaded onto 12% native polyacrylamide gels (run in 1/2X TBE (pH7.5)). Gels were then dried and exposed to either X-ray film or phosphoimager screens. Phosphoimager data was collected using a Storm840 phosphorimager and analyzed with ImageQuant Tools (Molecular Diagnostics). The data analysis was performed according to Berggrun and Sauer [168]. Briefly, we measured the fraction of the primary bound species at each SmpB concentration and determined the apparent equilibrium dissociation constant by curve fitting using the equation: $\Theta_{eq} = C/(1+K_d/[SmpB]_i)$. Where Θ_{eq} is the fraction of RNA bound at equilibrium, C is a constant representing the maximum fraction bound of the specific bound species, and $[SmpB]_i$ is the initial concentration of SmpB. Note that $C < 1$ because of the occurrence of multiple bound species at SmpB concentrations where the fraction free is greater than zero.

3.3.7 Ribosome Association Assays

Ribosomes were purified from ΔB(DE3)/pETBA or pETBA^{H6} as previously described, with modifications [160]. 750mL cultures were grown to OD_{600} ~ 1.0 under continuous low-level induction (1μM IPTG). Cells were harvested and resuspended in 4mL of buffer IIIA [10mM Tris (pH7.5), 100mM Ammonium

Acetate, 10mM Magnesium Acetate, 6mM β-Mercaptoethanol, and 100units/mL RNase inhibitor (Porcine, Amersham)]. Cells were lysed by sonication, and the lysate was centrifuged at 30,000XG for 1hr. The S30 supernatant was then centrifuged at 100,000XG for 1hour to isolate crude ribosomes. Crude ribosome pellets were then washed with 1mL of Buffer IIIB [20mM Tris (pH7.5), 800mM Ammonium Acetate, 10mM Magnesium Acetate and 6mM β-Mercaptoethanol], and resuspended in 1mL of buffer IIIA. Crude ribosomes were then layered onto 2mL of a 32% sucrose cushion containing: 32% sucrose, 20mM Tris (pH7.5), 500mM NH_4Cl, 10mM $MgCl_2$, 0.5mM EDTA, 6mM β-Mercaptoethanol, and 100 units/mL *RNase* Inhibitor. The sucrose cushions were then centrifuged at 100,000XG for 6hr. The ribosome pellets were again washed with 1mL of buffer IIIB and ribosomes were resuspended in 500μL of Buffer IIIA lacking RNase inhibitor.

For analysis of SmpB ribosome association, a fraction of this ribosome preparation was resolved on a 15% Tris-Tricine gel and western blots were developed using either α-his6-HRP (Santa Cruz) or α-SmpB (a rabbit polyclonal antibody raised against *E.coli* SmpB) and α-rabbit HRP (Santa Cruz). For analysis of tmRNA ribosome association, the RNA from 250μL of the ribosome preparation was extracted with 750 μL of Tri Reagent LS (MRC). RNA samples were run on a 1.5% formamide-formaldehyde denaturing agarose gel and northern blots were developed using a biotinylated full-length tmRNA dsDNA probe and a Biotin detection kit (Roche).

3.3.8 Analysis of λ-N trpAT reporter tagging

750mL cultures of ΔB(DE3)/pACYCDuet-λ/pETBAh6 (carrying different SmpB mutations) cells were grown to OD_{600} around 0.5 and induced with 1mM IPTG for 45 min. Cells were harvested and resuspended in lysis buffer 1 with 150mM KCl in place of NH_4Cl. Cells were lysed by sonication and λ-N protein was purified over Ni-NTA beads (Qiagen). Ni-NTA purification products were then separated by Gel filtration over an FPLC HiPrep Sephacryl S-100 column (Amersham). Five 3mL fractions around the λ-N peak were pooled and concentrated. Aliquots were desalted using C_{18} Zip Tips (Millipore). Desalted protein sample was mixed with Sinapinic acid matrix and myoglobin internal standard (Sigma) and spotted for MALDI-TOF MS analysis.

3.4 Results

3.4.1 SmpB C-terminal Tail Mutants are Defective in Supporting tmRNA Mediated Tagging

I set out to identify functionally relevant residues in the C-terminal tail of SmpB protein by introducing premature termination codons at various positions in the SmpB coding sequence, and assessing the ability of the mutant proteins to

support tmRNA-mediated endogenous tagging. Endogenous tagging activity was assayed using a plasmid that expresses SmpB and a tmRNA variant in which the mRNA segment is mutated to code for six histidine residues (changing the tmRNA tag sequence from ANDENYALAA to ANDEHHHHHH). SmpB mutants were generated in this construct and endogenous tagging activity was assayed in a W3110Δ*smpB1* deletion strain (hereafter called ΔB). We found that deletion of five amino acid residues from the unstructured C-terminal tail of SmpB ($SmpB^{155}$) had no adverse affect on tmRNA-mediated endogenous tagging activity. Indeed, a subtle increase in activity was observed with $SmpB^{155}$. In contrast, removal of two additional amino acids ($SmpB^{153}$) severely reduced tagging activity, while removal of one additional amino acid ($SmpB^{154}$) had a moderate effect on endogenous tagging, reducing it to the level of wild type protein (Fig. 3.1 and Table 3.1). Further truncations in the C-terminal tail, including $SmpB^{151}$, $SmpB^{148}$, $SmpB^{144}$, $SmpB^{139}$, $SmpB^{133}$, or $SmpB^{59}$ entirely abolished the ability of the protein to support $tmRNA^{H6}$-mediated endogenous tagging (Fig. 3.1, Table 3.1, and data not shown).

Stepwise deletion of residues in the unstructured C-terminal tail of SmpB revealed that loss of residues 155 and 154 led to a gradual decrease in tmRNA mediated endogenous tagging activity (i.e. $SmpB^{155}$ > $SmpB^{154}$ > $SmpB^{153}$). I, therefore, hypothesized that residues I154 and M155 may be important for SmpB function. To investigate the functional importance of this region, I generated substitution mutations of these residues in the context of the full-length protein and assayed the ability of mutant proteins to support endogenous $tmRNA^{H6}$ tagging activity. Single amino acid substitutions of residues 154 or 155 to alanine, glutamine, or glutamic acid had little effect on endogenous tagging activity (Table 3.1). Consequently, I explored double substitution mutations of residues I154 and M155. Conservative substitution of both residues (I154L/M155I, hereafter called $SmpB^{LI}$), as well as substitution to positively charged residues (I154R/M155K, or $SmpB^{RK}$), or to polar residues (I154Q/M155Q, or $SmpB^{QQ}$) had no adverse effect on endogenous tagging activity (Fig. 3.1, Table 3.1). Conversely, when negatively charged residues were introduced (I154D/M155E, or $SmpB^{DE}$), endogenous tagging activity was nearly abolished. Introduction of alanine at both positions (I154A/M155A or $SmpB^{AA}$) yielded a moderate reduction in tagging activity (Fig. 3.1, Table 3.1). It is possible that some SmpB mutations may lead to a change in expression level or stability of the protein within cells. To control for this possibility, I performed western analysis on S30 extracts from cells expressing each SmpB variant. I found the amount of soluble SmpB to be similar for all SmpB variants studied and not related to tagging efficiency (data not shown).

tmRNA and SmpB are required for induction of the lytic cycle in the λ*imm*P22 *dis c*2–5 hybrid phage [4, 112]. I assessed the ability of SmpB mutants to support lytic development of this phage. Data are summarized in Figure 3.1, and Table 3.1. As expected, the λ*imm*P22 phage phenotypes largely mirror the observed endogenous tagging phenotypes. SmpB mutants defective in $tmRNA^{H6}$-mediated endogenous tagging (i.e. $SmpB^{DE}$, $SmpB^{153}$, and all smaller truncation mutants

studied) were also severely impaired in supporting lytic growth of the hybrid phage. However, the modest loss of tmRNA tagging activity supported by $SmpB^{AA}$ was not observed in phage assays, also a very subtle decrease in efficiency of plating was observed with $SmpB^{155}$ and $SmpB^{154}$ (Fig. 3.1, Table 3.1). These phage induction assay data support the conclusion that $SmpB^{DE}$ and SmpB truncation mutants are defective in supporting SmpB•tmRNA mediated tagging activity.

3.4.2 SmpB Mutants are Fully Competent in Binding tmRNA

Next, I set out to identify the specific biochemical defect in SmpB function resulting from these mutations. The two known functions of SmpB are binding to tmRNA and promoting its stable association with ribosomes [4, 16, 127]. First, I assessed the ability of mutant SmpB proteins to bind tmRNA, both *in vivo* and *in vitro*. I purified the SmpB•tmRNA complex under near physiological conditions by affinity chromatography (over Ni^{2+}-NTA beads) followed by an ion exchange step using a FPLC monoQ (HR 10/10) column. Under these conditions, SmpB protein and tmRNA co-elute as a single peak around 600 mM KCl. The chromatographic behavior of the defective mutants $SmpB^{DE}$, $SmpB^{153}$, and $SmpB^{148}$ was indistinguishable from $SmpB^{WT}$ in this regard. That is, $SmpB^{153}$, $SmpB^{148}$ and $SmpB^{DE}$ remained bound to tmRNA through both steps of the purification process (not shown). These results suggest that the SmpB mutants that fail to support endogenous $tmRNA^{H6}$ tagging are not defective in binding tmRNA *in vivo*.

Binding of one SmpB molecule to one tmRNA molecule (presumably the most functionally relevant interaction) produces only a small and difficult to discern electrophoretic mobility shift (not shown). A large body of evidence including enzymatic footprinting, mutational analysis, and recent co-crystal structure models suggests that the tRNA-like domain of tmRNA is necessary and sufficient for specific binding of one molecule of SmpB protein [15, 70, 125, 126]. Therefore, I utilized a synthetic tmRNA variant ($tmRNA^{113}$, that includes the tRNA-like domain of tmRNA without the pseudoknots or the mRNA sequence) to assess the tmRNA binding affinities of $SmpB^{WT}$ and the severely defective mutants, $SmpB^{DE}$, $SmpB^{153}$, and $SmpB^{139}$. *In vivo*, SmpB must recognize tmRNA in the presence of a large excess of cellular RNAs. Therefore, to ensure that only specific interactions of SmpB protein with tmRNA were detected, I conducted gel mobility shift assays under high stringency binding conditions. Binding reactions were carried out in the presence of 300 mM KCl and 100 nM total *E. coli* tRNA (a 1000 fold molar excess of the structurally related competitor tRNA over $tmRNA^{113}$). The gel mobility shift assay data includes multiple bound species (Fig. 3.2). Co-crystal and Cryo-EM structural data both suggest that the functional SmpB•tmRNA complex contains one molecule of each binding partner (13, 24). Hence, for the purposes of data analysis, I have treated the primary bound species as the specific bound complex and the additional bound species, which appear at higher SmpB concentrations, as products of additional protein-protein and protein-RNA interactions (see Materials and Methods, and [168]). The binding data are very complex, as multiple bound species appear at SmpB

concentrations where the free RNA is greater than zero. I attempted several methods of data analysis but was unable to obtain a perfect fit. The curve fit analysis that is presented is meant to be an estimate of tmRNA binding affinity used to compare different SmpB variants.

Regardless of what data analysis method was used, I did not observe any difference in the affinities of $SmpB^{WT}$, $SmpB^{DE}$, $SmpB^{153}$, and $SmpB^{139}$ for tmRNA (Fig. 3.2, Table 3.1). The apparent equilibrium dissociation constants, K_d, of all mutants studied were similar to $SmpB^{WT}$ (K_d for $SmpB^{WT}$, $SmpB^{DE}$, $SmpB^{153}$, and $SmpB^{139}$ were calculated to be 1.17 +/- 0.18 nM, 0.623 +/- 0.13 nM, 0.848 +/- 0.22 nM and 0.519 +/- 0.095 nM, respectively). Furthermore, I observed no difference in the binding affinity of $SmpB^{WT}$ and the $SmpB^{153}$ truncation mutant to full-length tmRNA (not shown). Taken together, these data show that mutations to the unstructured C-terminal tail of SmpB affect neither the ability of the protein to bind full length tmRNA *in vivo*, nor the affinity of SmpB interactions with tmRNA under stringent *in vitro* conditions. Therefore, I conclude that $SmpB^{DE}$, $SmpB^{153}$, and $SmpB^{139}$, although defective in endogenous tagging, are fully capable of binding tmRNA with high affinity and specificity, both *in vivo* and *in vitro*.

3.4.3 C-terminal Tail Mutants Support tmRNA Association with Ribosomes

Next, I hypothesized that mutations in the C-terminal tail of SmpB might impair the protein's ability to stably associate with ribosomes and/or to support the association of tmRNA with ribosomes. To test this hypothesis, I purified tight coupled 70S ribosomes from cells expressing $SmpB^{WT}$ and the various mutants using a method modified from Vila Sanjurjo *et al*. [160], and analyzed them for the presence of SmpB protein and tmRNA. I used $SmpB^{WT}$ as a positive control and $SmpB^{59}$ (an SmpB truncation mutant with only residues 1-59, that does not support stable association of tmRNA with ribosomes) as a negative control throughout these experiments. I found by western blot analysis that $SmpB^{WT}$, $SmpB^{RK}$, $SmpB^{AA}$, and $SmpB^{DE}$ all stably associate with ribosomes (Fig. 3.3*E*). Northern blot analysis of these samples, using tmRNA specific probes, revealed that all of the aforementioned SmpB mutants also support stable association of tmRNA with ribosomes (Fig. 3.3*A*). The SmpB truncation mutants $SmpB^{155}$, $SmpB^{154}$, $SmpB^{153}$, $SmpB^{151}$, $SmpB^{148}$, $SmpB^{139}$ and $SmpB^{132}$ also displayed no defects in this regard (i.e. these SmpB truncation mutants associate with ribosomes, and are fully proficient in promoting stable association of tmRNA with ribosomes) (Fig. 3.3*B*,*F*). Ethidium bromide staining of the gels used for northern blot (Fig. 3.3*C*,*D*) is shown to demonstrate that a similar amount of ribosome preparation was loaded onto each lane.

Taken together, these data demonstrate that SmpB variants carrying specific mutations near their C-termini are fully capable of performing the two known functions of SmpB (i.e. specific binding of tmRNA and supporting its stable association with 70S ribosomes), nonetheless, these mutants fail to support tmRNA tagging activity.

3.4.4 SmpB Mutants Fail to Support Transpeptidation and Partial Tagging

Having identified tagging deficient SmpB mutants that retain the ability to bind tmRNA and promote stable association of the SmpB•tmRNA complex with stalled ribosomes, I set out to further define the mechanistic time frame of the defect. I wanted to know whether the defective SmpB mutants were capable of adding any part of the tag sequence (addition of the tmRNA-charged alanine or any portion of the tmRNA encoded tag) to the C-terminal end of incompletely synthesized protein fragments (i.e., partial tagging).

In order to assay for partial tagging, I utilized λ-*N-trpAt,* a synthetic gene construct that contains the N-terminal 93 residues of the Lambda cI repressor followed by a His6 epitope and a *trpAt* transcriptional terminator [4, 169]. Transcription of this gene yields a non-stop mRNA that leads to ribosome stalling and subsequent tagging by the SmpB•tmRNA system. I co-expressed the λ-N-trpAt protein (hereafter referred to as λ-N) along with $tmRNA^{H6}$ and the SmpB C-terminal tail mutants, then purified the λ-N protein and analyzed the product for the presence or absence of any tmRNA-encoded tag sequence by MALDI-TOF MS (Fig. 3.4 and Fig. 3.5). In the absence of functional SmpB•tmRNA a series of peaks were observed, the largest of which represents the major translation product from the *λ-N-trpAt* gene. The smaller flanking peaks represent minor translation products, most likely arising from *λ-N-trpAt* transcript degradation (Figure 3.4 and [169]). In the presence of functional SmpB•$tmRNA^{H6}$, a second set of peaks appears, corresponding to the major and minor translation products with the $tmRNA^{H6}$ encoded tag sequence at their C-termini (Fig. 3.4, Fig. 3.5 and [169]). If only the tmRNA-linked alanine is added to the λ-N protein product, one would expect a peak corresponding to the mass of the major untagged translation product plus the mass of an alanine residue.

The observed peaks in the MALDI-TOF spectra of λ-N protein purified from cells expressing the $SmpB^{DE}$, $SmpB^{153}$, and $SmpB^{148}$ mutants correspond to protein products encoded solely by the *λ-N-trpAt* construct; the major peak in each profile corresponds exactly to the predicted mass identity of untagged λ-N protein (Fig. 3.4 and Fig. 3.5). I did not observe any peaks corresponding to tmRNA tagged products. These results agree with the endogenous tagging and hybrid phage phenotypes of these mutants, demonstrating that the $SmpB^{DE}$, $SmpB^{153}$, and $SmpB^{148}$ mutants are unable to support the addition of the tmRNA encoded tag sequence to nascent polypeptides in response to ribosome stalling. Furthermore, I did not observe any peaks corresponding to partially tagged λ-N protein products that could result from the addition of either alanine alone, or any portion of the tag sequence, arising from the mRNA-like function of tmRNA (Fig. 3.4 and 3.5). Therefore, the MALDI-TOF analysis demonstrates that the $SmpB^{DE}$, $SmpB^{153}$, and $SmpB^{148}$ mutants are unable to support either the tRNA- or the mRNA-like function of tmRNA.

As expected, the MALDI-TOF spectrum of λ-N protein purified from cells expressing SmpB^{AA} includes the set of mass peaks corresponding to untagged products, along with an additional set of peaks corresponding to products containing the full tmRNA^{H6} encoded tag sequence (Fig. 3.4 and Fig. 3.5). Once again, no partially tagged products were observed with SmpB^{AA}.

Although the MALDI-TOF spectra in all experiments were of sufficient resolution to detect several previously characterized minor translation products (Fig. 3.4, Fig. 3.5, and [169]), I did not observe mass peaks in any spectrum that corresponded to 'partially tagged' proteins. One possible explanation for this observation is that addition of an alanine to the C-terminus of the λ-N-trpAt protein renders it unstable within cells. To control for this possibility I expressed a variant of the λ-N-trpAt gene that codes for a C-terminal alanine followed by a termination codon. I found this product to be stable and soluble when expressed (data not shown). Another possibility is that the MALDI-TOF-MS signal of a partially tagged product is masked by other proteins in the sample. The clearly defined mass peaks corresponding to minor translation products seen here, and by Williams *et al.* [169], argues against this possibility. It is also formally possible that a partially tagged product is not released from the ribosome, however considering the labile nature of the ester linkage between tmRNA and alanine I consider this to be unlikely. One would expect that a partially tagged protein bound to tmRNA would be released via the same mechanism that permits the release of the untagged species from P-site tRNA in the absence of tmRNA. The most likely explanation is that the SmpB^{DE}, SmpB^{153}, and SmpB^{148} mutants do not support either full or 'partial' tagging, and that the decreased tagging activity of SmpB^{AA} mutant is not due to partial tagging activity, but rather due to an overall reduction in the tagging proficiency of this mutant. Thus, the unstructured C-terminal tail of SmpB plays a crucial role after association of the SmpB•tmRNA complex with ribosomes but prior to transpeptidation of the tmRNA linked alanine and establishment of the tmRNA reading frame.

3.5 Discussion

These results demonstrate that the C-terminus of SmpB is involved in supporting a novel function of the protein in *trans*-translation. I have shown that mutations to specific C-terminal residues of SmpB protein impair the protein's ability to support endogenous tmRNA-mediated tagging, as well as induction of lytic development of a λ*imm*P22 hybrid phage (Fig. 3.1 and Table 3.1). Significantly, these mutants are not defective in binding tmRNA *in vivo* or *in vitro*, nor are they impaired in their ability to promote stable association of the SmpB•tmRNA complex with tight-coupled 70S ribosomes (Figs. 3.2, 3.3). Nonetheless, the mutant proteins are unable to support addition of the tmRNA-linked alanine to incompletely synthesized polypeptides (Fig. 3.4, and Fig. 3.5). Thus, the newly identified function of the C-terminal tail of SmpB is required for events in trans-translation downstream of SmpB•tmRNA complex formation and

its stable association with ribosomes, but prior to addition of the tmRNA-linked alanine to incomplete polypeptides.

Residues I154 and M155 are important in supporting this novel function. Interestingly, while the length of the C-terminal extension varies among bacterial SmpB genes, all known SmpB C-terminal tails include at least the amino acid that aligns with I154 in *E. coli*. Negative charges at residues 154 and 155 are not well tolerated, as introduction of negative charge at both residues 154 and 155 renders the protein severely defective in supporting tmRNA-mediated tagging (Fig. 3.1, Table 3.1).

It is also conceivable that the observed ribosome association phenotypes do not reflect functional binding of SmpB•tmRNA•EF-Tu•GTP quarternary complexes with the ribosomal A-site. SmpB and tmRNA may be co-purifying with ribosomes due to non-specific protein-RNA or RNA-RNA interactions. The fact that the ribosomes are purified under stringent salt conditions (0.5 M NH_4Cl) argues against this possibility. The observation that tmRNA fails to co-purify with ribosomes when the truncated SmpB variant $SmpB^{59}$ is expressed indicates, even more strongly, that the observed *in vivo* ribosome association results reflect specific, functional binding of quarternary complexes to ribosomes. I have performed ribosome purifications using three different high stringency protocols. All SmpB C-terminal tail mutants tested support association of the SmpB•tmRNA complex with ribosomes regardless of the purification protocol used. Even when ribosomes are purified by sedimenting tight-coupled 70S ribosomes through a high salt sucrose cushion (0.5 M NH_4Cl), followed by isolation of the 70S peak through a linear sucrose gradient in high salt (0.3 M NH_4Cl), the mutant SmpB•tmRNA complexes remain bound to the ribosome (data not shown). This provides strong evidence that the observed ribosome association phenotypes reflect specific interactions with the ribosome.

When a ternary complex of cognate tRNA with EF-Tu(GTP) is brought to the ribosomal A-site, proper codon-anticodon interactions somehow trigger conformational changes that activate the GTPase domain of EF-Tu. Rapid GTP hydrolysis is followed by release of EF-Tu(GDP) and accommodation of the tRNA acceptor stem into the peptidyl transferase center [23, 170, 171]. Considering the structural and mechanistic similarities between tRNAs and the tRNA-like domain of tmRNA, one would presume that accommodation of tmRNA would proceed much like that of tRNAs. However, tmRNA lacks a traditional anticodon stem loop, and thus codon-anticodon interactions are necessarily absent from the tmRNA accommodation step [16, 69, 74]. It is unclear what mechanistic events trigger GTP hydrolysis by EF-Tu when tmRNA is presented to the ribosomal A-site. It is possible that the C-terminal tail region of SmpB plays a direct or indirect role in GTPase activation. Karzai et al. [4] first suggested the hypothesis that SmpB might serve as a tRNA anticodon stem loop mimic, in a manner analogous to domain IV of EF-G. Our results demonstrate that the C-terminal tail of SmpB is not required for initial binding of the tmRNA-EF-Tu-GTP complex to stalled ribosomes. However, this region might gain structure in the context of the ribosome to mediate specific contacts with ribosomal elements necessary for

proper positioning or accommodation of tmRNA into the A-site. Deletion of residues I154 and M155 or introduction of negative charges at these positions may destabilize interactions of the SmpB C-terminal tail with rRNA or ribosomal proteins near the decoding center, explaining the observed loss of SmpB•tmRNA-mediated tagging activity of these mutants (Fig. 3.1, Table 3.1). Hence, one possible explanation for the observed phenotype of the SmpB C-terminal tail mutants is that they are defective in properly engaging ribosomal elements responsible for eliciting EF-Tu GTPase activity.

It is also possible that mutations to the SmpB C-terminus affect the protein's ability to support a step in *trans*-translation downstream of GTP hydrolysis. Contacts made by the SmpB C-terminal tail may be required for proper positioning of the tmRNA acceptor stem in the peptidyl transferase center during accommodation. In addition, it is possible that the SmpB C-terminal tail serves as an anchor for the tmRNA tRNA-like domain during the proofreading step that follows GTP hydrolysis. Alternatively, these contacts could play a role in establishment of the tmRNA encoded reading frame. The precise mechanistic role of the SmpB C-terminal tail remains to be elucidated, however the results presented in this study clearly demonstrate that SmpB performs an essential novel function in *trans*-translation that extends beyond specific association and transport of tmRNA to the ribosomal A-site.

There is extensive precedent in the literature to support the idea that a ribosome-associated protein may contain extensions that are unstructured in solution but gain structure within the context of the ribosome. For example both ribosomal proteins S4 and S19 contain extended termini which are disordered in NMR solution structures [151]. In the crystal structure of the 30S subunit, however, these extensions are structured and make important contacts with 16S rRNA [152]. In a recent report, Hoang and colleagues demonstrated that the C-terminal tails of ribosomal proteins S9 and S13 contribute to the binding of P-site tRNA to the 30S ribosomal subunit [153]. Hence, the extended termini of ribosomal proteins have been shown to play a functional role in translation. Therefore, it would not be surprising to discover that the SmpB C-terminus gains structure upon ribosome binding to make critical contacts required for tmRNA mediated tagging. Elucidating the structure of full-length SmpB and characterizing the nature of the contacts made by its C-terminal extension will require high-resolution structural data of SmpB•tmRNA in both the accommodated and un-accommodated state.

The results of this study demonstrate a novel role for SmpB in the *trans*-translation mechanism, a role beyond binding tmRNA, delivering it to the ribosome and promoting its stable association. Taken together with previous structural data, these results support the notion that the SmpB C-terminal extension plays a crucial role in proper engagement and accommodation of tmRNA in the ribosomal A-site.

3.6 Tables and Figures

Table 3.1

SmpB Mutant	Endogenous SsrA Tagging Activity[a,c]	EOP[b,c]	Apparent K_d(nM)[c]
$SmpB^{WT}$	100	1	1.17+/-0.018
$SmpB^{155}$	149+/-95	0.131+/-0.019	N/D
$SmpB^{154}$	99.4+/-29	0.291+/-0.098	N/D
$SmpB^{153}$	13.6+/-12	0.0000556+/-0.000039	0.848+/-0.22
$SmpB^{151}$	1.17+/-0.43	0.000306+/-0.0000077	N/D
$SmpB^{148}$	0.768+/-0.310	0.000207+/-0.000044	N/D
$SmpB^{139}$	0.469+/-0.25	0.000234+/-0.000031	0.519+/-0.095
$SmpB^{132}$	0.653+/-0.455	0.000208+/-0.000069	N/D
$SmpB^{59}$	3.12+/-4.9	0.0000365+/-0.000021	N/D
$SmpB^{I154A}$	116+/-9.1	0.956+/-.33	N/D
$SmpB^{I154Q}$	136+/-9.7	0.862+/-0.15	N/D
$SmpB^{I154E}$	164+/-81	0.700+/-0.354	N/D
$SmpB^{M155A}$	96.6+/-21	1.03+/-0.086	N/D
$SmpB^{M155Q}$	103+/-38	0.681+/-0.26	N/D
$SmpB^{M155E}$	71.3+/-41	0.700+/-0.022	N/D
$SmpB^{LI}$	95.1+/-4.9	0.881+/-0.23	N/D
$SmpB^{RK}$	152+/-23	0.313+/-0.047	N/D
$SmpB^{QQ}$	136+/-30	0.247+/-0.182	N/D
$SmpB^{AA}$	51.9+/-12	0.631+/-0.10	N/D
$SmpB^{DE}$	17.7+/-8.5	0.0000334+/-0.000018	0.623+/-0.13

[a] Endogenous Tagging activity is given as % of Tagging activity with $SmpB^{WT}$

[b] λ*imm*P22 phage induction phenotype is given as efficiency of plating (EOP) with plaque formation from cells expressing $SmpB^{WT}$ taken as EOP=1

[c] All values represent combined analysis of at least three independent experiments (N=3). N/D = not determined.

Figure 3.1

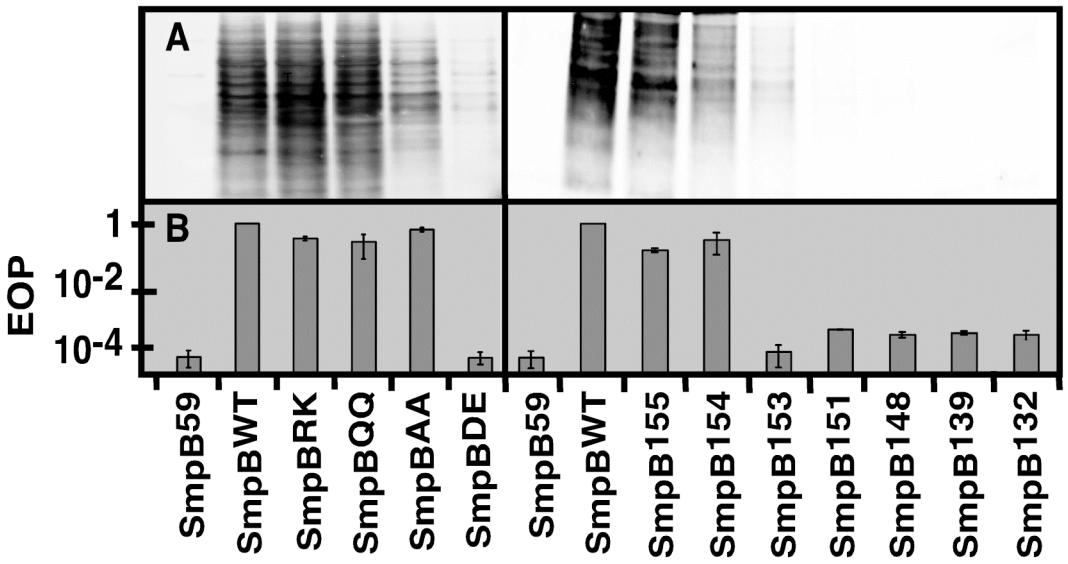

Figure 3.1. *Endogenous tagging phenotypes*. Panel (*A*) shows western blot analysis, using IR800 conjugated anti-his6 antibody, showing the pattern of proteins tagged by $tmRNA^{H6}$ in cells expressing different SmpB variants. Panel (*B*) is analysis of λ*imm*P22 hybrid phage induction supported by different SmpB mutants. Data are presented as efficiency of plating (EOP) where the number of plaques formed when $SmpB^{WT}$ was expressed is taken as EOP=1. Error bars represent the mean +/- standard deviation (three independent experiments). $SmpB^{WT}$ was used as a positive control and $SmpB^{59}$ (an SmpB truncation mutant with only residues 1-59) was used as a negative control throughout these experiments.

Figure 3.2

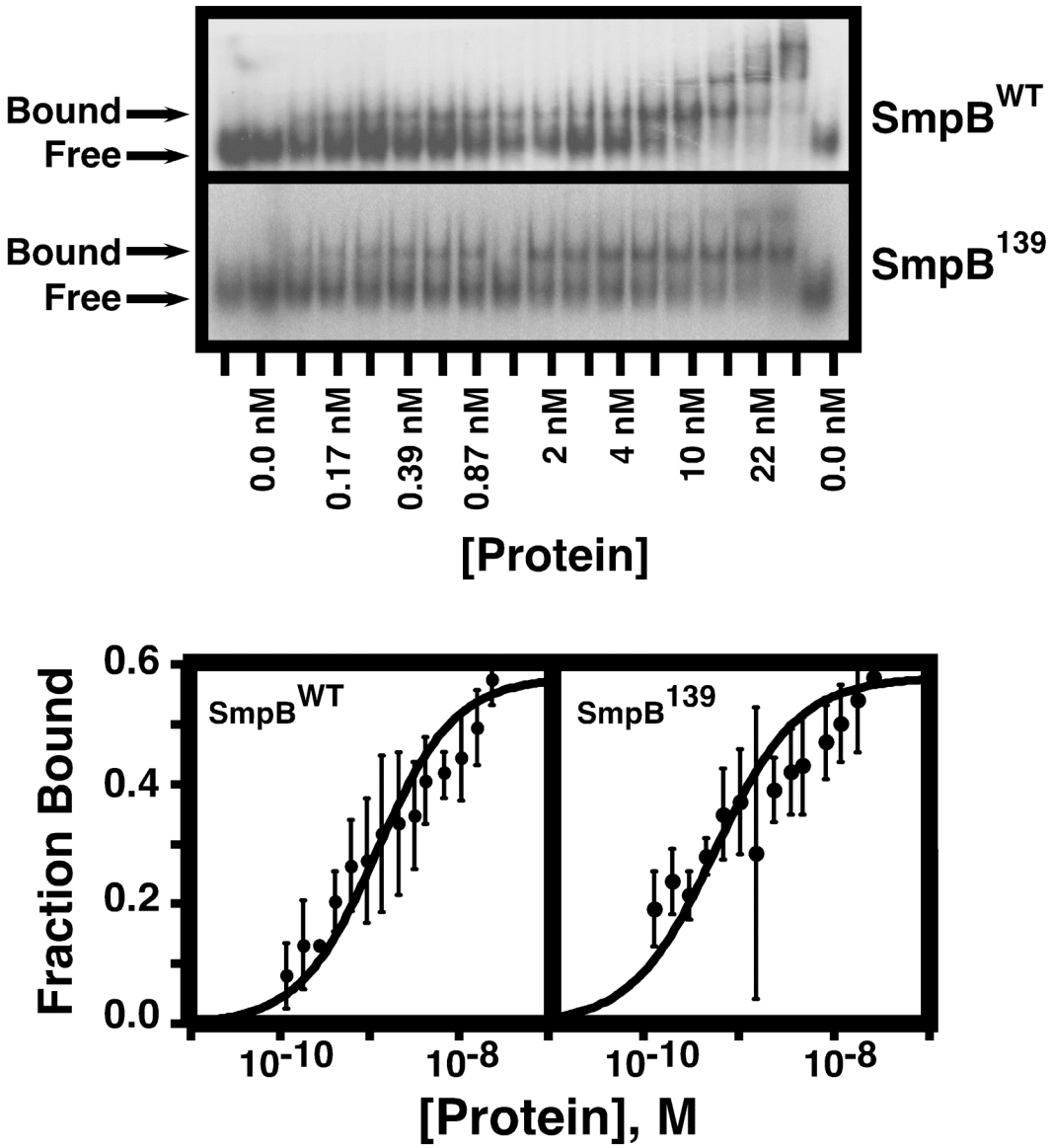

Figure 3.2. *tmRNA binding assays*. The top panel shows gel mobility shift assays of the tmRNA binding of $SmpB^{WT}$ and $SmpB^{139}$. The bottom panel is curve fit analysis that was used to determine the apparent equilibrium dissociation constants (K_d) of SmpB -$tmRNA^{113}$ interactions.

Figure 3.3

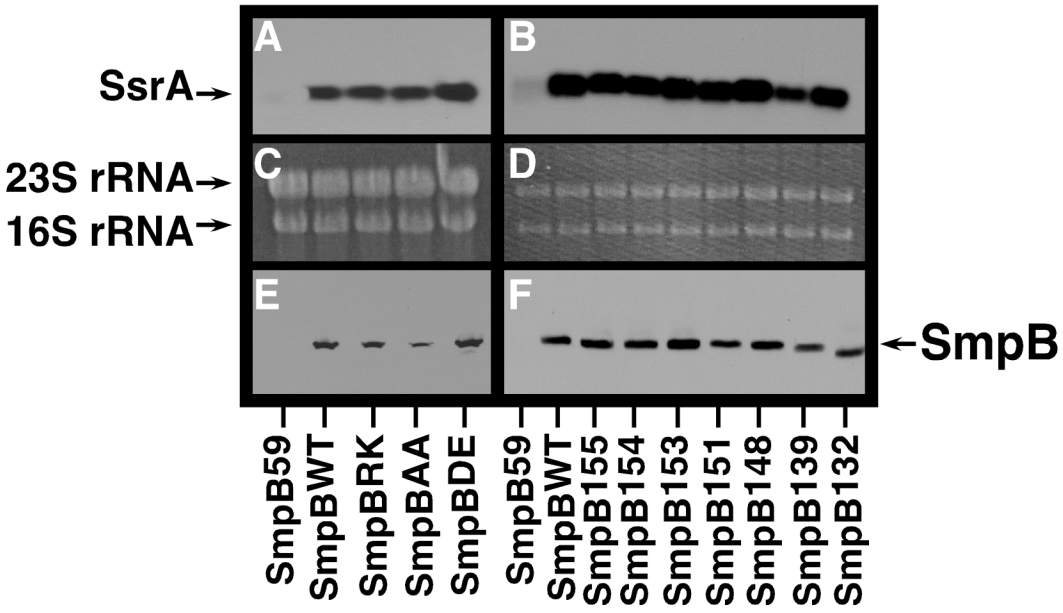

Figure 3.3. *Ribosome association*. Panels (*A, B*) are northern blot analysis using a tmRNA-specific probe to detect tmRNA in purified ribosome preparations. Panels (*C, D*) are ethidium bromide staining of the same gel as in panels (*A, B*), showning ribosomal RNA as a loading control. Panels (*E, F*) depict western blot analysis using anti-his6 antibody to detect his6-tagged SmpB protein in the same purified ribosome preparations used in panels (*A-D*). The SmpB variant expressed in the cells from which the ribosomes were purified is indicated on the horizontal axis.

Figure 3.4

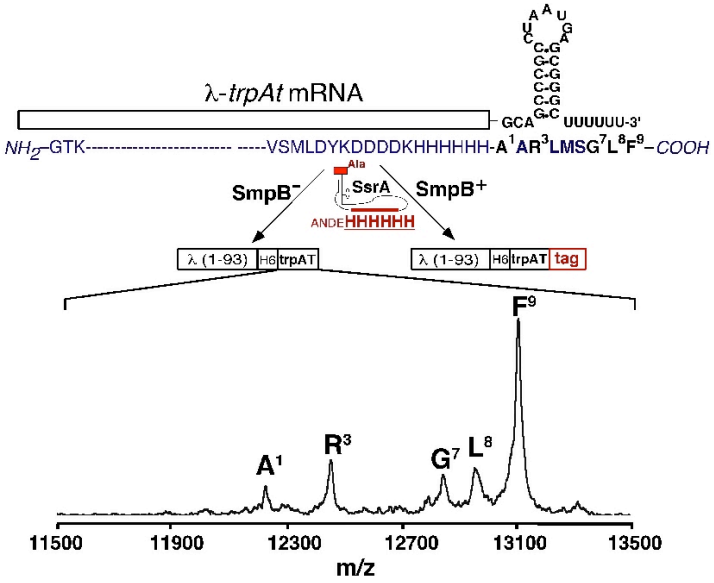

Figure 3.4. *MALDI-TOF MS analysis of λ-N-trpAT protein products***.** The top panel is a schematic representation of the λ-*N- trpAt* mRNA and the amino acid sequence of its protein product. Amino acid numbering begins at the alanine following the 6His epitope. In the presence of functional SmpB protein addition of the tmRNA^{H6} tag sequence to the C-terminus of a portion of the observed products is expected. In the absence of functional SmpB, only untagged products should be observed. The bottom panel is the MALDI-TOF MS spectrum of purified λ-N protein from cells expressing the SmpB^{DE} variant. Letter designations correspond to the C-terminal amino acid of the protein species. The pattern of the MALDI-TOF spectra for cells expressing the SmpB^{153} and SmpB^{148} mutants is similar to the SmpB^{DE} pattern; when SmpB^{AA} is expressed a series of peaks corresponding to λ-N protein products containing the full tmRNA^{H6} encoded tag sequence are observed (see Fig. 3.5).

Figure 3.5

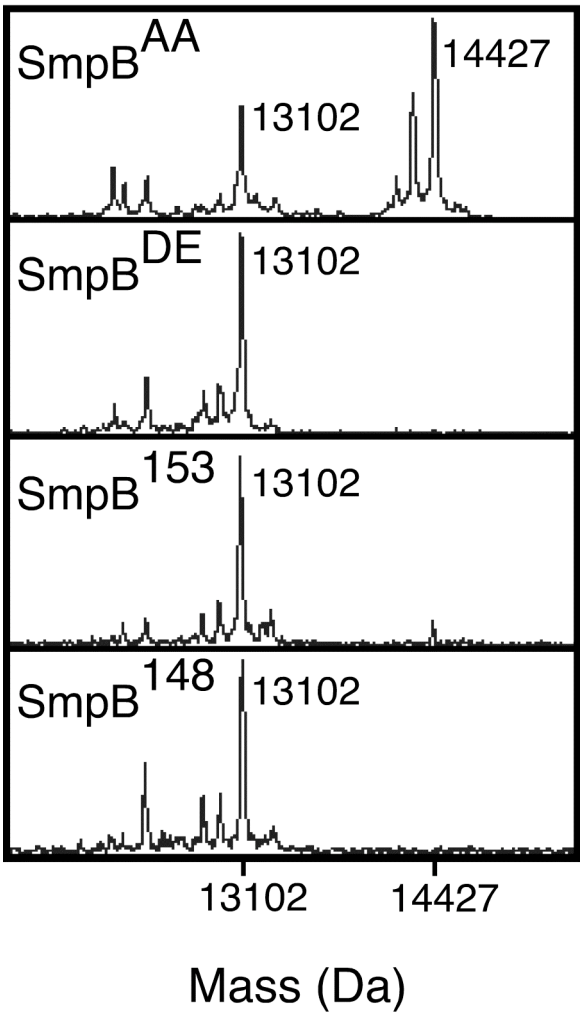

Figure 3.5. *MALDI-TOF MS spectra*. MALDI-TOF MS spectra of purified λ-N protein from cells expressing the $SmpB^{AA}$, $SmpB^{DE}$, $SmpB^{153}$, and $SmpB^{148}$ variants. The species with m/z = 13102 is the major untagged λ-N protein product, while the peak with m/z = 14427 corresponds to the major product with the full $tmRNA^{H6}$ encoded tag sequence.

Chapter 4: *In Vitro* Analysis of *trans*-Translation

4.1 Summary

tmRNA acts a both a tRNA and an mRNA to rescue ribosomes stalled on defective messages, direct the degradation of aberrant protein products, and facilitate the decay of the causative mRNA. The tRNA-like domain of tmRNA shares sequence and functional similarity with $tRNA^{Ala}$. However, due to the absence of an anticodon on tmRNA, the mechanism of the tRNA-like actions of tmRNA is necessarily different from that of canonical tRNAs. In order to dissect the mechanism of tmRNA-mediated translation elongation, I assembled an *in vitro* system for studying the tRNA-like actions of tmRNA. I found that EF-Tu and its GTPase activity are required to stimulate transpeptidation onto tmRNA. In addition, my results suggest that the unstructured C-terminal tail of SmpB is required to support transpeptidation, but not GTP hydrolysis. These results suggest that the SmpB C-terminal tail plays a role in accommodation of the tmRNA TLD after GTP hydrolysis.

4.2 Introduction

The mechanism of SmpB•tmRNA-mediated ribosome rescue and peptide tagging may be conceptually divided into three distinct phases. The first phase is the tRNA-like activity where SmpB•tmRNA recognizes stalled ribosomes, binds in the ribosomal A-site and the nascent polypeptide is transferred onto the tmRNA TLD [1, 5, 7, 16, 73]. The second phase is the mRNA-like action. During this phase the ribosomal reading frame shifts to translate the tmRNA encoded proteolysis tag, directing the degradation of the aberrant protein product [1, 5, 7, 16, 73]. The final phase is SmpB•tmRNA-mediated decay of the causative defective mRNA by RNaseR [8].

The tRNA-like phase of tmRNA activity is thought to proceed via a mechanism analogous to $tRNA^{Ala}$-mediated translation elongation. Like $tRNA^{Ala}$, tmRNA is charged with alanine by Ala-RS [79, 80]. Ala-$tmRNA^{Ala}$ then binds in the ribosomal A-site along with EF-Tu(GTP), however unlike the tRNA ternary complex the tRNA activity of tmRNA requires the additional protein factor SmpB [5, 76, 77, 79, 80]. The SmpB•tmRNA•EF-Tu(GTP) quarternary complex is accommodated into the

ribosomal A-site and the growing peptide is transferred onto the tmRNA TLD in a transpeptidation reaction.

The mechanism of $tRNA^{Ala}$ mediated translation elongation is well characterized. Upon binding in the ribosomal A-site (in a ternary complex with EF-Tu(GTP)), the anticodon of $tRNA^{Ala}$ base pairs with the A-site mRNA codon. This codon-anticodon interaction then drives conformational changes within the ribosome that lead to activation of the GTPase domain of EF-Tu, resulting in rapid GTP hydrolysis. The GDP form of EF-Tu exhibits reduced affinity for the tRNA, and EF-Tu(GDP) dissociates from the complex. This allows the aminoacyl acceptor stem of $tRNA^{Ala}$ to swing into the peptidyl tranferase center in the 50S ribosomal subunit, in a process known as accommodation. Transpeptidation onto $tRNA^{Ala}$ then occurs spontaneously.

The tmRNA TLD lacks an anticodon arm, therefore the mechanistic events that drive accommodation of and transpeptidation onto the tmRNA TLD are necessarily distinct from those of $tRNA^{Ala}$. Little is known about these mechanistic differences. Specifically, it has been demonstrated that EF-Tu can bind the tmRNA TLD [125, 126, 134-136, 138, 172], however it remains unclear whether this interaction, or EF-Tu GTPase activity are required for the tRNA-like phase of SmpB•tmRNA action. Two recent studies have addressed the importance of EF-Tu in transpeptidation onto tmRNA[137, 138]. The first study found that omitting EF-Tu from and *in vitro trans*-translation reaction resulted in partial loss of peptide bond formation activity[137]. A second study demonstrated that at reaction times of 30 minutes, neither the absence of EF-Tu or the presence of kirromycin had any effect on the extent of transpeptidation onto full length tmRNA[138]. In contrast, this same study measured peptide bond formation on an artificial tmRNA TLD construct and found that this reaction requires EF-Tu. Hence, the requirement of EF-Tu to support transpeptidation onto tmRNA remains an open question. Furthermore, no data regarding the role of EF-Tu mediated GTP hydrolysis in this process is currently available. In contrast to EF-Tu, it is clear that SmpB is required to support the tRNA-like activity of tmRNA. Three separate studies have established that SmpB is essential for tmRNA-mediated peptide bond formation [97, 137, 138]. This result is expected since SmpB is required to support stable association of tmRNA with ribosomes [4]. Furthermore, results presented in chapter 3 suggest that the SmpB C-terminal tail is required for peptide bond formation *in vivo*.

In this study, I set out to assess the role of EF-Tu and EF-Tu mediated GTP hydrolysis in supporting the the tRNA-like phase of *trans*-translation. I found that rapid peptide bond formation requires both EF-Tu and EF-Tu GTPase activity, as in the absence of EF-Tu or in the presence of a GTPase defective EF-Tu mutant, the rate of peptide bond formation is slowed severely. I also demonstrated that the C-terminal tail of SmpB is required for transpeptidation onto tmRNA, but not for EF-Tu mediated GTP hydrolysis. These results suggest that the SmpB C-terminal tail is important for accommodation of the tmRNA TLD in the early stages of *trans*-translation.

4.3 Experimental Procedures

4.3.1 Protein and RNA Purification

Protein translation factors IF-1, IF-2, IF-3, EF-Tu, EF-Ts, EF-G, AlaRS, MetRS, Phe-RS and MTF were purified as in chapter 2. Proteins were purified by batch affinity chromatography using Ni^{2+}-NTA agarose (Qiagen, Valencia,CA) followed by FPLC ion exchange using either a MonoS (HR 10/10) column or a MonoQ (HR 10/10) column (GE Healthcare, Piscataway, NJ) eluted with a linear salt gradient.

tmRNA was purified from cells as follows. 6L of BL21(DE3)/pLysS/pET28BA cells were grown to OD_{600} around 0.5-0.7. SmpB and tmRNA were simultaneously induced with 10μM IPTG for 2 hours. The SmpB•tmRNA complex was purified by affinity chromatography using Ni^{2+}-NTA agarose resin (Qiagen, Valencia,CA). tmRNA was separated from SmpB via RNA extraction using TriReagent LS (MRC, Cincinnatti,OH). tmRNA was further purified via FPLC anion exchange using a MonoQ (HR 10/10) column (GE Healthcare, Piscataway, NJ). A $tRNA^{fMet}$ enriched tRNA pool was purified by isolating total tRNA from cells expressing $tRNA^{fMet}$ from the plasmid ptrnfM. tRNA purification was performed as described [159].

Preparative purification of charged and formylated fMet-$tRNA^{fMet}$ was performed as follows. Charging and formylation reactions were performed in buffer A (25mM Tris (pH7.5), 100mM NH_4Cl, 30mM KCl, 8mM $MgCl_2$ and 1mM DTT). Reactions (10mL) included 20μM $tRNA^{fMet}$, 200μM L-methionine, 150μg N^{10}-formyl-tetrahydrofolate, 2μM Met-RS, 5μM MTF, and 3mM ATP, incubated at 37°C for 20min. For [^{35}S]fMet-$tRNA^{fMet}$, the concentration of L-methionine was reduced to 15μM and supplemented with 165μM [^{35}S]L-methionine. RNA was precipitated with isopropanol, washed with ethanol, and extracted with TriReagent LS and resuspended in buffer B (50mM potassium phosphate (pH7), 1.7M $(NH_4)_2SO_4$). The charged and formylated fraction was separated from uncharged tRNA via FPLC hydrophobic interactions chromatography using a Hi-Trap Phenyl Sepharose HP column (GE Healthcare, Piscataway, NJ) and a linear gradient from 10-50% buffer C (20mM MOPS (pH7.5)) in buffer B. This product was diluted 4 fold in buffer D (50mM sodium acetate (pH5)) and purified by FPLC anion exchange using a MonoQ (HR 10/10) column (GE Healthcare, Piscataway, NJ). fMet-$tRNA^{fMet}$ was eluted in a linear gradient of 0-100% buffer E (50mM sodium acetate (pH5), 1M NaCl) in buffer D. fM mRNA (5'-GGCAAGGAGGUAAAAAUGUUC-3') and fMF mRNA (5'-GGCAAGGAGGUAAAAAUGUUC-3') were purchased from Integrated DNA Technologies (Coralville IA)

4.3.2 Ribosome complexes

For GTP hydrolysis assays, fM 70S initiation complexes were assembled in buffer B (50mM Tris (pH 7.5), 70mM NH_4Cl, 30mM KCl, 10mM $MgCl_2$, and 20mM

DTT). The reaction mix contained 5μM fMet-tRNA^{fMet}, 3μM 70S ribosomes, 3μM IF-1, 3μM IF-2, 3μM IF-3, 1mM GTP and 8μM fM or fMF mRNA. Reactions were incubated at 37°C for 45 minutes. Initiation complexes were separated from free reaction components by pelleting (500μL reaction) through 1mL of a 10% sucrose cushion in buffer C (40mM Tris (pH 7.5), 300 mM NH_4Cl, 10mM $MgCl_2$, 2mM β-mercaptoethanol). Ribosomes were pelletted by centrifugation at 41,000 RPM for 16 hours in a TLA 100.3 rotor (Beckman Coulter, Fullerton, CA). Occupency of initiator tRNA in pre-*trans*-translation complexes was measured by quantitative Northern blotting with a probe specific to tRNA^{fMet}. Occupency of tRNA^{fMet} was close to 100%.

For peptide bond formation assays, fMF 70S initiation complexes were generated as above, except fMF mRNA and [^{35}S]fMet-tRNA^{fMet} were used. Figure 4.1 depicts the formation of fMF pre-*trans*-translation complexes schematically. Prior to purification, fMF 70S initiation complexes were subjected to a single round of *in vitro* transpeptidation and translocation by mixing equal volumes of initiation complex formation reaction and phenylalanine addition mix. Phenylalanine addition reaction mix contained 20μM EF-Tu, 5μM EF-Ts, 1mM GTP, 2mM ATP, 2μM Phe-RS, 1mM phospho(enol)pyruvate, 0.1mg/mL pyruvate kinase, 1mM L-phenylalanine, and 100μM total E. coli tRNA as a source of tRNA^{Phe}. Phenylalanine addition reactions were performed in buffer D (50mM Tris (pH7.5), 70mM NH_4Cl, 30mM KCl, 10mM $MgCl_2$, 20mM DTT, 0.5mM spermidine, and 8mM putrescine). The product of this reaction, fMF pre-*trans*-translation complexes was separated from free components by pelletting 500μL of reaction through 1mL of 10% sucrose cushion in buffer C as above.

4.3.3 *In vitro* peptide bond formation assays

Figure 4.2 describes the *in vitro* peptide bond formation assay. Peptide bond formation assays were performed in buffer D. Components were pre-incubated at 37°C for 10 minutes to assemble SmpB•tmRNA•EF-Tu(GTP) quarternary complexes. Unless otherwise noted, quarternary complex formation reactions (25μL) contained 2.4μM tmRNA, 2.4μM SmpB, 10μM EF-Tu, 7μM EF-Ts, 2μM Ala-RS, 100μM GTP, and 2mM L-alanine. At time = 0, 250nM fMF 70S pre-*trans*-translation complexes were added at 37°C. At the indicated time points, 5μL were removed and added to 250μL of 0.5N KOH to quench the reaction. To hydrolyze RNA, reactions were incubated at 37°C for 15 minutes. Reactions were neutralized by the addition of 50μL of 50% acetic acid. Insoluble material was removed by centrifugation (5 minutes at 18,000XG). [^{35}S]fMet-Phe was separated from [^{35}S]fMet-Phe-Ala by reverse phase HPLC over a C18 column (The Nest Group, Southborough, MA) using a gradient from 0-65% Acetonitrille in 1% TFA. The sum of the [^{35}S] radioactivity counts in fractions corresponding to the [^{35}S]fMet-Phe-Ala and [^{35}S]fMet-Phe peaks was calculated and the ratio of [^{35}S]fMet-Phe-Ala to [^{35}S]fMet-Phe counts was plotted.

4.3.4 *In vitro* GTP hydrolysis assays

EF-Tu([γ^{32}P]GTP) was generated in buffer E (50mM Tris(pH7.5), 50mM NH_4Cl, 10mM $MgCl_2$, and 1mM DTT). The nucleotide exchange reaction (50μL) contained 50μM EF-Tu, 1μM EF-Ts, 50μM GTP, 200nM [γ^{32}P]GTP, 2mM ATP, 3mM Phospho(enol)pyruvate, and 0.1mg/mL Pyruvate Kinase. Reactions were incubated at 37°C for 10 minutes. Free [γ^{32}P]GTP was removed by gel filtration through two sequential Sephadex G25 spin columns (GE Healthcare, Piscataway, NJ) equilibrated in buffer E.

SmpB•tmRNA•EF-Tu([γ^{32}P]GTP) quarternary complexes were formed by pre-incubating components for 5 minutes at 37°C in buffer D. Unless otherwise noted, quarternary complex formation reactions (20μL) contained 0.5μM tmRNA, 0.5μM SmpB, 2μM Ala-RS, 0.5μM EF-Tu([γ^{32}P]GTP), 1mM L-alanine, and 2mM ATP. At time = 0, an equal volume of 500nM fM 70S ribosome initiation complexes was added to start the GTP hydrolysis reaction. At the given time points, 5μL of this reaction was added to 10μL of 50% formic acid to quench. 1μL of this mix was spotted onto PEI cellulose TLC plates. [γ^{32}P]GTP and $^{32}P_i$ were resolved by thin layer chromatography in 0.5M Potassium phosphate (pH 3.5).

4.4 Results

4.4.1 SmpB C-terminal tail is required for transpeptidation onto tmRNA *in vitro*

In order to dissect the mechanism of tmRNA tRNA-like activity, I assembled and *in vitro* system to study *trans*-translation. Results presented in chapter 3 suggest that the unstructured C-terminal tail of SmpB is required for the tRNA-like activity of tmRNA *in vivo*. First, I set out to confirm this result using the *in vitro trans*-translation system. I performed peptide bond formation assays using either wild type SmpB or the SmpB C-terminal truncation mutants $SmpB^{153}$ and $SmpB^{139}$. $SmpB^{139}$ failed to support both endogenous tagging and peptide bond formation activity *in vivo* (chapter 3). $SmpB^{153}$ failed to support peptide bond formation *in vivo* and supported only minimal endogenous tagging activity (chapter 3).

For peptide bond formation assays, pre-*trans*-translation complexes were assembled *in vitro*. Figure 4.1 describes the assembly of pre-*trans*-translation complexes. Briefly, I generated 70S ribosome initiation complexes programmed on fMF mRNA. fMF mRNA contains only a ribosome binding site, an AUG initiation codon, and a UUC phenylalanine codon. These initiation complexes were subjected to a single round of translation elongation and translocation leaving 70S pre-*trans*-translation complexes with fMet-Phe-$tRNA^{Phe}$ in the P-site and an empty A-site, lacking both tRNA and mRNA. As such, these complexes are a model substrate for SmpB•tmRNA.

Figure 4.2 depicts the peptide bond formation reaction. Pre-*trans*-translation complexes were mixed with pre-formed SmpB•tmRNA•EF-Tu(GTP) quarternary complexes. Reactions were quenched at 0, 10 second, 30 second, and 10 minute time points with potassium hydroxide, and the fMet-Phe-Ala tripeptide was separated from fMet-Phe dipeptide by reverse phase HPLC. I performed peptide bond formation assays using either no SmpB, wild type SmpB, SmpB^{153}, or SmpB^{139}. Results are presented in figure 4.3. When wild type SmpB was included in the reaction, I observed rapid peptide bond formation. Greater than 90% of the fMet-Phe was converted to fMet-Phe-Ala by the 10 second time point. In contrast, in the absence of SmpB, no peptide bond formation was observed, even after 10 minutes. SmpB^{139} was entirely defective in supporting peptide bond formation, as no fMet-Phe-Ala was formed after 10 minutes. SmpB^{153} resulted in a severe decrease in the rate of transpeptidation, as less that 20% of the fMet-Phe was converted to fMet-Phe-Ala, and no peptide bond formation was observed until the 10 minute time point. These results are consistent with the results described in chapter 3, suggesting that the C-terminal tail of SmpB plays a role in supporting the tRNA-like function of tmRNA.

4.4.2 EF-Tu GTP hydrolysis is required for transpeptidation onto tmRNA

Since tmRNA lacks an anticodon, the mechanistic events that lead to its accommodation into the ribosomal A-site are necessarily distinct from cognate tRNAs. It has been shown that EF-Tu can bind the tRNA-like domain of tmRNA [125, 126, 134-136, 138, 172], however it is unclear whether this interaction, or EF-Tu mediated GTP hydrolysis are required for the tRNA-like activity of tmRNA. To assess the role of EF-Tu in this process, I performed peptide bond formation assays in the presence of EF-Tu, in the absence of EF-Tu, and in the presence of the GTPase deficient EF-Tu mutant H84A [173, 174]. I found that reactions lacking EF-Tu, or containing the mutant EF-Tu exhibited a severe defect in the rate of peptide bond formation (Figure 4.4). No tripeptide was formed at the 10 second time point either without EF-Tu or with the mutant EF-Tu (as compared to greater than 90% completion of the reaction with wild type EF-Tu in 10 seconds). After 10 minutes, the EF-Tu(H84A) and no EF-Tu reactions were able to convert only 30-40% of dipeptides to tripeptides. These results are consistent with a requirement for EF-Tu and EF-Tu mediated GTP hydrolysis to support rapid transpeptidation onto tmRNA *in vivo*.

4.4.3 SmpB C-terminal tail does not seem to be required for GTP hydrolysis

My results suggest that, like canonical tRNAs, tmRNA requires EF-Tu mediated GTP hydrolysis for peptide bond formation. Unlike canonical tRNAs, tmRNA lacks an anticodon, hence the mechanisms involved in stimulating EF-Tu GTPase activation must be different for tmRNA than for tRNAs. Thus, I hypothesized that the C-terminal tail of SmpB might make contacts within the ribosomal A-site that substitute for codon-anticodon interactions to stimulate GTP

hydrolysis. To test this hypothesis, I looked at the ability of SmpB C-terminal tail mutants to support EF-Tu mediated GTP hydrolysis.

The design of the GTP hydrolysis assay was similar to the peptide bond formation assay. Briefly, 70S ribosome pre-*trans*-translation complexes were first generated as a substrate for SmpB•tmRNA. SmpB•tmRNA•EF-Tu([γ^{32}P]GTP) quarternary complexes were formed by pre-incubating components. Quarternary complexes were mixed with pre-*trans*-translation complexes and reactions were quenched at 0, 10, and 60 second time points with formic acid. Liberated $^{32}P_i$ was then separated from [^{32}P]GTP by thin layer chromatography. Figure 4.5 depicts the results of GTP hydrolysis assays with and without tmRNA, with and without SmpB, and with the SmpB C-terminal truncation mutants SmpB^{153} and SmpB^{139}. In the presence of wt SmpB and tmRNA, I observed a rapid increase in the amount of liberated $^{32}P_i$ and a concurrent decrease in the amount of [^{32}P]GTP remaining between the 0 and 10 second time points. In the absence of either SmpB or tmRNA, I observed little or no increase in the amount of liberated $^{32}P_i$. The C-terminal tail truncation mutants SmpB^{153} and SmpB^{139} behaved like SmpB^{WT} in this assay. That is, in the presence of the mutant SmpBs rapid GTP hydrolysis occurred between the 0 and 10 second time points. Although these results are preliminary, they suggest that the C-terminal tail of SmpB is not involved in promoting GTP hydrolysis. Hence, the SmpB C-terminus appears to play a role in the tRNA-like function of tmRNA downstream of GTP hydrolysis, but prior to peptide bond formation.

4.5 Discussion

I have demonstrated that EF-Tu and GTP hydrolysis are required for transpeptidation onto tmRNA. Also, I've shown that while the C-terminal tail of SmpB is required to support tmRNA mediated peptide bond formation, it does not appear to be required to support activation of the EF-Tu GTPase and GTP hydrolysis. Therefore, the SmpB C-terminal tail seems to play a role in the tRNA-like function of tmRNA downstream of GTP hydrolysis, but prior to peptide bond formation.

Models of SmpB•tmRNA action predict that the tmRNA TLD behaves much like a canonical tRNA during the early stages of the *trans*-translation mechanism. The TLD is charged with alanine by Ala-RS and binds EF-Tu(GTP) *in vitro* [79, 80, 125, 126, 134-136, 138, 172]. However, due to the absence of codon-anticodon interactions, the mechanism of tmRNA entry into the ribosomal A-site is necessarily distinct from that of cognate tRNAs. Hence, discovering how the tmRNA TLD comes to be accommodated into the ribosomal A-site remains a key challenge in understanding the mechanism of *trans*-translation. Therefore, determining the role, if any, of EF-Tu and EF-Tu GTPase activity in this process is central to the understanding of tmRNA tRNA-like action. My results agree with

those of Hallier and colleagues, showing that omission of EF-Tu results in a dramatic defect in the rate of peptide bond formation [137]. My results further demonstrate that the GTPase activity of EF-Tu is also essential for rapid transpeptidation onto the tmRNA TLD. However, my results disagree with those of Shimizu and colleagues who reported no difference in the extent of peptide bond formation with and without EF-Tu [138]. In that study, however, the authors looked only at the extent of transpeptidation after a 30 minute incubation. On this time scale, peptide bond formation may have saturated even in the absence of EF-Tu.

The structure of canonical tRNAs exhibits a 90° angle, one side formed by the acceptor and T-arms, the other by the D-loop and anticodon arm. A recent co-crystal structure model of the tmRNA TLD in complex with SmpB suggests that the tmRNA TLD exhibits an extended conformation (around 120°) [81]. Interestingly, the positioning of SmpB in this co-crystal structure restores the 90° angle, with SmpB now occupying the position corresponding to the D-loop and anticodon arm of canonical tRNAs. This leads to the hypothesis that SmpB may serve as an anticodon mimic. That is, SmpB may make functional contacts in the ribosomal A-site that substitute for codon-anticodon interactions to drive GTP hydrolysis and accommodation of the tmRNA TLD. Also intriguing is the positioning of the SmpB C-terminus in this model. Although the unstructured C-terminal tail was truncated for crystallization, the base of the tail is positioned in close proximity to the decoding center when the co-crystal structure is docked into the ribosomal A-site [81]. This led me to hypothesize that the SmpB C-terminal tail may be making contacts near the decoding center analogous to codon-anticodon interactions. As such, I speculated that the tail might be required to promote activation of the EF-Tu GTPase. Preliminary results depicted in figure 4.5 suggest that that is not the case. Omitting the C-terminal tail completely (SmpB[139]) had no discernable effect on GTP hydrolysis activity.

Promoting GTPase activation is not the only function of codon-anticodon interactions in the canonical tRNA-mediated decoding process. Codon-anticodon interactions also serve to anchor the tRNA in the A-site during a post-GTP hydrolysis proofreading step. This, then, is one possible role for the SmpB C-terminal tail. It is possible that other regions of the SmpB protein or tmRNA are responsible for stimulating the ribosomal conformation switch that stimulates GTPase activation. In this case, the SmpB C-terminal tail could serve to prevent dissociation of the tmRNA TLD from the A-site after GTP hydrolysis.

Alternatively, contacts made by the C-terminal tail of SmpB may be required for accommodation of the tmRNA TLD. It seems unlikely that contacts made by this flexible region of the protein could drive the mechanical movement of the TLD acceptor arm into the peptidyl transferase center upon EF-Tu release. These contacts could, however, be required for proper positioning of the TLD during accommodation. That is, loss of the C-terminal tail could lead to a non-productive accommodation step where the acceptor stem does not fully reach the peptidyl transferase center. It is also possible that the SmpB C-terminal tail is required for release of EF-Tu(GDP) upon GTP hydrolysis. Analysis of the rates

of accommodation of the tmRNA TLD and release of EF-Tu(GDP) may help shed light on this issue. It is also formally possible that the SmpB C-terminal tail is important for GTPase activation, but the defect in the rate is subtle and can't be resolved on the 10 second time scale. This possibility seems unlikely in light of the complete loss of peptide bond formation activity with SmpB^{139} both *in vivo* and *in vitro*. Analysis of the rate of GTP hydrolysis utilizing rapid kinetics techniques will be required to fully clarify this point.

4.6 Figures

Figure 4.1

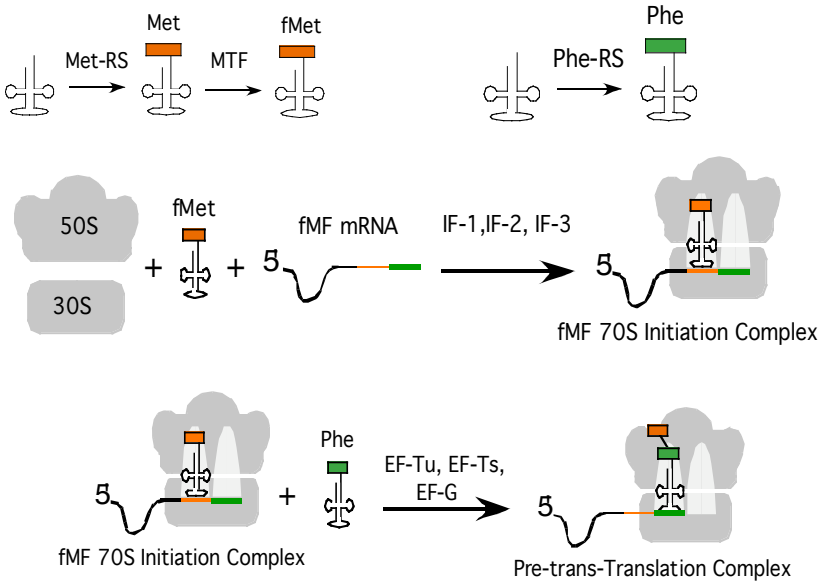

Figure 4.1: *Pre-trans-translation complex assembly.* Initiator tRNA is charged by Met-RS and formylated by MTF. $tRNA^{Phe}$ is charged by Phe-RS. 50S and 30S ribosomal subunits, fMet-$tRNA^{fMet}$, and fMF mRNA are assembled into 70S initiation complexes through the actions of IF-1, IF-2, and IF-3. The A-site phenylalanine codon is translated through the activities of Phe-$tRNA^{Phe}$, EF-Tu, and EF-Ts. The ribosome is translocated through the action of EF-G. This leaves a pre-*trans*-translation complex with fMet-Phe-$tRNA^{Phe}$ peptidyl tRNA in the P-site and an empty A-site, lacking both tRNA and mRNA

Figure 4.2

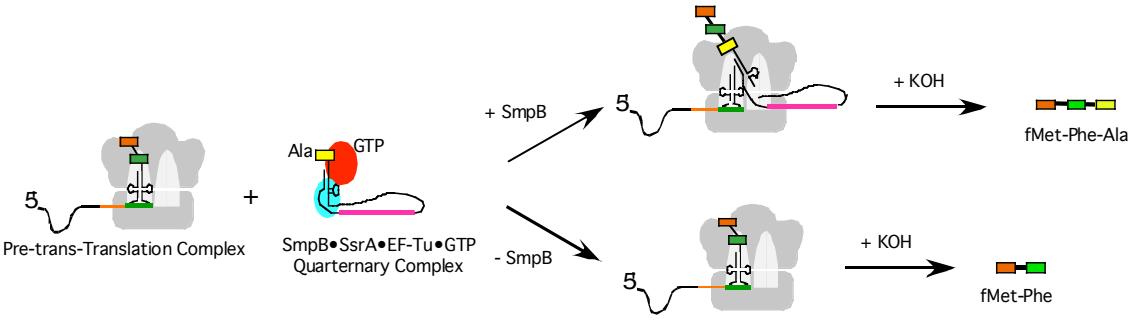

Figure 4.2: *Peptide bond formation assay.* fMF pre-*trans*-translation complexes are reacted with pre-formed SmpB-tmRNA-EF-Tu(GTP) quarternary complexes. In the presence of functional SmpB, fMet-Phe dipeptide is converted to fMet-Phe-Ala tripeptide. fMet-Phe and fMet-Phe-Ala may be separated by HPLC.

Figure 4.3

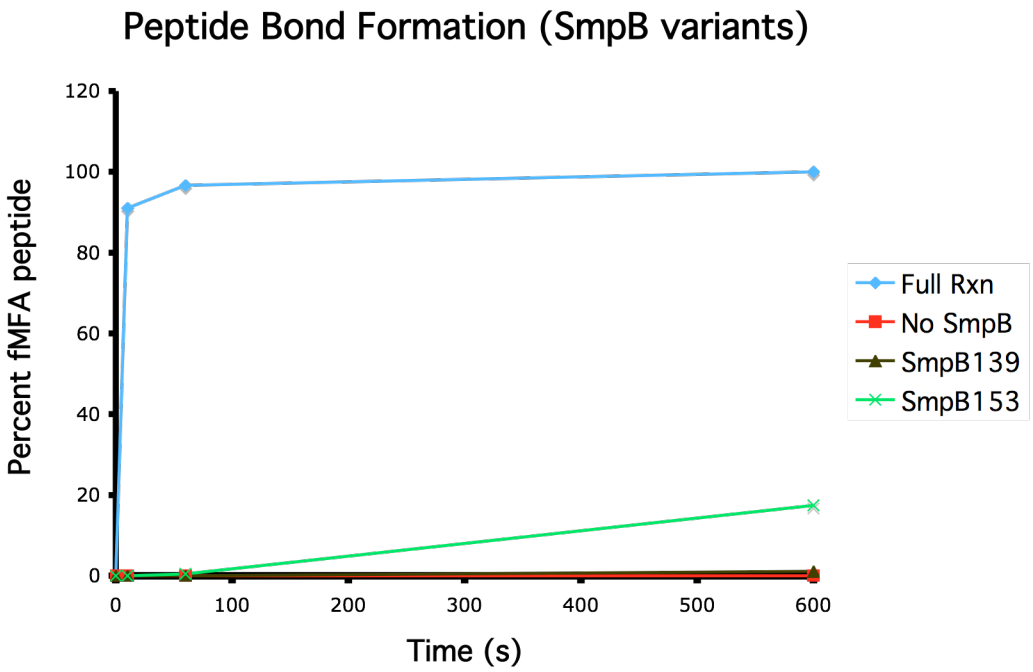

Figure 4.3: *Peptide bond formation activity supported by SmpB C-terminal tail mutants.* Peptide bond formation assay results. Peptide bond formation assays were performed using either no SmpB, $SmpB^{WT}$, or SmpB truncation mutants. The percent of fMF peptide that was converted to fMFA peptide was monitored by reverse phase HPLC.

Figure 4.4

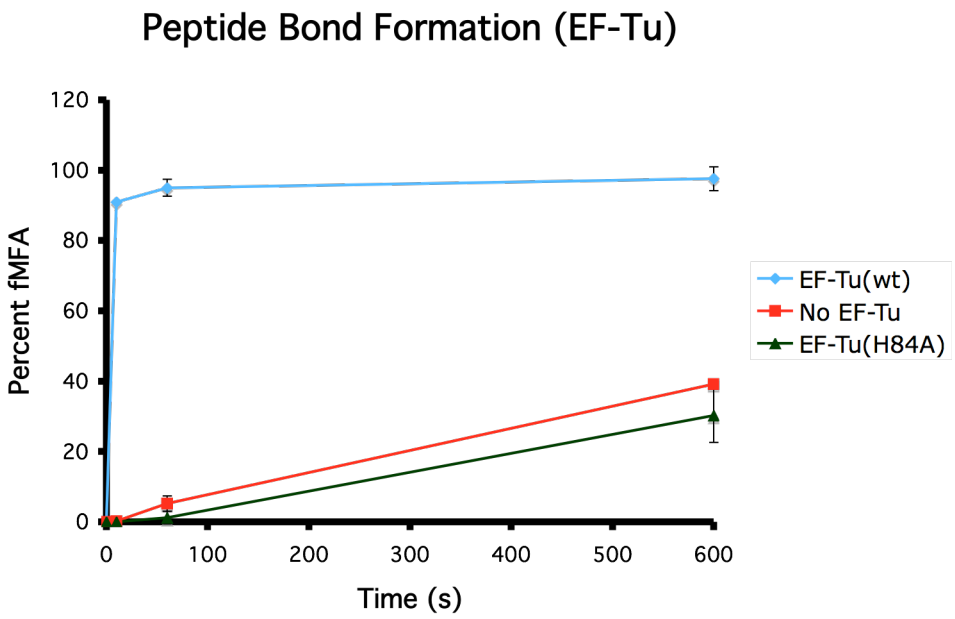

Figure 4.4*: EF-Tu dependence of tmRNA-mediated peptide bond formation activity.* Peptide bond formation assays were performed using either no EF-Tu, wild type EF-Tu, or the GTPase defective EF-Tu mutant EF-Tu(H84A). The percent of fMF dipeptide converted to fMFA tripeptide was monitored by HPLC.

Figure 4.5

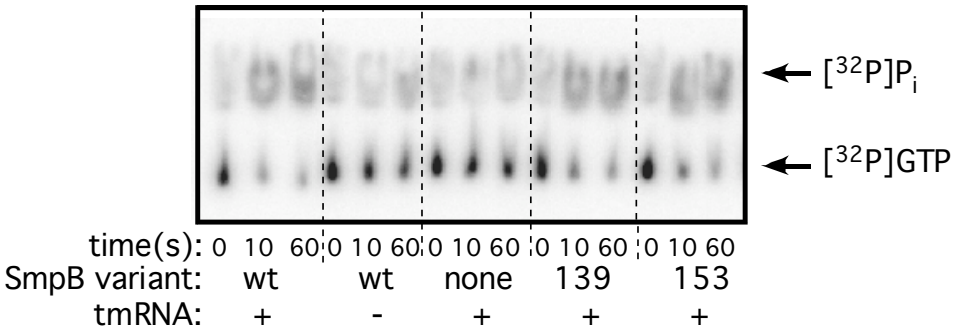

Figure 4.5: *GTP hydrolysis activity using SmpB truncation mutants.* SmpB•tmRNA•EF-Tu([γ^{32}P]GTP) quaternary complexes were mixed with stalled ribosomes *in vitro*. Thin layer chromatography was used to separate P_i from GTP. Accumulation of P_i and concomitant loss of GTP indicate GTP hydrolysis activity.

Chapter 5: Concluding Remarks

5.1 Summary

In this thesis, I have described experiments aimed at answering specific, key questions related to the mechanism of the SmpB•tmRNA mediated translational control mechanism known as *trans*-translation. In the early stages of the *trans*-translation mechanism, SmpB and tmRNA must recognize stalled ribosomes and bind in the ribosomal A-site. It is clear that SmpB is required to support association of tmRNA with stalled ribosomes[4, 123, 124], however two competing models are available to describe the assembly of this SmpB•tmRNA•stalled ribosome complex. In one model, free SmpB binds in the A-site of stalled ribosomes and recruits tmRNA to initiate *trans*-translation. The second model predicts that a pre-formed SmpB•tmRNA•EF-Tu(GTP) quarternary complex (analogous to the tRNA•EF-Tu(GTP) ternary complex) recognizes and binds stalled ribosomes. My results suggest that the reported free SmpB•ribosome complex (formed in the absence of tmRNA) is non-specific and physiologically irrelevant. Interaction of free SmpB with ribosomes is labile and salt sensitive both *in vivo* and *in vitro*. SmpB co-localizes with tmRNA in subcellular fractions. Most strikingly, tmRNA is required for enrichment of SmpB in stalled ribosomes. These results are inconsistent with the SmpB•ribosome pre-binding model, and argue strongly for binding of a pre-formed SmpB•tmRNA•EF-Tu(GTP) quarternary complex to stalled ribosomes to initiate the *trans*-translation mechanism.

SmpB protein possesses a C-terminal tail that lacks structure in solution [70, 81, 88, 89]. Through site directed mutagenesis and *in vivo* based functional assays, I've shown that deletion of the tail, or mutation of specific residues near the C-terminus abolishes SmpB's activity in supporting tmRNA mediated tagging. Interestingly, C-terminal tail mutants still retain their ability to support the two known functions of the protein, binding to tmRNA and promoting association of the SmpB•tmRNA complex with stalled ribosomes. Hence, I've identified a novel function of SmpB protein in *trans*-translation, a function that requires an intact C-terminal tail.

I've assembled an *in vitro trans*-translation system in order to pinpoint the specific function of the SmpB C-terminal tail in the molecular mechanism of *trans*-translation. I've shown that the C-terminal tail is required to support transpeptidation onto the TLD of tmRNA *in vitro*. This finding is consistent with

the results of an *in vivo* reporter tagging assay. Hence, the novel SmpB function performed by the tail occurs during the tRNA-like function of tmRNA. Utilizing the *in vitro trans*-translation system, I was able to demonstrate that, like canonical tRNAs, tmRNA requires EF-Tu-mediated GTP hydrolysis for peptide bond formation. However, the SmpB C-terminal tail does not appear to be involved in eliciting activation of the EF-Tu GTPase and GTP hydrolysis, as mutant SmpBs lacking the C-terminal tail were fully functional in supporting GTP hydrolysis activity *in vitro*.

The work presented in this thesis provides new insights into the mechanism of SmpB•tmRNA mediated *trans*-translation. *trans*-translation is a bacteria-specific mechanism required for survival and/or virulence in bacterial pathogens [106-109], making it a prime candidate for the rational design of antibacterial compounds. A more thorough understanding of the mechanism of SmpB•tmRNA action should bring to light new targets for design of such compounds. Perhaps more striking than the potential downstream clinical applications of the study of *trans*-translation, it represents a novel variation on the well-characterized protein synthesis mechanism. From a basic biochemical perspective, understanding how ribosome-catalyzed peptide bond formation may occur in the absence of codon-anticodon interaction will shed new light on basic ribosomal activity and perhaps lend to a deeper understanding of the canonical tRNA-mediated decoding process. Equally fascinating is the question of what mechanistic events mediate the ribosomal reading frame switch to initiate translation of the tmRNA ORF. The work presented in this thesis represents significant steps toward the understanding of this elegant translational control mechanism.

5.2 Remaining Questions

5.2.1 Does the C-terminal tail of SmpB effect the rate of GTP hydrolysis on a rapid time scale?

Results presented in chapter 4 suggest that the C-terminal tail of SmpB is dispensable for eliciting GTP hydrolysis. However, GTP hydrolysis occurs on a time scale of milliseconds when a cognate tRNA-containing ternary complex binds the ribosome. As such, it is formally possible that SmpB C-terminal tail mutants may exhibit a subtle defect in supporting GTP hydrolysis (up to one order of magnitude). Such a defect would not be detected on the 10 second time scale used for GTP hydrolysis assays in chapter 4. Repeating the GTP hydrolysis assays on a millisecond time scale using a quench flow rapid kinetics instrument would more firmly demonstrate whether or not the SmpB C-terminal tail is involved in activation of the EF-Tu GTPase.

5.2.2 Do SmpB C-terminal tail mutants affect the rate of accommodation of the tmRNA TLD?

The C-terminal tail of SmpB is required to support peptide bond formation, but is dispensable in supporting GTP hydrolysis. Therefore, it may be involved in accommodation of the tmRNA TLD into the peptidyl transferase center. The rate of accommodation of tRNAs is traditionally measured by fluorescence stopped-flow techniques. By covalently attaching an environmentally sensitive fluorophore to a specific modified base (dihydrouridine) in tRNAs, investigators are able to monitor accommodation by measuring the change in fluorescence associated with movement of the tRNA into the peptidyl transferase center. In principle, such an approach could be used to study accommodation of the tmRNA TLD, in order to look for potential differences in the rate of accommodation of the TLD when wild type SmpB or SmpB C-terminal tail mutants are added to an *in vitro* reaction. In practice this approach is complicated by the fact that tmRNA does not possess a dihydrouridine. Hence, measuring the rate of accommodation of the tmRNA TLD awaits the development of techniques to specifically label the tmRNA TLD (or SmpB) with an environmentally sensitive fluorophore.

5.2.3 Do SmpB C-terminal tail mutants support release of EF-Tu(GDP)

When a cognate tRNA binds the ribosomal A-site, GTP hydrolysis is followed by release of EF-Tu(GDP). This, then, allows the tRNA to swing into the peptidyl transferase center to participate in peptide bond formation. One possible explanation for the GTP hydrolysis and peptide bond formation phenotypes of SmpB C-terminal tail mutants is that EF-Tu(GDP) is unable to dissociate from the complex after GTP hydrolysis. The absence of the SmpB C-terminal tail may result in a ribosomal A-site conformation that does not permit EF-Tu release. The *in vitro trans*-translation system that I've developed is ideally suited to address this question. One could assemble a large-scale peptide bond formation reaction, pellet the ribosomes by centrifugation, and look for EF-Tu in the ribosomal and non-ribosomal fractions by western blotting. Alternatively, including [α-^{32}P]GTP in the reaction mix would allow the monitoring of release of GDP after GTP hydrolysis.

5.2.4 What contacts does the SmpB C-terminal tail make in the ribosomal A-site?

In concert with functional assays designed to identify the molecular mechanism of SmpB C-terminal tail activity, biochemical identification of the binding site(s) of the tail in the ribosomal A-site would be invaluable in determining its function. This could be accomplished via footprinting of ribosomal RNA as well as cross-linking to identify any protein-protein interactions involving the tail. Briefly, one could assemble peptide bond formation assays including either wild type SmpB or SmpB^{139}. The reaction could be stalled in a variety of ways (i.e. inclusion of kirromycin to block EF-Tu release, use of a non-hydrolyzable GTP analog such as GTPγS to block GTP hydrolysis, using the GTPase defective EF-Tu(H84A), or using an antibiotic such as chloramphenicol

that blocks peptide bond formation) in order to obtain a synchronized population of ribosomes. One could then perform crosslinking and footprinting experiments, comparing the patterns obtained with wild type and mutant SmpB and identifying unique contacts using mass spectrometry. Identification of the C-terminal tail binding site, in concert with our current knowledge of tRNA A-site interactions may provide valuable clues about the specific function of the C-terminal tail.

5.2.5 What events lead to EF-Tu GTPase activation when SmpB•tmRNA•EF-Tu(GTP) binds the A-site of a stalled ribosome?

Cognate codon-anticodon interactions are required to elicit GTPase activation in tRNA•EF-Tu(GTP) ternary complexes. However, with SmpB•tmRNA, GTP is hydrolyzed in the absence of a codon or anticodon. Preliminary results suggest that the SmpB C-terminal tail is not responsible for eliciting GTPase activation. However, considering the positioning of SmpB in a recent three dimensional model [81], it seems likely that SmpB is involved in this process. Since SmpB is required to bring tmRNA to the ribosome, omitting it from an *in vitro* GTP hydrolysis assay won't provide any insight into its putative role in GTPase activation. Instead, a useful approach would be to attempt to isolate SmpB mutants that are able to support tmRNA-ribosome association, but fail to support GTP hydrolysis. As the structure of SmpB is known, one might begin by mutating conserved surface residues on a surface of the protein with unknown function. Techniques outlined in this thesis, including use of the *in vitro trans*-translation system should lend themselves well to this aim. Upon isolation of such mutations, it would be useful to define the ribosomal binding site of the mutated region, using techniques described in the previous section.

5.2.6 What other protein factors are involved in *trans*-translation

In chapter two, I described the purification of a ribosome pool that is enriched for stalled ribosomes. Proteomics approaches, comparing the representation of proteins in stalled versus total ribosomes should permit the identification of other proteins present on stalled ribosomes. The in *vivo* functional assays described in chapter two, as well as the *in vitro trans*-translation system highlighted in chapter four may then be used to help define the role, if any, that newly identified factors play in *trans*-translation.

SmpB•tmRNA-mediated tagging occurs not only at the end of non-stop messages, but at internal mRNA positions that lead to ribosome stalling [5, 93-96]. For example, tandem rare arginine codons drive scarcity of cognate tRNAs, leading to ribosome stalling and *trans*-translation [6, 8, 95, 99]. It is thought that a co-translational mRNA cleavage event occurs to convert these mRNAs to non-stop mRNAs, making them substrates for SmpB•tmRNA [92, 97-100]. One major unanswered question, then, is what is the nuclease that is responsible for this cleavage event. Proteomic analysis of a purified pool of ribosomes stalled on a rare codon containing mRNA may help address this question.

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