FUNCTIONAL STUDIES OF TRANSFER RNA INTERACTIONS IN THE

RIBOSOME

DISSERTATION

Presented in Partial Fulfillment of the Requirements for

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By

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*****

The Ohio State University

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ABSTRACT

During translation, tRNAs must move rapidly to their adjacent sites on the ribosome, while maintaining precise pairing with mRNA. In this work, the interactions of tRNA with the ribosome were examined in order to gain insights into the mechanisms by which the ribosome maintains the correct reading frame, and those interactions which contribute to rapid translocation.

Mutant tRNAs with expanded anticodon loops have been isolated, which are capable of decoding a four-nucleotide sequence of mRNA. Using an in vitro assay with purified components, the position of mRNA was mapped with respect to the ribosome to determine the molecular basis by which these expanded-anticodon loop tRNAs recognize and position a quadruplet frameshift sequence. The results of these experiments suggest a new model for frameshift suppression by tRNAs with expanded anticodon loops, and provide insights into how the ribosome normally maintains the reading frame (Chapter 2).

Movement of tRNAs through the ribosome is thought to take place in a stepwise manner, with hybrid intermediates. This work demonstrates that movement of tRNA into one of these hybrid-tRNA binding sites, the P/E site, destabilizes the mRNA (Chapter 3). In order to determine how these hybrid states relate to kinetically defined events of
translocation, substitutions in tRNA or rRNA were examined which inhibited movement into either the P/E site or the A/P site. These data suggest that movement of tRNA into the P/E and A/P sites are separable events and allow tRNA movements with respect to both subunits to be integrated into a kinetic model for translocation (Chapter 4).
Dedicated to H. Norman Walker
ACKNOWLEDGMENTS

I would first like to express my gratitude to Kurt Fredrick for his time and dedication as an advisor, optimistic attitude, and commitment to thoroughly scrutinizing lab work, presentations, and writing. I feel fortunate to have been the first student to join the lab and to be given so many opportunities to pursue interesting projects, present data, and learn different techniques.

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I also thank Barry Cooperman (U Pennsylvania) and his lab (especially Dongli Pan) for hosting me in Philadelphia, sharing advice on measuring the GTPase activity of EF-G, and performing and editing kinetic modeling on the model proposed in Chapter 4. Dr. Cooperman also made important suggestions in revising the PNAS manuscript.
Thanks is also given to current and former members of the Fredrick lab for sharing data and for assistance on various projects: specifically Nimo Abdi for constructing the SQZ10 strains (Chapter 3); Kevin McGarry for performing Quickchange mutagenesis and collaborating to collect a full data set on our overlapping projects (as noted and discussed in Chapter 3 and published in Molecular Cell); Ichi Shoji for taking on the reverse translocation project and for performing kinetic modeling on the results of Chapter 4 (published in Molecular Cell and PNAS); Xiaofen Zhong for developing an \textit{in vitro} translation system used to compare activity of mutant ribosomes (data not discussed). I also thank Daoming Qin for discussions, critical reviews, and for sharing his story about pulling the safety shower, and Aishwarya Devaraj and Sean McClory for their friendship over the past year.

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<td>Å</td>
<td>angstrom (unit)</td>
</tr>
<tr>
<td>A site</td>
<td>aminoacyl/acceptor site</td>
</tr>
<tr>
<td>aa</td>
<td>aminoacyl</td>
</tr>
<tr>
<td>ASL</td>
<td>anticodon stem loop</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>basepair</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>cryo-EM</td>
<td>cryogenic electron microscopy</td>
</tr>
<tr>
<td>CTD</td>
<td>carboxy-terminal domain</td>
</tr>
<tr>
<td>da</td>
<td>Dalton (unit)</td>
</tr>
<tr>
<td>EF</td>
<td>elongation factor</td>
</tr>
<tr>
<td>E site</td>
<td>exit site</td>
</tr>
<tr>
<td>FRET</td>
<td>Forster resonance energy transfer</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
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<td>messenger RNA</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>P site</td>
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</tr>
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<td>Full Form</td>
</tr>
<tr>
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<td>-----------</td>
</tr>
<tr>
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<td>posttranslocation complex</td>
</tr>
<tr>
<td>PRE</td>
<td>pretranslocation complex</td>
</tr>
<tr>
<td>PTC</td>
<td>peptidyl transferase center</td>
</tr>
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<td>ribosomal protein</td>
</tr>
<tr>
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<td>ribosomal RNA</td>
</tr>
<tr>
<td>RSR</td>
<td>ratchet-like intersubunit rotation</td>
</tr>
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</tr>
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</tr>
<tr>
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<td>single-molecule Forster resonance energy transfer</td>
</tr>
<tr>
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<td>sparsomycin</td>
</tr>
<tr>
<td>suf</td>
<td>suppressor of frameshift</td>
</tr>
<tr>
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<td>transfer RNA</td>
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1.1. Overview of translation

The information content of genetic material is converted into specific functions by the transcription of DNA to RNA, and the translation of mRNA to protein. Translation is orchestrated by the ribosome, a 2.5 MDa RNA-protein machine, which engages transfer RNAs (tRNA) to synthesize a polypeptide chain corresponding to a defined sequence of mRNA. Translation is a complex process and can be divided into four phases: 1) initiation involves the assembly of a ribosome complex with an initiator tRNA paired to the correct start codon; 2) elongation is the phase in which the polypeptide is built according to the mRNA sequence; 3) termination occurs when a stop codon is read by a release factor, which then cleaves the polypeptide from the tRNA; and 4) recycling is the splitting of ribosomes into subunits following termination.

During elongation (Fig. 1.1), tRNAs interact with three defined binding sites on each subunit of the ribosome: the aminoacyl (A) site, the peptidyl (P) site, and the exit
(E) site. Elongation is a cyclic process of three steps: 1) decoding, the accurate pairing of a tRNA anticodon with a mRNA codon, 2) peptidyl transfer, the transfer of a peptide to an aminoacyl (aa) tRNA, and 3) translocation, the concerted movement of tRNA and mRNA. First, the correct aa-tRNA must pair with mRNA in the A site in the multistep GTP-dependent decoding process. Elongation factor Tu (EF-Tu) catalyzes this delivery of tRNA to the A site, as part of a ternary complex with GTP. Upon binding of a cognate tRNA, ribosomal elements recognize the geometry of the basepair and stimulate the GTPase activity of EF-Tu (1). This leads to release of EF-Tu, and movement of the acceptor end of the tRNA into the A site (2). The peptidyl transferase reaction occurs immediately after accommodation of the aa-tRNA in the A site, and is catalyzed by the rRNA of the large subunit (3). There is evidence that the ribosome acts as an entropic trap, meaning that it decreases the entropic component of the activation energy barrier for peptidyl transferase without appreciably affecting the activation enthalpy (4, 5). In other words, the ribosome contributes to catalysis by the positioning of substrates, rather than by lowering the heat of activation. After each peptide bond is formed, a deacylated tRNA remains in the P site and a peptidyl tRNA in the A site. The mRNA must be displaced by one codon concurrently with the movement of its corresponding tRNA to allow the cycle to continue. This passage of tRNAs through the ribosomal subunit interface occurs in a stepwise manner, with hybrid state intermediates (6). First the acceptor ends of the tRNAs move with respect to the large (50S) subunit, into hybrid binding sites (A/P for a tRNA bound in the 30S A site and 50S P site and P/E for a tRNA bound in the 30S P site
and 50S E site). Next, the anticodon ends move to the adjacent sites on the small (30S) subunit, along with mRNA, in an EF-G-dependent fashion.

![Diagram of the Hybrid States Model for Translation Elongation](image)

**Figure 1.1. The Hybrid States Model for Translation Elongation.** Described in text.

It was proposed that tRNAs move through hybrid binding sites long before the availability of any biochemical or structural data as a logical means to explain how tRNAs maintain the reading frame during translocation (7, 8). The first data that supported the possibility of hybrid binding sites came from chemical probing experiments performed before and after treating complexes with puromycin, a drug that mimics the 3’ end of an aa-tRNA by binding to the 50S A site and acting as an A-site substrate for peptidyl transfer. When puromycin binds, it reacts with the nascent peptide to deacylate P-site tRNA, and the resulting peptidyl-puromycin product dissociates from the ribosome. When complexes containing peptidyl-tRNA in the P site were treated with puromycin, the chemical protection patterns changed (6). 50S P-site protections disappeared, 50S E-site protections appeared, and 30S P-site protections remained mostly
unchanged. This suggested that when the peptidyl-tRNA became deacylated, it moved spontaneously with respect to the 50S subunit, but did not move with respect to the 30S subunit (e.g. P/E-site binding). In the same study, evidence was obtained to suggest that tRNA can occupy the A/P site. When aa-tRNA was delivered to the A-site of a complex containing peptidyl-tRNA in the P site, peptide bond formation left a complex with a peptidyl-tRNA in the A site, and a deacylated tRNA in the P site. Following peptidyl transfer, the 50S A site was not protected, and the 50S E site became protected, and the 30S A- and P-site protections were protected, suggesting that both tRNAs moved with respect to the 50S subunit into the P/E and A/P sites. Subsequent addition of EF-G•GTP resulted in movement of tRNA within the 30S subunit, as indicated by the loss of 30S A site protections. Together these data indicated that while tRNAs can move spontaneously with respect to the large subunit following peptide bond formation, EF-G•GTP is necessary to catalyze 30S subunit movement.

1.2. Ribosome structure

The prokaryotic ribosome is a 2.5 MDa machine comprised of 3 rRNAs (16S, 23S, and 5S) and more than 50 proteins (Table 1.1).
Table 1.1. Properties of *Escherichia coli* ribosomes. Table was adapted from (9).

<table>
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<tr>
<th></th>
<th>Ribosome</th>
<th>Small Subunit</th>
<th>Large Subunit</th>
</tr>
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<tbody>
<tr>
<td>Sedimentation coefficient</td>
<td>70S</td>
<td>30S</td>
<td>50S</td>
</tr>
<tr>
<td>Mass (kDa)</td>
<td>2520</td>
<td>930</td>
<td>1590</td>
</tr>
<tr>
<td>RNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Major</td>
<td>16S, 1542 nt</td>
<td>23S, 2904 nt</td>
<td></td>
</tr>
<tr>
<td>Minor</td>
<td>5S, 120 nt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA mass (kDa)</td>
<td>1664</td>
<td>560</td>
<td>1104</td>
</tr>
<tr>
<td>Proportion of mass</td>
<td>66%</td>
<td>60%</td>
<td>70%</td>
</tr>
<tr>
<td>Proteins</td>
<td>21</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Protein mass (kDa)</td>
<td>857</td>
<td>370</td>
<td>487</td>
</tr>
<tr>
<td>Proportion of mass</td>
<td>34%</td>
<td>40%</td>
<td>30%</td>
</tr>
</tbody>
</table>

The rRNA accounts for roughly 2/3 of the mass, and forms most of the core of the ribosome, while many of the proteins, which tend to be nonglobular and basic, bind the rRNA on the periphery. The two subunits join during initiation to form a spherical particle of ~250 Å in diameter. Transfer RNAs, the substrates of the ribosome, are also quite large (>25 kDa) and bind at the interface of the small (30S) and large (50S) subunits (Figure 1.2). There is compelling evidence that tRNA binding enhances the stability of the joined subunits in the 70S ribosome, the particle active in elongation. Several intersubunit bridges (i.e. contacts between the rRNAs and/or proteins that join the two subunits) also form within the 70S, and this bipartite structure purportedly facilitates the trafficking of tRNA molecules (7, 8, 10).
Figure 1.2. Crystal structure of the 70S ribosome with tRNAs and mRNA bound at 5.5 Å. Sliced view looking down on the tRNA binding sites. The 23S rRNA is pictured in gray, large subunit proteins are magenta, 16S is cyan, small subunit proteins are dark blue, mRNA is green, and the E-site, P-site, and A-site tRNAs are red, orange, and yellow, respectively. Adapted from reference (11).

While the structure of tRNA has been known since the 1970’s, little was known about the structure of the ribosome until recently. Several X-ray crystal structures of prokaryotic ribosomes, individual subunits, auxiliary factors, and complexes of the ribosome with ligands bound have become available in the last 8 years (reviewed in detail in reference (12)). High resolution structures of the 50S subunit [Haloarcula marismortui, 2.4 Å (3); Deinococcus radiodurans, 3.1 Å, (13)], 30S subunit [Thermus thermophilus, 3.05 Å (14)], and 70S ribosome [T. thermophilus, 2.9 Å (15); Escherichia
coli, 3.5 Å (16)] have contributed to the current level of understanding of the rRNA and r-protein interactions necessary for formation of the conserved globular structure.

Structures of the *T. thermophilus* 30S subunit are also available with cognate and near-cognate A-site substrates paired with mRNA [3.0-3.8 Å, (1)], initiation factors [IF1, 3.2 Å (17); CTD of IF3, 4.2 Å, (18)], and various antibiotics [*T. thermophilus*, 4.5 Å, (18); 3.0 Å, (19)]. These structures revealed ribosomal elements involved in recognition of correct codon-anticodon pairing in the A site and conformational changes which may contribute to selection of the initiator tRNA and start codon in the P site, although the placement of IF3 and tetracycline in structures from the Max-Planck/Weismann group are considered questionable (12, 18). A limitation of the currently available structures of the 30S subunit is the packaging in the crystals. The spur of one 30S subunit acts as a tRNA anticodon stem loop mimic in the P-site of another, occluding the binding of canonical tRNA anticodon stem loops and mRNA to the P site, and likely prevents the binding of most of the initiation and elongation factors (14). This may explain the placement of IF3 on the solvent face of the 30S (18), distal to the site mapped biochemically (20).

Structures of the 50S subunit with tRNA acceptor ends, transition state analogs, and various antibiotics bound [>10 structures from *H. marismortui* and *D. radiodurans* at ~2-4 Å, reviewed in (21)] have assisted in deciphering the roles of rRNA and tRNA in peptide bond formation, and the mechanisms by which antibiotics inhibit protein synthesis. Binding of tRNA–CCA ends and minihelices to the archaeal *H. marismortui* 50S subunit also revealed the first high-resolution view of the 50S E-site interactions
with tRNA (22). When the *H. marismortui* 50S structure was compared to the later structure of the *T. thermophilus* 70S ribosome with E-site tRNA bound (15), differences in the E sites of the bacterial and archaeal ribosomes became apparent, which contribute to the differential susceptibility of bacteria and higher organisms to E-site binding antibiotics (23).

Perhaps most relevant to studies on translocation, however, are several structures of the intact 70S ribosome bound with mRNA and tRNAs [*T. thermophilus*: 5.5 Å, (11), Figure 1.2; 2.8 Å, (15); 3.7 Å, (24); 4.5 Å, (25); *E. coli*, 3.5 Å, (26)], which reveal the orientations of the tRNA molecules with respect to rRNA elements, and the placement of the mRNA as it moves through the 30S subunit. The early *T. thermophilus* 70S structure provided conclusive evidence that tRNA binds to an exit site on both the 30S and 50S subunits, which had been a matter of controversy, as no 30S E-site specific base protections had been observed prior to that point (11). More recent structures of the 70S ribosome also shed light on the subject of codon-anticodon pairing in the E-site, as it became apparent that when a Shine-Dalgarno (SD) helix was paired with the complementary end of the 16S rRNA (the anti-Shine-Dalgarno sequence, ASD), E-site codon-anticodon pairing was prohibited (15, 25). In the absence of SD-ASD interactions, E-site codon-anticodon pairing was more likely to occur (25). However, there are no available high-resolution crystal structures of a canonical translation elongation complex, or of the ribosome with elongation factors bound.

Cryogenic-electron microscopy (Cryo-EM) images lend a lower resolution view (~10-20 Å) of the ribosome, but have greatly extended our knowledge of the dynamics of
tRNA and ribosomal elements during translation by capturing images of various functional translation complexes that have yet to be crystallized.

Fig. 1.3. Cryo-EM reconstruction of the 70S ribosome with deacylated tRNA(s) bound in the presence (right) or absence (left) of EF-G. In the left image, deacylated tRNA (red) is bound to the E/E site and P/P site (not visible), while in the right image, deacylated tRNA (green) is bound to the P/E site. The large subunit is in cyan, while the small subunit is in yellow, with EF-G in red. The hatched arrow indicates inward movement of L1, while the solid arrow indicates the direction of 30S movement during the ratchet-like-intersubunit-reorganization (RSR). CP, central protuberance; sb, L7/L12 stalk base (remainder of L7/L12 stalk is not shown); h, head of 30S subunit; sp, spur (helix 6) of the small subunit. Image adapted from reference (27).

In particular, cryo-EM images of the ribosome bound with EF-G gave the first glimpse of large-scale rearrangements of the ribosome that occur (Fig 1.3, references (27-29)). Binding of EF-G resulted in several movements. First of all, the two subunits of the ribosome had undergone a ratchet-like subunit rotation (RSR), in which the small subunit was rotated with respect to the large subunit (Figure 1.3) (29). Another noticeable
movement was that of the two stalks of the 50S subunit (28). The L7/L12 stalk, a region of the 50S known to interact with the GTPase translation factors entering the A site, appeared bifurcated, whereas the L1 stalk, a mushroom shaped domain composed of L1 and its rRNA binding site which makes up the majority of the 50S E site contacts, had moved inward towards the subunit interface (Figure 1.3). Structures of 70S•EF-G complexes containing deacylated tRNA in the P site indicated that in this ratcheted state of the ribosome, tRNA occupied the hybrid P/E site, and the L1 arm of the ribosome appeared to contact the P/E site tRNA (27).

In addition to structures of ribosomal complexes, there are also several structures available of isolated elongation factors. Several structures of EF-Tu and EF-G became available in the mid-1990’s (30-33). What was immediately clear upon comparison was that the structure of the ternary complex of EF-Tu•GDPNP•aa-tRNA and that of EF-G are strikingly similar. EF-G is a five-domain protein with two domains that are homologous to EF-Tu, as well as three additional domains (Figure 1.4). When the homologous domains of EF-G were overlaid onto EF-Tu, the extensions of EF-G occupied a similar position to that of the aa-tRNA in the ternary complex. One of these additional domains (domain IV) overlapped both the position and charge distribution of the anticodon stem loop (ASL) of the tRNA, suggesting that EF-G acts a molecular mimic of the ternary complex.
Figure 1.4. Comparison of prokaryotic elongation factors. Comparison of the crystal structure of the ternary complex aa-tRNA\textbullet{}EF-Tu\textbullet{}GTP (PDB1TTT) with that of EF-G (PDB1WDT) and a homology model for *E. coli* LepA [Note: homology model shown here places domains III and IV of LepA in slightly different orientations than shown in a recently published crystal structure of LepA (see reference (34)), although overall domain placement is similar]. The domains of EF-G are indicated with roman numerals, except for the G’ subdomain of domain I. Figure adapted from reference (35).

The structure of a third elongation factor, LepA (also referred to as EF4), was recently published (35). This factor is also a homolog of EF-G, but lacks an equivalent to domain IV and the G’ domain, and contains a C-terminal extension not found in EF-G. LepA is thought to catalyze reverse translocation, and the extended domains of LepA and EF-G are predicted to confer specificity in catalyzing forward or reverse translocation (Fig. 1.4; references (34, 35)).
Figure 1.5. Side view of the crystal structure of the 70S ribosome with tRNAs bound. Sliced view looking into A site. Coloring as in Figure 1.2. Adapted from reference (11).

A view of the A-site side of the 70S ribosome reveals a funnel-shaped area which serves as the entrance channel of the ribosome, allowing aa-tRNAs, mRNA, and translation factors to bind (Fig 1.4) (11). From this view, it is apparent that the ASLs of tRNAs interact with the 30S subunit, while the remainder of the tRNA contacts are formed on the 50S subunit. A rotated view looking down on the tRNAs reveals the active sites on each subunit (Figure 1.2). The anticodon of the A-site tRNA pairs with mRNA in the decoding center on the small subunit, whereas the acceptor ends of the A and P site tRNAs meet in the large subunit peptidyl transferase center (PTC).
Less obvious are those elements which contribute to translocation. Because tRNAs are displaced by 20-70 Å during each movement event, it is unlikely that a single active site is responsible for their movement. The binding site of the prokaryotic translocase, EF-G, was mapped by chemical probing and directed hydroxyl radical probing to the region near the entrance channel, where the factor appeared to contact the parts of the A site, Sarcin-ricin loop (SRL), and the L7/12 stalk of the large subunit, and extended into the A site of the small subunit (36, 37). Cryo-EM reconstructions of various complexes showed EF-G in similar locations that agree with these biochemical data, and also revealed conformational changes induced by high-affinity binding of the factor (29, 38). These large-scale conformational changes involve the RSR (described in section 1.2 and figure 1.3), which would entail extensive reorganization of the subunit interface and intersubunit bridges, and movement of tRNA into the P/E site (27, 39).

1.3. tRNA interactions during translation

Efficient translocation was observed when EF-G•GTP was added to PRE complexes containing an anticodon stem loop (ASL) in the A site, suggesting that 50S contacts to the A-site substrate were not essential for translocation (40). However, replacement of full-length peptidyl-tRNA with an ASL in the A site of the PRE complex resulted in a >300-fold decrease in the apparent translocation rate (41). This suggests that interactions between the A-site substrate and the 50S subunit are important. There is also evidence that the length of the peptidyl group on the A site tRNA influences the rate of translocation (41-43). Replacement of a peptidyl group with an aminoacyl group on the
tRNA moving out of the A site decreased the rate and the extent of translocation (Table 4.1) (42, 44, 45). Chemical protection and single-molecule FRET experiments also provided evidence that aminoacyl-tRNA is defective in A/P state formation (6, 46-48), which suggests that the defects conferred by aminoacyl-tRNA are due to its inability to occupy the A/P state. Residue G2553 of the 50S A site forms a Watson-Crick basepair with C75 of the tRNA to stabilize its binding. Mutation G2553U (predicted to destabilize tRNA in the A/A site) suppressed the inhibitory effect of the aminoacyl group, further suggesting that this group inhibits the A/A-to-A/P transition (45). When the rate of translocation was monitored in ribosomes harboring G2553U, it was actually increased relative to wild-type, suggesting that it A/P-site binding may be conformationally linked to the rate-limiting step (43). These results imply an important role for A/P site binding in maintaining rapid translocation, potentially by destabilizing tRNA in the A site, as has been suggested (42).

While an ASL can suffice as an A-site substrate, no translocation was observed when the PRE complex contained an ASL in the P site (40). Moreover, modification or truncation of the 3’ terminal adenosine (A76) of the tRNA moving out of the P site decreased the rate of translocation and inhibited binding to the E site, suggesting that interaction of A76 of the P-site tRNA with the E site is necessary for rapid translocation (49). The observation that the 2’ hydroxyl groups at positions 71 and 76 of P-site tRNA^{fMet} are important for translocation further indicated a role for the terminus of the P-site tRNA (50). It was hypothesized that the importance of these tRNA interactions stems from their contribution to P/E site binding. Within the 50S E site, A76 stacks between
G2421 and A2422 and forms hydrogen bonds with C2394 of the 23S rRNA, and truncation of A76 or modification of C2394 prevents binding of tRNA to the E/E site (15, 46, 51, 52). Cryo-EM (27) and smFRET (53) experiments suggest that the 50S contacts made in the P/E site could be maintained in the E/E site, so the crystal structure data of tRNA bound to the E/E site may be similar to that observed in the P/E site. The importance for these interactions between P-site tRNA and the 50S E site was recently addressed directly, by mutating the 23S rRNA of the 50S E site at position 2394. Mutation C2394G was shown to prevent protection of the E-site rRNA under conditions which favored E/E or P/E site binding (54). Mutations of C2394 conferred defects in growth, translocation, and E-site binding, further implying a role for the P/E site in translocation (Chapters 3 and 4)(54).

Within the 50S P site, the 3’ terminal nucleotides interact with conserved bases of the 23S rRNA. Two Watson-Crick basepairs were identified between tRNA bases C74, C75 and rRNA bases G2252, G2251, respectively, which were essential for growth and important for positioning of tRNA for peptidyl transfer. Mutation of either of these rRNA bases disrupted the interaction with the 50S P site (46, 55) and decreased the apparent rate of translocation by ~2-fold (43). However, the presence of either rRNA mutation with the compensatory mutation on the tRNA moving out of the A site (predicted to restore base-pairing in the A/P site while forcing P-tRNA into the P/E site) resulted in a ~2-fold rate enhancement over wild-type. This suggests that both A/P- and P/E-site binding contribute to rapid translocation.
1.4. Models for translocation.

1.4.1. Thermodynamics of translocation

A long-standing view in the field has been that translocation is inherently exergonic. The thermodynamic gradient model was proposed based primarily on the observations that slow translation of polyU mRNA can take place in the absence of elongation factors (56, 57). According to this model, forward translocation is an inherent property of the ribosome driven by the energy of peptide bond formation that is stored in the PRE complex and thermodynamically coupled to tRNA movement (58). The model postulates that the directionality of translocation results from the changes in acylation state of the A- and P-site tRNAs that occur upon peptidyl transfer. Transpeptidation converts P-site peptidyl-tRNA to deacylated tRNA and A-site aa-tRNA to peptidyl-tRNA, and alters the relative binding affinities of the tRNAs, such that each has a higher affinity for its adjacent site than its resident site (59). This model is contingent upon the idea that the POST state is more thermodynamically stable than the PRE state, making translocation an exergonic (i.e. thermodynamically downhill) process (58).

This model was recently refuted by data from our group, which showed that in some ribosomal complexes, the PRE state is actually more stable than the POST state (See Chapter 3, Fig. 3.6), and depletion of EF-G from these complexes results in spontaneous reverse translocation (60). In these complexes, EF-G was necessary for forward movement, as no spontaneous forward translocation was observed. These data
argued that tRNA-mRNA movement may be endergonic, and were supported by complementary data from another group (61).

How then can poly-Phe synthesis in the absence of EF-G and GTP be explained? In the previous experiments, poly-Phe was formed in reactions with excess Phe-tRNA present. Binding of Phe-tRNA to the A-site results in peptidyl transfer, an exergonic reaction. Regardless of whether the POST state was more thermodynamically favorable than the PRE state, it is likely that in this situation, translation would be driven in the overall forward direction by peptidyl transfer, which is irreversible, so long as the energy barrier for translocation could be breached.

1.4.2. Kinetics of translocation.

During the last ~30 years, the mechanism by which EF-G interacts with the ribosome to drive tRNA-mRNA movement has been extensively analyzed by a variety of biochemical and biophysical techniques, leading to a detailed kinetic model for translocation.

It was observed early on that translocation can occur in the presence of EF-G•GDPNP, and this was interpreted as evidence that EF-G acted as a GTPase switch (58). Canonical GTPase switch proteins bound to GTP have a high affinity for the product state, and hydrolysis of GTP reduces this affinity to allow product release. In the case of EF-G, it was posited that binding of the factor catalyzes translocation, while GTP hydrolysis stimulates a change in conformation necessary for dissociation of the factor.
When the rates of translocation and GTP hydrolysis were measured by pre-steady-state methods, GTP hydrolysis was rapid and immediately followed EF-G binding to the PRE complex, while translocation was comparatively slow (62). Hydrolysis of GTP accelerated codon-anticodon movement to more than 50-fold faster than the rate observed when translocation was performed with non-hydrolyzable analogs, arguing against the idea that EF-G was acting as a switch protein. The lag between GTP hydrolysis and translocation suggested that hydrolysis induces a rearrangement of the factor prior to translocation. Instead of being active in the GTP form as a switch protein, it was proposed that EF-G functions as a motor protein, which is active in the GDP or GDP•Pi form (i.e. activated by GTP hydrolysis).

Later experiments monitored the rates of tRNA movement and phosphate (Pi) release in the presence of various antibiotics and mutations (Figure 1.6)(63-65). Deletion of domains 4 and 5 of EF-G slowed codon-anticodon movement and Pi release identically, suggestive of a rate-limiting step that precedes both events. This extension of the motor protein model stated that GTP hydrolysis incites a conformational rearrangement, termed unlocking, that limits both codon-anticodon movement and Pi release (63). Effects of antibiotics and ribosomal mutations indicated that, although both events are limited by the unlocking step, codon-anticodon movement and Pi release are independent of each other and probably occur in random order (63-65). The fact that Pi release follows unlocking supports the idea that EF-G (GDP•Pi) is the active form of the factor that promotes translocation. Finally, ribosomal rearrangements must occur to
"relock" the tRNAs in their new sites, followed by release of EF-G•GDP from the POST complex, although the kinetics of these events have yet to be fully characterized.

Figure 1.6. Wintermeyer model for EF-G dependent translocation. Described in text. Steps following translocation have not been studied in detail and are not shown. Figure adapted from (63).

A limitation of this model is that it does not incorporate movements of tRNAs. Recently, the movement of tRNAs with respect to both subunits was addressed by single-turnover translocation assays. In the work of Pan et al., which examined translocation of proflavin-labeled tRNAs, two intermediates could be detected that were hypothesized to correspond to individual movements of the A and P-site tRNAs with respect to the large subunit (Figure 1.7) (66). Addition of viomycin stabilized a state that was predicted to correspond to the P/E state, while spectinomycin stabilized an intermediate called the INT complex, thought to correspond to the hybrid state.
Independent movement of the A- and P-site tRNAs on the large subunit was consistent with results of smFRET experiments (48) which showed that at equilibrium, tRNAs in a PRE complex rapidly moved between three FRET states that were structurally consistent with the classical state (A/A-, P/P-site bound tRNAs), P/E state (A/A-, P/E-site bound tRNAs), and hybrid state (A/P-, P/E-site bound tRNAs).

Further support for the independent movement of tRNAs within the large subunit was drawn from single-turnover translocation assays described in this work (Chapter 4). In these experiments, movement of tRNA into the A/P or P/E site was inhibited by tRNA and rRNA substitutions, and the effects of these substitutions on translocation parameters
were measured. When movement of tRNA into the P/E site was inhibited by mutations of C2394 of the 50S E site, defects in both the maximal translocation rate and the apparent affinity of EF-G for the PRE complex were observed. In contrast, when movement of tRNA into the A/P site was inhibited by substitution of the peptidyl group on the A-site tRNA with an aminoacyl group, a defect was observed in maximal translocation rate, but no defect was observed in the apparent affinity of EF-G for these PRE complexes. This difference suggests that movement into the P/E and A/P sites are kinetically separable events, and movement of tRNA into the P/E site contributes to high-affinity interaction of EF-G with the PRE complex. The rate of GTP hydrolysis was also measured in this study, and was not inhibited in ribosomes defective in P/E state formation. This indicates that EF-G binding and GTP hydrolysis precede P/E state formation, and led us to propose a model much like that proposed by the Cooperman group (described in detail in Chapter 4).

1.4.3. Structural basis for translocation.

The current structural models for translocation are based on several movements suggested from cryo-EM and X-ray crystallographic studies. Cryo-EM reconstructions of 70S ribosomes with EF-G bound demonstrated the *ratchet-like-intersubunit rotation* (RSR), which involved a rotation of the ribosomal subunits with respect to each other (27, 29). This rotation occurred in the direction of translocation, suggesting that it may contribute to tRNA movement, and was only observed in complexes in which tRNA could occupy the P/E site. Experiments using site-specific cross-links between ribosomal
proteins of the two subunits provided strong evidence that intersubunit rotation is required for translocation (10). Complexes containing peptidyl-tRNA, which binds the P/P site but not the P/E site, were unable to undergo RSR and had low affinity for EF-G (27, 67). Because the RSR took place in all structures in which EF-G was bound, it was posited that RSR is a prerequisite for EF-G binding. Recent experiments monitored FRET between labeled ribosomal proteins of the small and large subunits of reconstituted ribosomes, and one of these FRET states was proposed to correspond to RSR. These studies demonstrated that RSR only occurred under conditions when tRNA moved into the P/E site, and it was gleaned from these data that RSR and P/E site binding are coupled (68). A second movement inferred from a comparison of several independent forms of 70S ribosomes is the swiveling of the head of the small subunit (Figure 1.10)(16). This rotation follows the trajectory of tRNA-mRNA movement, and is proposed to direct their movement. Accompanying the head rotation is an opening of the space between the P and E sites, which is ordinarily sterically blocked by several 16S rRNA nucleotides involved in binding P-site tRNA. The opening of this gate was proposed to correspond to the unlocking event identified by single-turnover kinetic techniques (63).
Figure 1.8. Movement of the head domain of the 30S subunit. Position of the head domain of the 30S subunit in *E. coli* ribosome II from reference (16) compared to the *T. thermophilus* 70S ribosome. Differences in the position of corresponding phosphorus atoms (light blue) and Cα positions for S13 and S19 (dark blue) in the two head domains are shown as vectors pointing in the direction of the arrows. Figure adapted from (16).

The overall model that has been proposed for how these structural changes contribute to translocation is a combination of the three movements: 1) Ratcheting of the subunits, which may accompany P/E formation, 2) swiveling of the head, and 3) opening of the gate between the 30S P and E sites, which may correspond to the rate-determining unlocking step identified biochemically (16).
CHAPTER 2

RECOGNITION AND POSITIONING OF MESSENGER RNA IN THE RIBOSOME BY TRANSFER RNAs WITH EXPANDED ANTICODONS

2.1. Introduction

In the 1970's, Roth and coworkers isolated many mutations that suppressed single base pair insertion (+1 frameshift) mutations in the histidine operon of Salmonella typhimurium. (reviewed in (69)). These suppressor of frameshift (suf) mutations were classified by distinct suppression activities and mapped to separate loci (70-72). Interestingly, most of the characterized suf alleles were found to encode tRNAs with anticodon loops expanded by 1 nucleotide (nt). Alleles of sufA, sufB, sufD, sufG, and sufJ encoded 8-nt anticodon loop variants of tRNA\textsuperscript{Pro1}, tRNA\textsuperscript{Pro2}, tRNA\textsuperscript{Gly1}, tRNA\textsuperscript{Gln1}, and tRNA\textsuperscript{Thr3} (8-nt loop tRNAs) (73-76). Analogous tRNA variants have since been isolated in Escherichia coli and Saccharomyces cerevisiae (77-79). Among the first characterized was a sufD allele of S. typhimurium encoding a mutant tRNA\textsuperscript{Gly1} with anticodon CCCC instead of CCC that induced ribosomal frameshifts at runs of guanosines (74). This led to
an elegant quadruplet-pairing model to explain +1 frameshift suppression in which the expanded anticodon pairs with 4 bases of the mRNA and facilitates translocation of the mRNA by 4 nucleotides, thus allowing the ribosome to re-enter the correct reading frame. Consistent with this model, optimal suppression activity typically requires complementarity between the 4-nt expanded anticodon and the 4-nt frameshift sequence in the mRNA. A notable exception is the sufJ suppressor, which functions efficiently without the potential to pair with the fourth nucleotide of the frameshift sequence (75, 80).

This classical quadruplet-pairing model has been challenged (81). Molecular analysis of tRNAs encoded by the sufA6 and sufB2 alleles revealed that in each case, the 3’-most base of the putative quadruplet anticodon was methylated on its Watson-Crick face. Base methylation was therefore predicted to preclude quadruplet pairing. An alternative model was proposed in which the 8-nt loop tRNA alters the balance of isoacceptors in the cell and allows a near-cognate wild-type tRNA to enter the A site and cause a +1 frameshift event to restore the correct reading frame.

Although sufA6 and sufB2 may promote frameshifting without quadruplet pairing, the suggestion that 8-nt loop tRNAs act indirectly to promote frameshifting is inconsistent with the dominant nature of most suf alleles (70). Furthermore, it has been shown in vitro and in vivo that 8-nt loop tRNAs can direct incorporation of unnatural amino acids in response to a complementary quadruplet mRNA sequence (82-84). These studies demonstrate that 8-nt loop tRNAs can act directly to promote +1 frameshifting and lend support to the classical quadruplet-pairing model.
According to the quadruplet-pairing model (Fig 2.1, panel A), the expanded anticodon forms four base pairs with the mRNA in the A site, and this 4 bp 'codon-anticodon' helix is then translocated to the P site, resulting in restoration of the correct reading frame (69). However, no biochemical evidence for four base pairs between tRNA and mRNA has been reported, and whether an additional base pair can be accommodated in either ribosomal site remains unclear. An alternative possibility consistent with the genetic data is that the first and fourth bases of the mRNA frameshift sequence are recognized sequentially in the A and P sites, respectively (Fig 2.1, panel B). Here, we use purified components to test the ability of tRNA analogs with expanded anticodons to recognize and position mRNA in the ribosome. This study provides new insights concerning the 30S subunit A and P sites and the molecular mechanism of frameshift suppression.
Figure 2.1 Base pairing between expanded anticodon tRNAs and mRNA according to the classical quadruplet-pairing model (a), or the differential-pairing model (b).
2.2. Materials and Methods

2.2.1 Reagents

Tight-couple ribosomes from \textit{E. coli} strain MRE600 and EF-G were purified as described (85). Hexahistidine-tagged T7 RNA polymerase was over-expressed and purified using Ni\textsuperscript{2+}-NTA resin (Qiagen).

Derivatives of tRNA\textsubscript{Ala} with mutant anticodons were made by \textit{in vitro} transcription and gel purified. Mutations were constructed in a plasmid containing the tRNA\textsubscript{Ala} gene downstream of the T7 promoter using QuikChange\textsuperscript{TM} (Stratagene) as described (86). These mutant plasmids were digested with BstN1, which cuts the template strand precisely at the 3' end of the gene, and used as templates for \textit{in vitro} transcription by T7 RNA polymerase. Derivatives of T4 gene 32 mRNA (m408, m409, m410, m411, m412, m413, m424, and m425) were made as described previously (85). ASL\textsubscript{Val} (5'-CCUCCUUACAAAGGAGG-3'), derivatives of ASL\textsubscript{Val} and ASL\textsubscript{Ala1B} containing mutations in the anticodon, and short mRNAs [m401 (5'-GGCAAGGAGGUAAAAAUACCGGAAGGCACGU-3'), m402 (5'-GGCAAGGAGGUAAAAAUACCGGAAAGGCACGU-3'), m403 (5'-GGCAAGGAGGUAAAAAUACCGGAAAGGCACGU-3'), and m404 (5'-GGCAAGGAGGUAAAAAUACGUAAAAAGCAGCGU-3')] were purchased from Dharmacon.
2.2.2. Toeprinting

Toeprinting experiments were performed as described previously (85, 87). The P-site binding experiments of Fig. 3 were performed in both TNM [50 mM Tris-HCl (pH 7.6), 100 mM NH₄Cl, 20 mM MgCl₂, and 6 mM β-mercaptoethanol] and polymix [5 mM potassium phosphate (pH 7.3), 95 mM KCl, 5 mM NH₄Cl, 0.5 mM CaCl₂, 5 mM magnesium acetate, 8 mM putrescine, 1 mM spermidine, and 1 mM DTT] (88) buffer, and virtually identical toeprint patterns were observed.

2.2.3. Filter Binding

ASLs were radiolabeled using γ-[³²P]-ATP (3000 Ci/mmol) and T4 polynucleotide kinase as recommended by the manufacturer (New England BioLabs). The enzyme was heat-inactivated, and free γ-[³²P]-ATP was removed by passing the reaction over a Sephadex G-15 (Amersham) spin column. Each radiolabeled ASL was then diluted into TNM buffer containing unlabeled ASL such that the concentration of ASL was 450 nM (56 μCi/mmol). For certain experiments, antibiotic (500 μM paromomycin or tetracycline) and/or competitor (2.25 μM ASL Val1) were added to the radiolabeled ASL stock prior to its addition to ribosomal complexes.

To determine the affinity of the ASLs for the A site, filter binding was performed on a Schleicher and Schuell 96-well dot-blot apparatus using the double-filter method (89, 90). First, the P site was filled by incubating 70S ribosomes (2 μM) with tRNA Tyr2 (4 μM) and mRNA (6 μM; m401, m402, m403, or m404) in TNM buffer for 20 minutes at 37°C, and the complex was placed on ice. Next, the complex was serially diluted using
TNM buffer, and 25 µL of the complex at each of 7 concentrations was mixed with 5 µL of 450 nM radiolabeled ASLs in a 96-well plate and incubated for 30 minutes at 37°C. Finally, reactions were diluted with 100 µL of cold TNM buffer and immediately filtered through a bi-layer of nitrocellulose and Hybond N+ membranes. The membranes were separated, dried, and exposed to a phosphorimager screen for quantification. The fraction of ASL bound for each reaction was determined as the fraction of counts trapped on the nitrocellulose and was the mean of three experiments. The data were plotted in Kaleidagraph, and the $K_D$ was determined using the equation $[70S\cdot ASL] = [70S]_{\text{input}} + [ASL]_{\text{input}} + K_D - ((([ASL]_{\text{input}} + [70S]_{\text{input}} + K_D)^2 - 4[ASL]_{\text{input}}[70S]_{\text{input}}))^{1/2}/2$ as described (Pleiss and Uhlenbeck, 2001). Values for $B_{\text{max}}$ were obtained from the curve-fitting and correspond to the maximal fraction of ASL bound.

2.3. Results

2.3.1. Positioning of mRNA by tRNA$^{\text{Ala}}_{\text{ACC}}$ and tRNA$^{\text{Ala}}_{\text{GCC}}$ bound to the P site

Two tRNA$^{\text{Ala}}_{\text{ACC}}$ variants were generated containing expanded anticodons ACCG and GCCG (Fig. 2.2). (Throughout this manuscript, both codon and anticodon sequences are written 5' to 3'). Analogous variants of yeast tRNA$^{\text{Phe}}$ were shown previously to promote efficient (>20%) +1 frameshifting during translation in vitro (82). We chose tRNA$^{\text{Ala}}_{\text{GCC}}$ because its anticodon stem-loop (ASL) lacks modifications and is not used as a specificity determinant for AlaRS (91, 92). Control 7-nt anticodon loop variants with anticodons ACC, GCC, and CCG were also made. A 9-nt anticodon loop variant with the
sequence GCCCG between U33 and A37 was fortuitously obtained during the
mutagenesis. Earlier work demonstrated that certain tRNAs with 9-nt anticodon loops can
also promote frameshifting (83, 93).

Fig 2.2. Derivatives of tRNA^{Ala2} (a), ASL^{Ala1B} (b), and ASL^{Val1} (c) used in this study.
Nucleotides of 8-nt anticodon loops are numbered as shown (d).
Using a set of model mRNAs derived from phage T4 gene 32 mRNA, we bound each tRNA^{Ala2} derivative to the P site and mapped the position of mRNA in the resulting ribosomal complexes by toeprinting (Fig. 2.3, 2.4). Because toeprints map precisely 16 nucleotides from the first nucleotide of the P codon, this method allows the mRNA nucleotides that physically occupy the P site to be assigned (40, 94, 95). Three pairs of mRNAs were used for this analysis (Fig. 2). Messages m408 and m410 contain two nearby GGU codons, but only m408 has a C at position +6 to generate the predicted frameshift sequence CGGU. Analogously, m412 and m409 contain two nearby CGG codons, but only m412 has a U at position +9 to generate CGGU. The frameshift sequence CGGU has the potential to form 4 base pairs with the expanded anticodon of either tRNA^{Ala2}_{ACCG} (4 Watson-Crick) or tRNA^{Ala2}_{GCCG} (3 Watson-Crick, 1 G-U wobble).

Finally, m411 and m413 contain two nearby GGC codons, but only m411 has the predicted frameshift sequence CGGC. CGGC has the potential to form 4 base pairs with the expanded anticodon of tRNA^{Ala2}_{GCCG} only.
Figure 2.3. Sequences of mRNAs used in the P-site binding experiments.
The Shine-Dalgarno (SD) sequence is underscored. Position +1 is assigned as the first nucleotide of the coding region of T4 gene 32, from which these mRNAs are derived. GGU, CGG, and CCG codons are boxed in red, blue, and orange, respectively.

When either tRNA\(^{\text{Ala2}}\)\(_{\text{ACC}}\) or tRNA\(^{\text{Ala2}}\)\(_{\text{GCC}}\) was bound to ribosomes programmed with message m408, we observed a strong toeprint at +17 and a weak toeprint at +22 (Fig. 2.4A, lanes 3 and 4). This pattern indicates that ~93% of the ribosomal complexes contained the upstream GGU codon in the P site while ~7% contained the downstream GGU codon in the P site. Positioning of the upstream GGU codon is thermodynamically favored because this codon is more optimally spaced from the Shine-Dalgarno sequence.

Binding of tRNA\(^{\text{Ala2}}\)\(_{\text{CCG}}\) to ribosomes in the presence of m408 resulted in a single toeprint at +21 (Fig. 2.4A, lane 5), which corresponds to positioning of the sole CGG codon in the P site. When either tRNA\(^{\text{Ala2}}\)\(_{\text{ACCG}}\) or tRNA\(^{\text{Ala2}}\)\(_{\text{GCCG}}\) was bound, toeprints were observed at +17 and +22, but not at position +21 (Fig. 2.4A, lanes 6 and 7). Thus, both expanded anticodons preferentially positioned the GGU codons of m408 in the P site, while CGG did not occupy the P site to any appreciable degree. Moreover, when either tRNA\(^{\text{Ala2}}\)\(_{\text{ACCG}}\) or
tRNA$_{\text{GCCG}}^{\text{Ala2}}$ was bound, the relative intensity of the +22 toeprint increased such that it was comparable to the +17 toeprint, indicating that about half the complexes in each reaction contained the downstream GGU in the P site. This increase can be explained by a fourth base pair between G36 of the expanded anticodon and C+6 of the frameshift sequence (CGGU), which cannot be formed by the control 7-nt loop tRNAs. Consistent with this interpretation, a C to A substitution at position +6 of the mRNA caused a large reduction in the relative intensities of the +22 toeprints directed by tRNA$_{\text{ACCG}}^{\text{Ala2}}$ and tRNA$_{\text{GCCG}}^{\text{Ala2}}$ (Fig. 2.4B, lanes 6 and 7). In ribosomes programmed with m410, < 5% of the complexes contained the downstream GGU in the P site whether tRNA$_{\text{ACC}}^{\text{Ala2}}$, tRNA$_{\text{GCC}}^{\text{Ala2}}$, tRNA$_{\text{ACCG}}^{\text{Ala2}}$, or tRNA$_{\text{GCCG}}^{\text{Ala2}}$ were bound.
Figure 2.4. Toeprints of ribosome complexes containing each of several tRNA$_{\text{Ala}}$ derivatives bound to the P site. The sequence of the anticodon is shown above each lane, and the relevant sequence of mRNA is shown below the gel. Position +1 of each mRNA is indicated by bold type. For each complex, the codon inferred to occupy the P site is underscored by a colored line that corresponds to the color of the anticodon sequence. Broken lines indicate toeprints of lower intensity.
The toeprint pattern directed by tRNA$_{\text{Ala2 ACCG}}$ was distinguished by an additional toeprint at +16 (Fig. 2.4A, B, E, F; lane 6). The +16 toeprint predicts that, in a fraction of the complexes, the mRNA sequence in the P site was AGG, which can only form two canonical base pairs with tRNA$_{\text{Ala2 ACCG}}$. One possibility is that an alternative conformation of the anticodon loop of tRNA$_{\text{Ala2 ACCG}}$ allows P-site positioning of AGG.

When tRNA$_{\text{GCCG}}$ or tRNA$_{\text{GCCG}}$ were bound to the P site in the presence of m412, toeprints were observed at positions +16 and +22 (Fig. 2.4C, lanes 6 and 7). The toeprints at +22 indicate that, as with m408, the GGU portion of the frameshift sequence CGGU was preferentially positioned in the P site by the 8-nt loop tRNAs. The toeprints at +16 correspond to positioning of the upstream CGG in the P site, as was observed for P-site tRNA$_{\text{CCG}}$ (Fig. 2.4C, lane 5). Thus each of these 8-nt loop tRNAs can position CGG in the P site when U is not immediately downstream. For both tRNA$_{\text{GCC}}$ and tRNA$_{\text{GCCG}}$, an additional toeprint was observed at position +17, which corresponds to positioning of GGA in the P site (Fig. 2.4C, lanes 4, 7). GGA is a near-cognate codon for tRNA$_{\text{GCC}}$, and presumably C36 and C35 pair with G+2 and G+3, respectively, in the P site. By analogy, C35 and C34 of tRNA$_{\text{GCCG}}$ likely pair with G+2 and G+3. Positioning of near-cognate GGA in the P site became more prevalent with m409, where the downstream GGU codon is absent (Fig. 2.4D).

We also tested the ability of the various tRNAs to position message m411, which contains the sequence CGGC (Fig. 2.3). Binding of tRNA$_{\text{GCCG}}$ to the P site resulted in strong toeprints at positions +17 and +22, indicating positioning of GGC in the P site.
(Fig. 2.4E, lane 7). This toeprint pattern was most similar to that generated by P-site tRNA$_{GCC}^{Aha2}$. However, weak toeprints were also observed at positions +16 and +21, corresponding to P-site positioning of AGG and CGG, respectively. When tRNA$_{ACCG}^{Aha2}$ was bound to the P site, relatively weak toeprints were observed at +16 and +22, corresponding to P-site positioning of AGG and GGC, respectively (Fig. 2.4E, lane 6). As mentioned above, positioning of AGG in the P site by tRNA$_{ACCG}^{Aha2}$ cannot be readily explained but probably results from an unusual interaction between the tRNA and mRNA. The fact that GGC was positioned in the P site rather than CGG is noteworthy because the latter is complementary to the expanded anticodon. These data suggest that the mRNA-tRNA helix physically positioned in the P site contains a mismatch between C+9 of m411 and A33.5 of tRNA$_{ACCG}^{Aha2}$. Interestingly, the +22 toeprint was dependent on C+6 of m411 (Fig. 2.4F, lane 6), suggesting that 3 Watson-Crick base pairs do form between tRNA$_{ACCG}^{Aha2}$ and m411, but one base pair (between G36 and C+6) is physically positioned toward (or in) the 30S E site.

The 9-nt loop variant tRNA$_{GCCCG}^{Aha2}$ was also included in this analysis. When tRNA$_{GCCCG}^{Aha2}$ was bound to the P site of ribosomes programmed with m408, m410, m411 or m413, toeprints at +15 and +16 were observed. These toeprints are distinct from those observed in the 7-nt loop control tRNAs and correspond to P-site positioning of AAG and AGG. In the case of m413, a weak toeprint at +20 was also observed, indicating positioning of the downstream AAG in the P site. When ribosomes contained m412 and m409, a single predominant toeprint at +16 was observed, corresponding to positioning
of CGG in the P site. The ability of tRNA$_{\text{Ala2}}^{\text{GCCCG}}$ to position such dissimilar codons in the P site suggests that the 9-nt anticodon loop adopts different conformations to facilitate unconventional tRNA-mRNA interactions.

It has been shown that movement of tRNA into the P/E state destabilizes the codon-anticodon helix (45). Thus, we were concerned that the toeprint patterns might be influenced by the ability of these deacylated tRNAs to occupy the P/E state. To address this, we repeated the experiments using ribosomes containing mutation C2394A, which disrupts tRNA-rRNA contacts in the 50S E site (22, 45, 54). We observed identical toeprint patterns with C2394A ribosomes, suggesting that none of the toeprints depended on the ability of tRNA to occupy the P/E state.

### 2.3.2. Translocation of tRNA$_{\text{Ala2}}^{\text{ACCG}}$ from the A site to the P site

The ability of tRNA$_{\text{Ala2}}^{\text{ACCG}}$ to act as an A-site substrate in EF-G-dependent translocation was tested in several contexts. Ribosomes were first incubated with m408 and tRNA$_{\text{Val}}$ to bind the P site. Toeprints were observed at +18 and +23, corresponding to P-site positioning of the upstream or downstream GUA, respectively (Fig. 2.5A, lanes 1, 5, and 9). In the +18 complex, the codon CGG occupies the A site. When either tRNA$_{\text{Ala2}}^{\text{ACCG}}$ or tRNA$_{\text{Ala2}}^{\text{ACCG}}$ was added to bind the A site, the relative intensity of the +19 toeprint increased (Fig. 2.5A, lanes 6 and 10), characteristic of A-site binding of cognate tRNA (96). After addition of EF-G and GTP, translocation of m408 was observed, indicated by toeprints at +21 and +22, respectively (Fig. 2.5A, lanes 8 and 12). The toeprint at +22 corresponds to positioning of the downstream GGU in the P site. Thus, translocation of
tRNA$_{\text{Ala}}^{\text{A}}_\text{ACCG}$ results in movement of mRNA by 4-nt, rather than 3-nt as observed for tRNA$_{\text{Ala}}^{\text{A}}_\text{CCG}$. The appearance of toeprints at positions +16 and +17 was also observed after translocation of tRNA$_{\text{Ala}}^{\text{A}}_\text{ACCG}$ (Fig. 2.5A, lane 12). These toeprints correspond to positioning of the upstream AGG and GGU in the P site and are presumably due to mRNA slippage caused by P/E-state occupation of the deacylated tRNA$_{\text{Ala}}^{\text{A}}_\text{ACCG}$(45). As expected, addition of non-cognate tRNA$_{\text{Ala}}^{\text{A}}_\text{ACC}$ and EF-G•GTP did not promote translocation (Fig. 2.5A, lane 4).

In a similar experiment, complexes were formed containing P-site tRNA$_{\text{Val}}$ paired to the GUA codon m424 (Fig. 2.5C). These +18 complexes also contained the codon CGG in the A site, but in this case, the mRNA nucleotide immediately 3' was G rather than U. Addition of tRNA$_{\text{Ala}}^{\text{A}}_\text{CCG}$ resulted in an obvious toeprint at +19 (Fig. 2.5C, lane 6), indicative of A-site binding. Subsequent addition of EF-G(+GTP) caused a toeprint at +21 (Fig. 2.5C, lane 8), corresponding to the posttranslocation complex with CGG in the P site. tRNA$_{\text{Ala}}^{\text{A}}_\text{ACCG}$ was also able to act as an A-site substrate in translocation, albeit less efficiently. In this case, the predominant toeprint of the posttranslocation complex was at +22 (Fig. 2.5C, lane 12), corresponding to positioning of GGG in the P site. Thus, tRNA$_{\text{Ala}}^{\text{A}}_\text{ACCG}$ promoted translocation of mRNA by 4 nucleotides despite the inability of A33.5 to pair with mRNA.

The ability of tRNA$_{\text{Ala}}^{\text{A}}_\text{ACCG}$ to recognize GGU in the A site and promote its translocation to the P site was also tested. In complexes containing P-site tRNA$_{\text{Tyr}}$ and m408, tRNA$_{\text{Ala}}^{\text{A}}_\text{ACCG}$ behaved similarly to tRNA$_{\text{Ala}}^{\text{A}}_\text{ACC}$ as an A-site substrate (Fig. 2.5B). Although both posttranslocation complexes contained GGU in the P site, the efficiency of
translocation was lower in the case of tRNA$_{\text{ACCG}}^{\text{Ala2}}$. In a different context, tRNA$_{\text{ACCG}}^{\text{Ala2}}$ was unable to promote translocation of GGU from the A site to the P site (Fig. 2.5D, lane 12).
Figure 2.5. Translocation of tRNA analogs containing expanded anticodons.
The position of mRNA in ribosomal complexes was mapped by toeprinting after each of several additions. Pretranslocation complexes were made by incubating ribosomes with (a), (c) and (d) mRNA (as indicated) and tRNA\textsuperscript{Val} or (b) tRNA\textsuperscript{Tyr} to fill the P site (P lanes) and then adding tRNA analogs (as indicated) to bind the A site (A lanes). Next, each reaction was further incubated with either GTP alone as a control (– lanes) or with EF-G plus GTP (+ lanes). Position +1 of each mRNA is indicated by bold type.
2.3.3. Translocation of ASL\textsubscript{Ala1B}^{\text{ACCG}} and ASL\textsubscript{Val1}^{\text{ACCG}} from the A site to the P site

During the course of this work, it was reported that derivatives of tRNA\textsubscript{Ala2} containing transplanted anticodons exhibit highly reduced affinity for the A and P sites due to base pair A32-U38, which acts as a strong negative determinant of binding (97). Because A32-U38 is rare (observed only in tRNA\textsubscript{Ala2} and tRNA\textsubscript{Pro2}), it was unclear how the data regarding tRNA\textsubscript{Ala2}^{\text{ACCG}} would relate to other suppressor tRNAs. To investigate whether the expanded anticodon ACCG conferred similar activities when transplanted into other tRNA species, we analyzed analogous derivatives of ASL\textsubscript{Ala1B}^{\text{ACCG}} and ASL\textsubscript{Val1}^{\text{ACCG}} (Fig. 2.2).

The ability of ASL\textsubscript{Ala1B}^{\text{ACCG}} and ASL\textsubscript{Val1}^{\text{ACCG}} to act as an A-site substrate in translocation was similar to that of tRNA\textsubscript{Ala2}^{\text{ACCG}} (Fig. 2.5). ASL\textsubscript{Ala1B}^{\text{ACCG}} and ASL\textsubscript{Val1}^{\text{ACCG}} were able to recognize either CGG or GGU in the A site and promote subsequent translocation to similar extents. With either codon, the efficiency of translocation of ASL\textsubscript{Ala1B}^{\text{ACCG}} or ASL\textsubscript{Val1}^{\text{ACCG}} was less than that of the cognate 7-nt loop ASL control. Interestingly, translocation of m425 paired to ASL\textsubscript{Val1}^{\text{ACCG}} resulted in toeprints at positions +20 and +21 (Fig. 2.5D, lane 36), corresponding to AGG and GGU in the P site, reminiscent of P-site positioning of m408 and m410 by tRNA\textsubscript{Ala2}^{\text{ACCG}} (Fig. 2.4). As observed for tRNA\textsubscript{Ala2}^{\text{ACCG}}, ASL\textsubscript{Ala1B}^{\text{ACCG}} promoted 4-nt translocation of m408, which contains the frameshift sequence CGGU (Fig. 2.5A, lane 24). However, unlike tRNA\textsubscript{Ala2}^{\text{ACCG}}, ASL\textsubscript{Ala1B}^{\text{ACCG}} did not direct efficient 4-nt translocation of m424, which contains CGGG (Fig. 2.5C, lane 24).
2.3.4. Codon dependence of ASL$_{\text{Ala}}$ and ASL$_{\text{Val}}$ binding to the A site

The efficiency of translocation was lower than expected in several experiments, even with control substrates. This could be explained by defects in either A-site binding or translocation. To distinguish these possibilities, we tested the ability of each ASL variant to bind the A site programmed with various codons. First, the experimental system was established using ASL$_{\text{Val}}$, which promoted efficient translocation (Fig. 2.6). Ribosome complexes containing P-site tRNA$_{\text{Tyr}}$ and either a GUA or CGG codon in the A site were mixed at various concentrations with $[^{32}\text{P}]-$ASL$_{\text{Val}}$, incubated for 30 minutes at 37°C, and filtered through a membrane bi-layer to determine the fraction of ASL$_{\text{Val}}$ bound. A substantial fraction ($B_{\text{max}} = 0.72$) of ASL$_{\text{Val}}$ bound the complex containing the cognate A codon GUA with a dissociation constant ($K_D$) of $\sim 37$ nM. An increase in binding was observed when paromomycin (100 µM) was included in the reaction ($K_D = 4.4$ nM; $B_{\text{max}} = 0.80$). In similar experiments, tetracycline (100 µM) decreased the affinity of ASL$_{\text{Val}}$ for the A site by $\sim$3-fold and the extent of binding by $\sim$15% (data not shown). In complexes where a non-cognate codon was positioned in the A site, a low extent of binding was observed ($B_{\text{max}} < 0.10$) that was not appreciably affected by the presence of paromomycin or tetracycline. These data indicate that most of the binding can be attributed to codon-dependent interaction of ASL$_{\text{Val}}$ with the A site.

To further analyze the low but significant levels of binding to complexes containing the non-cognate A codon, we bound each ASL$_{\text{Val}}$ variant to 50S subunits in the absence of mRNA (data not shown). In each case, $B_{\text{max}}$ values were similar to those
obtained using 70S complexes programmed with a non-cognate A codon \(0.09 < B_{\text{max}} < 0.16\), consistent with some non-specific interaction between these ASLs and ribosomal particles under these conditions.

**Figure 2.6. A-site binding and translocation of ASL\(^{\text{Val}}\).** (a) Ribosomal complexes with tRNA\(^{\text{Tyr}}\) in the P site and the cognate codon GUA (filled symbols) or the non-cognate codon CGG (open symbols) in the A site were incubated at various concentrations with 90 nM \(^{32}\text{P}\) ASL\(^{\text{Val}}\) in the absence (circles) or in the presence (squares) of paromomycin, and the fraction of bound ASL\(^{\text{Val}}\) was determined. Inset: Pretranslocation complexes were made by incubating ribosomes with m291 and tRNA\(^{\text{Phe}}\) to fill the P site (lane P) and then adding ASL\(^{\text{Val}}\) to bind the A site (A lane). The reaction was further incubated with either GTP alone as a control (– lane) or with EF-G plus GTP (+ lane).
Next, we analyzed the ability of each ASL variant to bind the A site of ribosome complexes programmed with various mRNAs. In these experiments, 0.5 µM unlabeled tRNA$_{Val}^{Val}$ (non-cognate for all A codons tested) was included to reduce background due to non-specific ASL-ribosome interactions. In the absence of paromomycin, these ASLs generally bound the A site poorly, if at all. Marginal binding of cognate control ASLs was detected in some complexes. In the presence of paromomycin, ASL$_{ACCG}^{Ala}$ and ASL$_{ACCG}^{Val}$ were observed to bind ribosomes programmed with either CGG or GGU in the A site (Fig. 2.7, Table 2.1). An exception involved binding of ASL$_{ACCG}^{Val}$ to the complex containing m401, which has CGGA immediately 3′ of the P codon (Fig. 2.7E). However, binding of ASL$_{ACCG}^{Ala}$ to the identical complex was readily detected (Fig. 2.7B), indicating that the anomalous data of Fig. 2.7E is specific to ASL$_{ACCG}^{Val}$. In general, the ability of these ASLs to bind the A site correlates well with their translocation efficiency, and binding of the 8-nt loop ASLs was generally reduced compared to that of the cognate 7-nt loop controls. These data suggest that the expanded anticodon ACCG can interact with either CGG or GGU in the A site.
Figure 2.7. A-site binding of 8 nt loop ASLs. Ribosomal complexes containing P-site tRNA$^{Tyr}$ were formed in which the mRNA sequence immediately 3’ of the P codon was (a) and (d) CGGU, (b) and (e) CGGA, or (c) and (f) GGUA. These complexes were incubated at various concentrations with radiolabeled derivatives of (a)–(c) ASL$_{Ala1B}$ or (d)–(f) ASL$_{Val1}$ in the presence (filled symbols) or in the absence (open symbols) of paromomycin, and the fraction of bound ASL was determined. The anticodon sequences tested were ACC (circles), CCG (squares), and ACCG (triangles).
Table 2.1. Parameters obtained from the binding of ASL variants to the A site of ribosomes programmed with different mRNAs in the presence of paromomycin

<table>
<thead>
<tr>
<th>Variant</th>
<th>Sequence of mRNA 3’ of P codon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CGGU</td>
</tr>
<tr>
<td>ASL_{Ala}^{1B}_{CCC}</td>
<td>140, 0.64</td>
</tr>
<tr>
<td>ASL_{Ala}^{1B}_{ACC}</td>
<td>940, 0.47</td>
</tr>
<tr>
<td>ASL_{Ala}^{1B}_{ACC}</td>
<td>84, 0.32</td>
</tr>
<tr>
<td>ASL_{Val}^{11}_{CCG}</td>
<td>120, 0.64</td>
</tr>
<tr>
<td>ASL_{Val}^{11}_{ACC}</td>
<td>510, 0.36</td>
</tr>
<tr>
<td>ASL_{Ala}^{1B}_{ACC}</td>
<td>200, 0.44</td>
</tr>
</tbody>
</table>

Reported values correspond to the estimated dissociation constant $K_D$ (nM), and maximal fraction bound $B_{max}$, and were obtained from experiments performed in triplicate. –, $B_{max} < 0.2$. 
2.4. Discussion

2.4.1. The expanded anticodon positions the 3' three-quarters of the frameshift sequence in the P site.

Curran and Yarus described two alternative models to explain how tRNAs with 8-nt anticodon loops could promote a +1 shift of the reading frame (98). The first model posits that the 3' bases of the expanded anticodon (nt 36, 35, and 34) determine the mRNA register with respect to the ribosome. Accordingly, translocation of an 8-nt loop tRNA to the P site results in movement of the mRNA by 3 nt. Nucleotide 33.5 of this P-site tRNA forms a fourth base pair with mRNA, thereby occluding the fourth nucleotide of the frameshift sequence and causing the A codon to be functionally re-assigned as the next available triplet. Consequently, decoding of this out-of-phase A codon and subsequent translocation results in a +1 shift of reading frame. The second model posits that translocation of 8-nt loop tRNA results in a 4 nt movement of the mRNA, changing the mRNA register with respect to the ribosome. In this case, the base pair formed by nt 33.5 moves into the P site, occupying the position that would normally be occupied by the wobble pair (tRNA nt 34 and P codon nt 3). We can readily distinguish between these proposed mechanisms because the toeprinting technique allows the position of mRNA with respect to ribosome to be determined. Our data, which are corroborated by Joseph and colleagues (99), clearly rule out the first model and support the second model.

Structural studies show that the mRNA backbone is kinked by ~45° between the A and P codons, which allows codon-anticodon interactions to take place in each site.
(100, 101). In the P site (Fig. 2.8), 16S rRNA nt C1400 stacks with nt 34 of the anticodon, while m²G966 contacts the adjacent tRNA backbone at the U-turn (11, 19). In all cases that we tested, 8-nt anticodon loops position the 3′ three-quarters of the frameshift sequence in the P site, suggesting that the 5′ bases of the expanded anticodon (nt 33.5, 34, and 35) pair with mRNA in the P site.

Figure 2.8. View of 30S P-site rRNA (gray) interactions with mRNA (cyan) and tRNA (lilac) analogs. Figure made in Ribbons using 30S subunit structure coordinates (PDB1FJF) from reference (14).
Positioning of mRNA is the same whether the 8-nt loop tRNA is translocated or directly bound to the P site. Presumably, 16S rRNA nucleotides C1400 and m^2G966 interact with the base and backbone, respectively, of nt 33.5, analogous to the contacts made by these 16S rRNA bases to nt 34 of wild-type tRNA. We propose that these contacts stabilize a cognate tRNA-mRNA base pair immediately adjacent to U33, which largely determines the mRNA register within the ribosome. The importance of the nucleotide immediately 3' of U33 in determining the mRNA register is evident when complexes containing 8-nt loop tRNA bound to the P site are compared. In no case is the inability of nt 36 to pair with mRNA sufficient to alter the mRNA register, but in many cases the inability of nt 33.5 to pair with the mRNA is sufficient to shift the register.

It is interesting to consider establishment of the reading frame in light of these observations. The only codons decoded in the P site are start codons, and among natural start codons, degeneracy is observed at the first position. This suggests that nt 34 of the initiator tRNA plays a more critical role than nt 36 in establishment of the reading frame, as would be predicted from this study.

2.4.2. Evidence for four base pairs between P-site tRNA and mRNA

For most 8-nt loop tRNAs, the efficiency of frameshift suppression depends on complementarity between the expanded anticodon and the frameshift sequence in the mRNA. Although this has often been given as evidence for formation of a quadruplet codon-anticodon helix, biochemical evidence for 4 base pairs between tRNA and mRNA in either ribosomal site has not been reported. Here, we show that the relative affinity of
GGU for the P site is increased when G36 of tRNA$^{\text{Ala}2}_{\text{ACCG}}$ can pair with a C immediately 5' of GGU. These data provide strong evidence for 4 base pairs between P-site tRNA$^{\text{Ala}2}_{\text{ACCG}}$ and mRNA. The fourth base pair involving G36 of tRNA$^{\text{Ala}2}_{\text{ACCG}}$ lies toward (or in) the E site and does not change the mRNA register within the ribosome. Additional evidence that P-site tRNA can form four base pairs with mRNA comes from a wild-type tRNA, tRNA$^{\text{fMet}}$ from the chloroplast of *Chlamydomonas reinhardtii* (102). In this case, A37 of tRNA$^{\text{fMet}}$ pairs with mRNA at position -1 to stimulate initiation.

Although our study shows that quadruplet pairing by the P-site tRNA can occur, we do not see evidence to support quadruplet pairing by A-site tRNA. Binding of the 8-nt loop ASLs is generally reduced compared to that of the cognate 7-nt loop controls, and the potential to form four base pairs between tRNA and mRNA does not consistently increase the observed binding affinity. These data predict that expansion of the anticodon confers a general decoding defect, consistent with experiments that compared levels of nonsense and frameshift suppression by variants of Su7 tRNA with 7-nt and 8-nt anticodon loops (98). Concurrent structural studies also suggest that an A-site ASL containing an expanded anticodon is unable to form four Watson-Crick base pairs with mRNA(103).

### 2.4.3. Recognition of either the 5' or 3' three-quarters of the frameshift sequence in the A site implies positional flexibility of the expanded anticodon.

To promote a +1 shift of the reading frame, a suppressor tRNA must be able to decode the 5' three-quarters of a frameshift sequence in the A site. Consistent with this
requirement, we find that tRNA analogs with the expanded anticodon ACCG are able to recognize CGG (the 5' three-quarters of the frameshift sequence) in the A site. An unexpected observation is that these analogs are also able to recognize GGU (the 3' three-quarters of the frameshift sequence) in the A site. These data suggest that the 8-nt anticodon loop can adopt alternative conformations to facilitate A-codon recognition. Recognition of the 3' three-quarters of the frameshift sequence is not predicted to result in a subsequent frameshift event, which may explain why this activity has not been identified previously. Although the ability of an ASL to bind the A site and act as an A-site substrate in translocation should correlate with the ability of the corresponding aminoacyl-tRNA to decode during translation, it will be important to directly test the effects of anticodon expansion on the process of aminoacyl-tRNA selection.

2.4.4. A revised quadruplet-pairing model of frameshift suppression.

On the basis of our findings, we propose a revised model (Fig. 2.9) to explain frameshift suppression by tRNAs with 8-nt anticodon loops, which is similar to “model P” proposed previously (104). Although this model may not be pertinent in all cases (e.g. sufB2 (81)), it may be pertinent for many. According to our model, the 3' nucleotides of the expanded anticodon recognize the frameshift sequence in the 30S A site, and this interaction is sufficient for GTPase activation of the ternary complex, accommodation of the aminoacyl-tRNA, and peptidyl transfer. Upon translocation of the 8-nt loop peptidyl-tRNA, an extended 4 bp helix forms between the expanded anticodon and the complementary frameshift sequence. In the posttranslocation complex, nt 33.5, paired to
the fourth base of the frameshift sequence, occupies the P-site position normally occupied by nt 34 of wild-type tRNA. Thus, translocation of the 8-nt anticodon loop results in a 4-nt movement of mRNA, changing the mRNA register in the ribosome to restore the correct reading frame. The A codon of the posttranslocation complex is positioned normally in the A site, and translation continues in the correct frame.

![Diagram](image)

**Figure 2.9. Revised quadruplet-pairing model for frameshift suppression by tRNAs with expanded anticodons.**

Although it is clear that anticodon size is a major determinant of reading frame maintenance, there are examples of 8-nt loop tRNAs that do not shift the reading frame and 7-nt loop tRNAs that do (105). These examples indicate that determinants of frame maintenance apart from codon-anticodon interactions remain to be elucidated.
CHAPTER 3

DESTABILIZATION OF THE P-SITE CODON-ANTICODON HELIX RESULTS FROM MOVEMENT OF tRNA INTO THE P/E HYBRID SITE WITHIN THE RIBOSOME

3.1 Introduction

Transfer RNA interacts with three sites on each subunit of the ribosome during translation, the aminoacyl (A) site, the peptidyl (P) site, and the exit (E) site. According to the hybrid states model (Fig 1.1), each round of elongation begins with a complex containing peptidyl-tRNA in the P site in the classical P/P state(6). To this EF-Tu•GTP delivers an aminoacyl (aa) tRNA to the A site, as part of the ternary complex. Cognate interactions stimulate GTP hydrolysis, release of EF-Tu, and movement of the acceptor end of the aa-tRNA into the 50S A site. At this point the tRNA is positioned in the A/A state and peptidyl transfer occurs immediately. Upon transfer of the peptide from the P-site tRNA to the A-site tRNA, the newly deacylated P-site tRNA moves spontaneously with respect to the 50S subunit into the E site to occupy the P/E hybrid site, and the A-site tRNA moves into the A/P site. EF-G•GTP then catalyzes translocation of the tRNA
and mRNA with respect to the 30S subunit, and the tRNAs again occupy the classical E/E and P/P sites, leaving the ribosome ready for another round of elongation.

Several studies have provided independent evidence for the hybrid-states model. Based on forster resonance energy transfer (FRET) measurements, a ~20 Å movement of the 5' end of the newly deacylated tRNA toward ribosomal protein L1 was inferred after peptide bond formation, yet the position of the peptidyl group changed little (106). Because L1 lies near the 50S E site, these data are consistent with formation of the P/E hybrid state. In recent single-molecule FRET studies that monitored the distance between probes attached to the elbow regions of ribosome-bound tRNAs, two alternative tRNA binding configurations were observed that were structurally consistent with the classical and hybrid states (47). An oscillation between these two distinct configurations in single ribosomes further suggested the existence of an equilibrium between the classical and hybrid states of bound tRNA. Additional evidence for the hybrid-states model comes from the ability of puromycin to react at 37°C with peptidyl-tRNA bound to the 30S A site (107, 108). Because puromycin is an aminoacyl-tRNA analog that binds the 50S A site, it was deduced that peptidyl-puromycin formation could only have resulted from movement of peptidyl-tRNA into the A/P site. Finally, cryo-electron microscopy (cryo-EM) studies have provided direct evidence for the P/E hybrid site (27, 28, 109).

Although a growing body of evidence supports the hybrid-states model, it remains an issue of some debate. According to the α-ε model, the tRNAs move to their adjacent ribosomal sites during translocation without passage through hybrid sites (110, 111). This model also posits (i) a strict coupling between selection of aminoacyl-tRNA in the A site
and ejection of deacylated tRNA from the E site and (ii) a mobile rRNA domain at the subunit interface that ferries the tRNAs from the A and P sites to the P and E sites during translocation. Which aspects of the hybrid-states or $\alpha$-$\epsilon$ model correctly reflect the movement of tRNA through the ribosome has been a question of considerable interest for many years.

Retention of the reading frame in ribosomal complexes after single-round translocation depends on the acylation state of the tRNA. When tRNA lacking a peptidyl group is translocated to the P site, the mRNA is susceptible to repositioning, allowing the tRNA to re-pair with a nearby codon. Here, we show that this activity results from movement of tRNA into the P/E hybrid site. Repositioning of mRNA is suppressed by 3'-truncation of the translocated tRNA, increased MgCl$_2$ concentration, and mutation C2394A of the 50S E site, and each of these conditions inhibits movement of tRNA into the P/E-site. Mutation G2252U of the 50S P site stimulates mRNA repositioning, suggesting that decreased affinity of tRNA for the P/P state also destabilizes mRNA in the complex. This work uncovers a functional attribute of the P/E state crucial for understanding translation. These data provide new evidence for the hybrid-states model and have mechanistic implications for translation.
3.2 Materials and Methods.

3.2.1. Strains and plasmids

Mutations were engineered into plasmid p278MS2, which contains a version of the *rrnB* operon that encodes 23S rRNA with an aptamer tag in place of nucleotides 2797-2799. This aptamer tag has high affinity for phage MS2 coat protein. K. Fredrick and K. McGarry performed mutagenesis and constructed derivatives of *E. coli* strain POP2136 recA.

Strains expressing homogeneous populations of ribosomes harboring each substitution of C2394 were constructed by N. Abdi by transforming plasmid p278MS2 (AmpR) and its derivatives pKM1 (C2394A), pKM2 (C2394U), and pKM6 (C2394G) (45, 112) into the Δ7prrn strain SQZ10 (kindly provided by C. Squires and S. Quan, Tufts University, Boston, MA). Transformants were grown in liquid media and spread onto LB plates containing Amp (100 µg/mL) and sucrose (5%) to select for the p278MS2 plasmid, and against pHKrrnC-sacB (KanR), the resident plasmid of SQZ10. Replacement of the pHKrrnC-sacB plasmid with each p278MS2 derivative yielded isolates that were sensitive to Kan, but resistant to both sucrose and Amp. Plasmid replacement was confirmed by purification of plasmid DNA from each strain and sequencing the relevant region of the 23S rRNA gene.

3.2.2. Biochemical reagents

MS2-tagged ribosomes from *E. coli* strain POP2136 recA harboring either G or U at 23S rRNA position 2252 were provided by K. Fredrick and K. McGarry. Tagged
ribosomes were prepared by passing the crude ribosome fraction over a GSTrap FF column pre-bound with GST-tagged MS2 coat protein (45, 112). Tight-coupled ribosomes, various forms of tRNAs, defined mRNAs, and EF-G were prepared as described (85, 87, 113).

To radiolabel the 3' end of tRNA, ATP(CTP):tRNA nucleotidyltransferase was used to add nucleotides back onto 3'-truncated tRNA (tRNAΔ3') in the presence of CTP and α-[32P]-ATP. In 200 µL reactions containing 50 mM glycine (pH 9.0) and 10 mM MgCl₂, tRNAΔ3' preparations (0.5 µM) were incubated for 5 minutes at 37°C in the presence of CTP (10 µM), α-[32P]-ATP (10 µM; <300 Ci/mmol), and ATP(CTP):tRNA nucleotidyltransferase (3 µg/mL). The reactions were then extracted with phenol and chloroform, free ATP was removed by passage through 2 sequential Sephadex G-25 (Amersham Biosciences) spin columns, and 75 µl of [3'-32P]-tRNA was added to 6 nmols of the identical unlabeled tRNA and aminoacylated as described (85, 87, 113).

3.2.3. Toeprinting

Toeprinting experiments were performed as described (85, 87). Typically, [32P]-labeled primer was annealed to mRNA (1 µM) in 50 mM Tris-HCl (pH 7.6) and 100 mM NH₄Cl by heating to 60°C and placing on ice. MgCl₂ (to 20 mM), β-mercaptoethanol (to 6 mM), ribosomes (0.7 µM), and tRNA (to 1 µM) were added and incubated at 37°C for 20 minutes to bind the P site, and a 2 µL aliquot "P" was removed to ice. Next, tRNA was added and incubated for 10 minutes to fill the A site, and a 2 µL aliquot "A" was removed to ice. A portion of the pretranslocation complex was then diluted by 20% into
buffer [50 mM Tris-HCl (pH 7.6), 100 mM NH₄Cl, 20 mM MgCl₂, 6 mM βME] containing GTP (1.5 mM) or GTP and EF-G (5 µM), such that the final concentrations of GTP and EF-G were 300 µM and 1 µM, respectively. After 10 minutes at 37° C, 2 µL aliquots were removed, and all aliquots were extended in parallel as described (40). The percentage of posttranslocation complexes that retain the correct reading frame (% frame retention) was determined as described (85).

3.2.4. Determination of the extent of deacylation of ribosome-bound aa-tRNA following translocation

The extent of deacylation was determined after translocation of Tyr-[3'-32P]-tRNAᵀyr² or Val-[3'-32P]-tRNA₉al to the P site. Complexes were made in an analogous manner to those in the corresponding toeprinting experiments described above. In the experiment of Fig. 5A, pretranslocation complexes were made by incubating control or G2252U ribosomes (1 µM) with message m5 (5'-GGCAAGGGAGGUAUAUGUACUUUGUAAAUAU-3'; 2 µM) and tRNAᵀ₉et (2 µM) to fill the P site and subsequently adding Tyr-[3'-32P]-tRNAᵀyr² or deacylated [3'-32P]-tRNAᵀyr² to bind the A site. In the experiment of Fig. 5B, pretranslocation complexes were made by incubating control or G2252U ribosomes (1 µM) with message m4 (5'-GGCAAGGGAGGUAUAACGUAAAAGCAGGU-3'; 2 µM) and tRNAᵀ₉et (2 µM) to fill the P site and subsequently adding Val-[3'-32P]-tRNA₉al or deacylated [3'-32P]-tRNA₉al to bind the A site. To each pretranslocation complex, EF-G (1 µM) and GTP (300 µM) were added, and the resulting posttranslocation complex was purified from free
tRNA by passage over 0.5 mL Sephacryl S-200 HR (Sigma) spin columns, pre-equilibrated in the buffer of the translocation reaction. Eluted samples were diluted 2-fold with acid loading buffer [7 M urea, 100 mM NaOAc (pH 5.2), 0.05 % bromophenol blue] and loaded (10 µL) onto an acid gel [6.5 % polyacrylamide, 8 M urea, 100 mM NaOAc (pH 5.2)], which was run for 12 hours at 10W in 100 mM NaOAc (pH 5.2).

In 85 µL reactions containing 50 mM Tris-HCl (pH 7.6), 20 mM MgCl₂, 100 mM NH₄Cl, and 6 mM βME, control or G2252U ribosomes were incubated for 20 minutes at 37°C with message m5 (5'-GGCAAGGAGGUAAAUAUGUACUUUGUAAAAU-3'; 2 µM) and fMet-[³⁵S]-tRNAˢᵗMet (1 µM) to bind the P site. Complexes were purified from free fMet-[³⁵S]-tRNAˢᵗMet by passage over 0.5 mL Sephacryl S-200 spin columns. One 7.5 µL aliquot of the excluded volume was immediately removed to 0.8 µL 3M NaOAc (pH 5.2) on ice, and a second 7.5 µL aliquot was incubated with puromycin (1.7 mM) for 10 minutes at room temperature prior to addition of NaOAc (pH 5.2) to 300 mM. The remainder of the excluded volume was incubated at room temperature and samples were removed at various time points and deacylation was quenched by addition of NaOAc (pH 5.2) to 300 mM on ice. Each quenched sample (2 µL) was spotted directly onto a glass-backed silica TLC plate (Analtech), and the TLC plate was developed in solvent (butanol: acetic acid: water; 3:1:1). Under these conditions, intact fMet-[³⁵S]-tRNAˢᵗMet remains at the origin, while formyl-[³⁵S]-methionine and fMet-[³⁵S]-puromycin migrate up the plate.
3.3. Results

3.3.1. Deacylation of $N$-acetyl-aminoacyl-tRNA in the posttranslocation complex results in mRNA repositioning.

In experiments that mapped the position of mRNA in ribosomal complexes before and after translocation by toeprinting, it was shown that retention of the reading frame depends on the presence of a peptidyl group on the tRNA translocated to the P site (85, 87). When tRNA lacking a peptidyl group was translocated, uncoupled movement of the mRNA was observed. Instead of moving in the 5' direction by 3 nucleotides as expected, the mRNA was repositioned in the 3' direction to allow re-pairing of the tRNA with an upstream out-of-frame codon, more optimally spaced from the Shine-Dalgarno sequence. This efficient in vitro frameshift event was only observed when (i) the translocating tRNA lacked a peptidyl group and (ii) the mRNA sequence contained an additional codon cognate for the translocating tRNA to provide opportunity for its re-pairing.

It was unclear from these experiments whether the mRNA slippage event occurred during or after EF-G-dependent movement of deacylated tRNA. Relevant to this question, the position of mRNA in ribosomal complexes was mapped after translocation of $N$-acetyl-aminoacyl-tRNA and subsequent treatment with puromycin (Fig. 3.1). To form pretranslocation complexes, tRNA$^{f\text{Met}}$ was first bound to the P site of ribosomes programmed with message m299. A single toeprint was observed at position +16 (Fig. 3.1, lane P), indicating that the AUG codon was positioned in the 30S P site. $N$-acetyl-Tyr-tRNA$^{\text{Tyr}_2}$ was then added to pair with the UAC codon in the A site, and this complex
was characterized by a strong toeprint at position +17 (Fig. 3.1, lane A). This single nucleotide shortening of the toeprint depends on cognate A-site tRNA and has been attributed to a conformational change that occurs upon A-site binding (96). Addition of EF-G and GTP resulted in a large reduction in the +16/17 toeprints and appearance of a strong toeprint at +19 (Fig. 3.1, lane G).

**Figure 3.1 Deacylation of N-acetyl-aminoacyl-tRNA in the posttranslocation (POST) complex results in mRNA slippage.** The position of mRNA in ribosomal complexes was mapped by toeprinting after each of several additions. A pretranslocation (PRE) complex was made by incubating ribosomes with m299 and tRNA$_{fMet}$ to fill the P site (lane P) and then adding N-acetyl-Tyr-tRNA$_{Tyr2}$ to fill the A site (lane A). Next, this PRE complex was incubated with EF-G plus GTP to form the POST complex (G lane). Finally, this POST complex was further incubated without or with addition of puromycin (1.7 mM) as indicated (Result first observed by Kurt Fredrick in a similar experiment).
The toeprint at +19 corresponds to the posttranslocation complex containing $N$-acetyl-Tyr-tRNA$^{Tyr_2}$ paired to UAC in the P site. The posttranslocation complex was then divided into two portions, puromycin was added to one portion, and aliquots were removed from each portion and toeprinted as a function of time. Addition of puromycin caused the appearance of a toeprint at position +15 (Fig. 3.1, +Pm lanes), which corresponds to positioning of the upstream UAU tyrosine codon in the P site. Thus, deacylation of $N$-acetyl-Tyr-tRNA$^{Tyr_2}$ in the posttranslocation complex results in repositioning of m299 in the 3’ direction by 4 nucleotides. In other words, deacylation allows the anticodon of P-site tRNA$^{Tyr}$ to disengage from the UAC codon and pair with the upstream UAU codon. The toeprint pattern observed after puromycin addition in this experiment is identical to that observed after translocation of deacylated tRNA$^{Tyr_2}$ in the same context ([87]), suggesting that in the latter case, mRNA repositioning could occur after tRNA translocation.

In previous footprinting experiments, addition of puromycin to ribosomes programmed with polyuridylic acid (poly-U) and containing $N$-acetyl-Phe-tRNA$^{Phe}$ in the P site resulted in loss of protections within the 50S P site and gain of protections within the 50S E site ([6]). It was inferred that after peptide bond formation, the newly deacylated tRNA moved spontaneously into the P/E hybrid site. We hypothesized that movement of tRNA into the P/E site destabilizes its interaction with mRNA (Fig. 3.2). This hypothesis could readily explain why mRNA repositioning was observed after either (i) deacylation of $N$-acetyl-aminoacyl-tRNA in the P site by addition of puromycin (Fig. 3.1) or (ii) translocation of deacylated tRNA to the P site ([85, 87]).
To evaluate this hypothesis, we used several strategies to alter the relative affinity for the P/E site and measured their effects on frame retention.

**Figure 3.2. The destabilization of mRNA observed is hypothesized to result from movement of tRNA into the P/E hybrid site.** Translocation of tRNA within the ribosome is believed to occur in a step-wise fashion (Pathway 1). After peptide bond formation, the newly deacylated tRNA and newly formed peptidyl-tRNA move first with respect to the 50S subunit into the hybrid P/E and A/P sites, respectively. EF-G (+GTP) then catalyzes the movement of tRNA and mRNA with respect to the 30S subunit. Because interaction of tRNA in the E/E site is kinetically labile (114, 115), peptidyl-tRNA bound to the P/P site is presumably sufficient to retain stable mRNA interaction in this POST complex. EF-G(+GTP) can also catalyze the translocation of deacylated tRNA within the ribosome (Pathway 2), although it is unclear whether this reaction involves the hybrid state intermediate. In this posttranslocation complex, tRNA can occupy either the P/P or the P/E site. It is hypothesized that movement of tRNA into the P/E site destabilizes mRNA in this posttranslocation complex, which results in repositioning of mRNA. Deacylation of peptidyl-tRNA in the posttranslocation complex of Pathway 1 by addition of puromycin also allows movement of tRNA into the P/E site, and destabilization of mRNA is also observed in this case (see Figure 3.1).
3.3.2. Repositioning of mRNA observed upon translocation of deacylated tRNA is partially suppressed by increasing the concentration of MgCl$_2$ in the translocation reaction.

Chemical probing experiments have shown that occupation of the P/E site depends on the concentration of Mg$^{2+}$ in the reaction (6). At 20-25 mM Mg$^{2+}$, the P/P site is favored, while at 10 mM Mg$^{2+}$, the P/E site is favored. To determine the effect of Mg$^{2+}$ concentration on reading frame retention, we mapped the position of mRNA in ribosome complexes after EF-G-dependent translocation at various concentrations of MgCl$_2$ (Fig. 3.3).

**Figure 3.3.** Repositioning of mRNA conferred by the translocation of deacylated tRNA is partially suppressed by increasing the MgCl$_2$ concentration in the translocation reaction. PRE complexes containing either N-acetyl-aminoacyl-tRNA (open symbols) or deacylated tRNA (closed symbols) bound to the A site were mixed into translocation reactions containing EF-G and GTP such that the final concentration of MgCl$_2$ differed as indicated. Reading-frame retention was determined after translocation of m291 with N-acetyl-Val-tRNA$^{Val}_1$ (△) or tRNA$^{Val}_1$ (○), m299 with N-acetyl-Tyr-tRNA$^{Tyr}_2$ (□) or tRNA$^{Tyr}_2$ (■), and m301 (87) with N-acetyl-Phe-tRNA$^{Phe}_8$ (△) or tRNA$^{Phe}_8$ (▲).
A pronounced effect of Mg$^{2+}$ was observed when deacylated tRNA$^{\text{Val1}}$ was translocated to the P site in ribosomes programmed with message m291. When the MgCl$_2$ concentration in the translocation reaction was increased from 10 mM to 25 mM, reading frame retention increased from ~20% to ~80%. A similar effect was observed when tRNA$^{\text{Phe}}$ was translocated with message m301. A 28% increase in frame retention was observed as the concentration of MgCl$_2$ increased from 10 to 25 mM in the translocation reaction. For complexes in which tRNA$^{\text{Tyr2}}$ and m299 were translocated, a ~10% increase in frame retention was observed when the MgCl$_2$ concentration was increased from 15 mM to 25 mM. Unlike translocation of deacylated tRNA, translocation of N-acetyl-aminoacyl-tRNA resulted in high-level frame retention throughout the range of MgCl$_2$ concentrations, although a 10-20% reduction in frame retention was observed at 10 mM MgCl$_2$. These data are consistent with the hypothesis that mRNA destabilization results from movement of tRNA into the P/E site.

It has been suggested that hybrid-state binding of tRNA depends on the use of conventional buffers (111). Although more recent cryo-EM data argues against this notion (27), it was of interest to compare reading frame retention in conventional buffer and polymix, a polyamine-containing buffer that enhances the activity and fidelity of ribosomes in vitro (88). Therefore, we prepared ribosome complexes in either conventional or polymix buffer and mapped mRNA before and after translocation.
Regardless of the buffer system used, frame retention depended on the presence of a peptidyl group analog on the tRNA translocated to the P site (data not shown). Thus, repositioning of mRNA attributed to occupation of the P/E site can be observed in either buffer system.

3.3.3. Repositioning of mRNA is suppressed by mutations of E-site nucleotide C2394.

To directly test the hypothesis that movement of tRNA into the P/E site destabilizes its interaction with mRNA, it was necessary to obtain ribosomes harboring a mutation in the 50S E site. Because two hydrogen bonds are formed between base C2394 of 23S rRNA and nucleotide A76 of tRNA in the 50S E site (22), we chose to mutagenize C2394. We generated each base substitution at position 2394 of the 23S rRNA gene cloned on plasmid p278MS2 (116). Plasmids encoding _rrnB_ without or with mutation C2394A, C2394G, or C2394U were moved into an _E. coli_ strain lacking all chromosomal _rrn_ operons (Δ7 prrn), replacing the resident plasmid containing _rrnC_ (selection was performed by N. Abdi).

We purified ribosomes harboring the C2394A mutation and assayed frame retention after EF-G-dependent translocation of various tRNA substrates (Fig. 3.4). In control ribosomes, translocation of _N_-acetyl-Phe-tRNA^{Phe} resulted in high-level retention of the reading frame. When _N_-acetyl-Phe-tRNA^{Phe} was translocated in C2394A ribosomes, a slight increase in frame retention was observed compared to control ribosomes. Translocation of tRNA^{Phe} in control ribosomes resulted in a substantial
decrease in reading frame retention, consistent with previous studies (85). In C2394A ribosomes, translocation of tRNA\textsuperscript{Phe} was further inhibited, providing direct evidence that the 50S E site is involved in the mechanism of translocation (49, 50). In addition, C2394A increased frame retention by ~2-fold. These data indicate that C2394A can suppress the mRNA repositioning conferred by translocation of N-acetyl-Phe-tRNA\textsuperscript{Phe} or tRNA\textsuperscript{Phe}. Similar results were obtained by H. Wang and K. McGarry with C2394A-containing ribosomes programmed with messages m299 and m291 (45), indicating that this phenomenon is not limited to a specific tRNA isoacceptor. The simplest interpretation of these results is that mRNA repositioning results from an interaction between the 50S E site and the tRNA translocated to the 30S P site.

Figure 3.4. Effects of 23S rRNA mutation C2394A on reading frame retention after translocation of two forms of tRNA\textsuperscript{Phe}. PRE complexes were made by incubating ribosomes with m301 and tRNA\textsuperscript{Tyr2} to fill the P site (P lanes) and then adding N-acetyl-Phe-tRNA\textsuperscript{Phe}, Phe-tRNA\textsuperscript{Phe}, or tRNA\textsuperscript{Phe} to bind the A site (A lanes) as indicated. Complexes were then incubated in the presence of GTP (- lanes) or EF-G plus GTP (+ lanes). At each stage of the experiment, the position of mRNA was mapped by toeprinting. Ability of C2394A to suppress mRNA repositioning was first observed by Huanyu Wang using ribosomes from another strain and a different mRNA-tRNA context.
3.3.4. Mutation G2252U stimulates mRNA repositioning.

G2252 of 23S rRNA forms a Watson-Crick base pair with C74 of tRNA in the 50S P site (55, 117). Mutation of G2252 will disrupt this base pair and presumably destabilize tRNA interaction in the P/P site. Ribosomes containing G2252U were used to assay reading frame retention after EF-G-dependent translocation of N-acetyl-Val-tRNA<sub>Val</sub>, Val-tRNA<sub>Val</sub>, and tRNA<sub>Val</sub>. Mutation G2252U decreased reading frame retention from 88% to 79%, from 72% to 50%, and from 60% to 41% upon translocation of N-acetyl-Val-tRNA<sub>Val</sub>, Val-tRNA<sub>Val</sub>, and tRNA<sub>Val</sub>, respectively. In other words, G2252U promoted mRNA repositioning in these cases. In addition, G2252U increased the efficiency of Val-tRNA<sub>Val</sub> translocation such that inhibition by this substrate was no longer observed. The effect of G2252U was first observed by K. Fredrick with a different tRNA-mRNA context.

3.3.5. Effect of G2252U on deacylation of tRNA within the ribosome.

Considerable mRNA repositioning was observed after translocation of aminoacyl-tRNA in control ribosomes or after translocation of aminoacyl-tRNA or N-acetyl-aminoacyl-tRNA in G2252U ribosomes. Because deacylation of tRNA can promote mRNA slippage (Fig. 3.1), it was important to determine whether mRNA slippage in these cases resulted from deacylation of tRNA and whether G2252U increased repositioning by stimulating deacylation of tRNA. To investigate these possibilities, we performed several experiments.
Figure 3.5. Effect of G2252U on deacylation of tRNA within the ribosome.
(A) G2252U increases the extent of deacylation of Val-[3'-32P]-tRNA\textsuperscript{Val1} in the POST complex. PRE complexes were formed by incubating programmed ribosomes with tRNA\textsuperscript{fMet} to fill the P site and subsequently adding either deacyl-[3'-32P]-tRNA\textsuperscript{Val1} (da) or Val-[3'-32P]-tRNA\textsuperscript{Val1} (aa) to bind the A site. Then, EF-G and GTP were added to catalyze translocation, and the resulting complexes were purified from free tRNA and subjected to acid gel electrophoresis to resolve aminoacylated from deacylated tRNA (as indicated).
(B) G2252U does not affect the extent of deacylation of Tyr-[3'-32P]-tRNA\textsuperscript{Tyr2} in the POST complex. This experiment is similar to that of panel A, although in this case, either deacyl-[3'-32P]-tRNA\textsuperscript{Tyr2} (da) or Tyr-[3'-32P]-tRNA\textsuperscript{Tyr2} (aa) was translocated to the P site prior to complex purification and acid gel electrophoresis. (C) G2252U does not affect the rate of deacylation of fMet-[35S]-tRNA\textsuperscript{fMet} bound to the P site. Control (□) or G2252U (○) ribosomes containing fMet-[35S]-tRNA\textsuperscript{fMet} bound to the P site were purified from free tRNA and the amount of intact fMet-[35S]-tRNA\textsuperscript{fMet} remaining was determined as a function of time. In control reactions, the amount of intact fMet-[35S]-tRNA\textsuperscript{fMet} remaining after addition of puromycin (1.7 mM) to control (■) and G2252U (●) complexes was also determined.
In the first experiment, the extent of deacylation was determined after translocation of Val-[3'-$^{32}$P]-tRNA$^{Val1}$ in control and G2252U ribosomes (Fig. 3.5A), in complexes analogous to those of the toeprinting experiments. First, CCA-adding enzyme and $\alpha$-[32P]-ATP were used to radiolabel the 3' terminal nucleotide of tRNA$^{Val1}$. Next, ribosomes with or without G2252U were used to make PRE complexes containing tRNA$^{fMet}$ in the P site and either Val-[3'-$^{32}$P]-tRNA$^{Val1}$ or deacyl-[3'-$^{32}$P]- tRNA$^{Val1}$ in the A site. EF-G and GTP were then added, and the resulting POST complexes were purified from unbound tRNA by Sephacryl S-200 chromatography. Finally, the aminoacylation state of the tRNA in each complex was assessed by acid gel electrophoresis (Fig. 3.5A). In this experiment, 93% of the tRNA remained aminoacylated after translocation in control ribosomes, while 92% remained aminoacylated in G2252U ribosomes. In other words, low and comparable levels of deacylation were observed, inconsistent with the hypothesis that mRNA repositioning observed after translocation of Val-tRNA$^{Val1}$ in either control or G2252U ribosomes is a consequence of deacylation.

For comparison, we determined the extent of deacylation after translocation of Tyr-tRNA$^{Tyr2}$ to the P site, which had been shown to behave similarly to Val-tRNA$^{Val1}$ when translocated from the A to P site of ribosomes with or without the G2252U mutation (45). We found that 77% of the tRNA$^{Tyr2}$ remained aminoacylated after translocation in control ribosomes, while 63% remained aminoacylated after translocation in G2252U ribosomes (Fig. 3.5B), indicating that G2252U can stimulate deacylation of Tyr-tRNA$^{Tyr2}$ to some degree. The levels of deacylation observed in control and G2252U
ribosomes suggest that deacylation of Tyr-tRNA\textsuperscript{Tyr2} contributes to, but cannot completely account for the mRNA repositioning observed in analogous toeprinting experiments.

To further investigate whether G2252U can stimulate deacylation, we compared the rate of deacylation of \[^{35}\text{S}]-\text{fMet-tRNA}^{\text{fMet}} bound to the P site in control and G2252U ribosomes. In this experiment, \[^{35}\text{S}]-\text{fMet-tRNA}^{\text{fMet}} was bound to the P site of programmed ribosomes, complexes were purified from unbound \[^{35}\text{S}]-\text{fMet-tRNA}^{\text{fMet}} by Sephacryl S-200 chromatography, and then the amount of intact \[^{35}\text{S}]-\text{fMet-tRNA}^{\text{fMet}} present was quantified as a function of time (Fig. 3.5C). Although equivalent amounts of \[^{35}\text{S}]-\text{fMet-tRNA}^{\text{fMet}} bound to control and G2252U ribosomes, the presence of G2252U decreased the puromycin reactivity of bound \[^{35}\text{S}]-\text{fMet-tRNA}^{\text{fMet}} from 88\% to 50\%. This is consistent with the role of G2252 in positioning P-site tRNA for peptidyl-transfer (116). Importantly, no difference in the rate of deacylation was apparent. Thus, G2252U does not generally stimulate deacylation of tRNA in the ribosome, which argues against the idea that increased deacylation causes increased mRNA repositioning in these ribosomes.

3.3.6. Depletion of EF-G from POST complexes results in reformation of the PRE complex but does not affect mRNA repositioning

In order to address recent suggestions that hybrid state formation (27) and P/E site-attributed mRNA repositioning (118) require EF-G binding, we next considered the effects of EF-G on P/E site-attributed mRNA repositioning.
Figure 3.6 Depletion of EF-G from POST complexes results in reformation of the PRE complex, but does not affect mRNA repositioning. The position of mRNA m299 (see Fig. 3.1) in ribosome complexes was mapped after each of several additions. PRE complexes were assembled by first binding tRNA\textsuperscript{fMet} to the P site, and then adding N-acetyl-Tyr-tRNA\textsuperscript{Tyr2} to bind the A site. The complexes were then further incubated with GTP and buffer in the absence (-) or presence (+) of EF-G. This reaction was divided into two portions. The first aliquot was further divided into 2 tubes, the first was incubated with DMSO, and the second was incubated with Thiostrepton (Thio). These reactions were then incubated in the presence or absence of puromycin and toeprinted. The second portion of the G reaction was run over an S200 spin column to remove free EF-G, GTP, and tRNA. The filtered reaction (EF-G depleted) was then treated with DMSO, Thio, and Puro as with the first portion of the G reaction and each reaction examined by toeprinting as indicated. The +16/17 toeprints indicate PRE complex with UAC in the A site, while the +19 toeprint indicates POST complex with UAC in the P site. The +15 toeprint is the product of mRNA repositioning, and corresponds to the upstream UAC codon.

We first formed PRE complexes containing tRNA\textsuperscript{fMet} in the P site and N-acetyl-Tyr-tRNA\textsuperscript{Tyr2} in the A site, and observed a strong toeprint at +17 (Fig 3.6, lane 1). Upon addition of EF-G, we saw a reduction of the +17 toeprint corresponding to the PRE complex and appearance of a toeprint at +19, corresponding to the POST complex, with N-acetyl-Tyr-tRNA\textsuperscript{Tyr2} in the P site (lane 2).

Further incubation of the EF-G-containing POST complexes with thiostrepton, an antibiotic known to decrease the apparent affinity of EF-G for the ribosome (119),
resulted in the unexpected reappearance of the +17 toeprint, corresponding to the PRE complex (Fig. 3.6, lanes 5 and 6). We did not observe this pattern in the no thiostrepton control, in which POST complexes were further incubated with DMSO (Fig. 3.6, lanes 3 and 4). In parallel, we depleted EF-G from a portion of these POST complexes by Sephacryl S200 (lane 7). This also resulted in the appearance of the toeprint at +17, corresponding to reformation of the PRE complex. Thus, either removal or inhibition of EF-G results in spontaneous reformation of the PRE complex. These unanticipated data were the first indication that the POST state is less stable than the PRE state in certain ribosomal complexes (60).

Each complex was also treated with puromycin. Regardless of whether EF-G was present or depleted, treatment of POST complexes with puromycin resulted in the appearance of a toeprint at +15, demonstrating that removal of the peptidyl group destabilized the mRNA (Fig. 3.6, lanes 4, 9). The same pattern was observed when puromycin was added to POST complexes that had been preincubated with thiostrepton, both in the presence or absence of EF-G (lanes 6, 11). From this it can be inferred that repositioning of mRNA dependent on P/E site binding was independent of EF-G. This is in disagreement with the conclusions of another study in which POST complexes assembled with a different mRNA were treated with thiostrepton prior to puromycin addition, and no repositioning was observed. Because thiostrepton is thought to interfere with binding of EF-G, it was suggested that EF-G was required to observe repositioning (118).
It is clear from our experiments that EF-G is not required for repositioning (Fig. 3.6), and the explanation for the result observed with thiostrepton in the former work is unclear (118).

3.3.7. Direct binding of deacylated tRNA to the P site causes upstream toeprints corresponding to near-cognate codons.

Upstream toeprints corresponding to near-cognate codons were observed upon direct binding of deacylated tRNA to the P site (87). These near-cognate codons were spaced suboptimally from, or within the Shine-Dalgarno sequence. Because these toeprints depended on deacylated tRNA, we hypothesized that they may be due to P/E-site binding. To address this question, we bound several forms of tRNA\textsuperscript{Tyr\textsubscript{2}} that differed in their relative affinity for the P/E site to \textit{E. coli} MRE600 ribosomes programmed with message m293 (Fig. 3.7). In the absence of tRNA, no toeprints were visible, indicating that there were no contaminating tRNAs in the ribosome preparations (Fig 3.7, lane 1). When the position of mRNA was mapped upon binding of N-acetyl-Tyr-tRNA\textsuperscript{Tyr\textsubscript{2}} (Fig 3.7, lane 2), the predominant toeprint was +19 (85% downstream toeprint), corresponding to pairing with a cognate UAC codon. However, when tRNA\textsuperscript{Tyr\textsubscript{2}}, predicted to bind the P/E site, was bound (Fig 3.7, lane 3), the distribution of toeprints changed, so that roughly half of the complexes contained a near-cognate AAU codon in the P site (56% downstream toeprint). Furthermore, when Tyr-tRNA\textsuperscript{Tyr\textsubscript{2}} was bound to the P site (Fig 3.7, lane 4), the distribution of +15/+19 toeprints was intermediate to that observed with deacyl or peptidyl tRNA (79% downstream toeprint), reminiscent of an intermediate level
of mRNA retention previously observed upon translocation of aa-tRNA (45, 85). When the 3’ terminal nucleotides were removed from the tRNA, however, the majority of the complexes contained a cognate UAC codon in the P site (91% downstream toeprint). Removal of the 3’ terminal -CA decreases the affinity of tRNA for the E site by >100-fold (49), and renders tRNA defective in P/E site binding (46).

Figure 3.7. Upstream toeprints corresponding to near-cognate codons are observed upon direct P-site binding, but only if the tRNA can occupy the P/E site. The position of mRNA was monitored after binding tRNA to the P site of ribosomes programmed with m293 (A) or m301 (B). The nucleotide in bold is designated as +1 based on spacing from the SD sequence (underlined), and generates a corresponding toeprint at +16. Similar results were obtained by K. McGarry.

Next, we monitored the toeprint pattern in m301-programmed ribosomes. No toeprints were observed in the absence of added tRNA (Fig. 3.7, lane 6). Upon binding of
N-acetyl-Tyr-tRNA\textsuperscript{Tyr2}, a toeprint was observed at +18, corresponding to positioning of the cognate UAC codon in the P site (Fig 3.7, lane 7). In this case, binding of deacyl tRNA\textsuperscript{Tyr2} results in the appearance of several toeprints (Fig. 3.7, lane 8). In addition to the predominant toeprint at +18 corresponding to positioning of the cognate UAC codon in the P site, toeprints were also observed at +9 and +12, corresponding to positioning of AAU in the P site. Similar to the patterns observed upon binding to m293-programmed ribosomes, the presence of upstream toeprints corresponding to near-cognate codons correlated with the ability of the tRNA to bind the P/E site. These data raise the hypothesis that mRNA is repositioned within these complexes during the primer extension analysis.

3.3.8. Mutations of C2394 decrease the intensity of upstream toeprints corresponding to near-cognate codons.

To confirm that the appearance of upstream toeprints was related to movement of the tRNA into the P/E site, we employed ribosomes harboring mutations in the 50S E site rRNA at position 2394. We bound tRNA\textsuperscript{Tyr2} to the P site of m293- or m301-programmed ribosomes harboring each substitution at 2394 (Fig 3.8). We found that mutation of the 50S E site decreased the intensity of the upstream toeprints, where the magnitude of the effect followed the trend A>G>U>WT. The same trend was observed for defects in growth rate (Chapter 4), rate and extent of sparsomycin-catalyzed translocation (see below section 3.12), apparent $K_m$ of sparsomycin for the PRE complex (data not shown), maximal rate of EF-G dependent translocation (Chapter 4, Table 4.1), and $K_{1/2}$ of EF-G for the PRE complex (Chapter 4, Table 4.1).
Figure 3.8. Mutations of C2394 suppress the appearance of upstream toeprints corresponding to near-cognate codons. The position of mRNA was monitored after binding tRNA to the P site of ribosomes from Δ7prrn strains as indicated. Complexes in panel A were programmed with message m293, and complexes in B were programmed with message m301.

3.3.9. Repositioning of mRNA is affected by the type and concentration of reverse transcriptase.

When POST complexes were treated with puromycin, the apparent rate of mRNA repositioning could not be resolved by toeprinting (Fig 3.1), which suggested two possibilities: 1) Repositioning occurs spontaneously upon P/E site binding at a rate that
cannot be resolved by manual techniques such as toeprinting, or 2) repositioning occurs in a reverse transcriptase-dependent manner. Two lines of evidence supported the latter. First, toeprints resulting from mRNA repositioning always corresponded to a site upstream from the initial codon positioned in the P site, consistent with the directionality of the primer extension reaction. Second, mRNA repositioning could not be detected by a fluorescence stopped-flow assay in a context similar to that in which it has previously been observed by toeprinting (data not shown). We hypothesized that the toeprints corresponding to upstream near-cognate codons and dependent on deacylated tRNA may also be the result of mRNA repositioning. In this case, the cognate codon would be initially present in the P site, but the mRNA would be repositioned by reverse transcriptase. In order to test whether reverse transcriptase could affect toeprint patterns, we bound tRNA\textsuperscript{Tyr2} or tRNA\textsuperscript{Tyr2} (Δ3') to the P site of ribosomes programmed with message m293 pre-annealed with labeled primer, and performed the extension reaction with several concentrations of either AMV or MMLV reverse transcriptase (Fig 3.9A).

When tRNA\textsuperscript{Tyr2} was bound to the P site, extension with any tested concentration of AMV reverse transcriptase yielded two toeprints: one at +19, corresponding to a cognate UAC codon, and a second at +15, corresponding to a near-cognate AAU codon (Fig 3.9A, AMV lanes). With AMV reverse transcriptase, there were no stops corresponding to non-cognate codons, which would suggest premature transcription termination or pausing. However, when the extension reaction was performed with MMLV reverse transcriptase, a different toeprint pattern was observed that was dependent on the concentration of reverse transcriptase.
Figure 3.9. Repositioning of mRNA observed by toeprinting is dependent on both P/E site binding and reverse transcriptase. (A) The position of m293 was mapped following direct binding of tRNA\textsubscript{Tyr2} or tRNA\textsubscript{Tyr2}(Δ3'), using increasing concentrations of AMV or MMLV reverse transcriptase (RT). (B) Model for RT and P/E site dependent mRNA repositioning. It is possible that the downstream (cognate) codon is initially positioned in the P site, but RT can promote repositioning of mRNA in those complexes in which tRNA can occupy the P/E site.

At lower concentrations of MMLV, a toeprint corresponding to the +19 UAC codon was observed, as well as several downstream stops that likely corresponded to pausing or termination by reverse transcriptase, as the non-cognate codons were predicted to occupy the P-site if these were the result of RT encountering ribosomes. At higher concentrations of MMLV, the toeprint at +15 corresponding to AAU became more intense, and the +19 toeprint became less apparent. At the highest concentrations tested,
+15 was the major toeprint observed, demonstrating that the distribution of the upstream and downstream toeprints could be altered by changing the concentration of reverse transcriptase. These data show that mRNA repositioning can occur in a reverse transcriptase-dependent manner.

Next we bound tRNA$^{Tyr2}$ (Δ3'), which is defective in P/E site binding, to the P site and mapped the mRNA position with varying amounts of AMV or MMLV reverse transcriptase in the extension mix. When primer extension was performed using AMV, a single toeprint was observed at +19 at all concentrations of reverse transcriptase. With MMLV, the +19 toeprint is prominent under all conditions, although the shorter extension products are still observed at low MMLV concentrations, and some RT-dependent mRNA repositioning is evident at high MMLV concentrations.

Together these data suggest a model in which mRNA repositioning depends on both P/E occupation and reverse transcriptase. We hypothesize that the downstream (cognate) codon is initially positioned in the P site, but reverse transcriptase promotes repositioning in those complexes in which tRNA can occupy the P/E site (Figure 3.10B).

3.3.10. Antibiotics that perturb toeprint patterns may alter the P/P to P/E equilibrium.

It is clear that the toeprinting pattern for certain mRNAs (e.g. m293, m301) depends on the ability of the tRNA to occupy the P/E site. To screen antibiotics for those that affect the P/P to P/E equilibrium, we next considered the effects of several antibiotics that are known to inhibit translocation.
Figure 3.10 Effects of antibiotics on reverse transcriptase-dependent mRNA repositioning. The position of message m293 was mapped after incubating tRNA$^{\text{Tyr2}}$ bound ribosomes with drugs as indicated.

To ascertain effects, we determined the ratio of toeprints at position +15 and +19 when tRNA$^{\text{Tyr2}}$ was bound to the P site of m293-programmed ribosomes and subsequently incubated with each antibiotic. In the first two lanes, tRNA$^{\text{Tyr2}}$ bound ribosomes were incubated in the absence of drug (Fig. 3.10, no drug, DMSO lanes). Upon extension, a strong toeprint was observed at +19, as well as a less intense toeprint at +15. Addition of antibiotics to these P-site bound complexes revealed several that decrease or increase the ratio of the +19 toeprint. Streptomycin and spectinomycin decreased the +19/+15 ratio, indicating that they may stimulate P/E site binding.

Conversely, binding of the aminoglycoside antibiotics neomycin, paromomycin, and gentamycin had the opposite effect, which may indicate that these antibiotics stabilize the classical conformation of the ribosome, with tRNA bound in the P/P site. The remaining
antibiotics did not induce a change in the ratio of the +19/+15 toeprints. Further experiments will be necessary to determine whether these antibiotics alter the P/P to P/E equilibrium or destabilize/stabilize the mRNA directly.

3.3.11 Substitutions of C2394 decrease the apparent rate of sparsomycin-catalyzed translocation.

Translocation can be induced in the absence of EF-G by the antibiotic sparsomycin (Sps) \((87)\). There is compelling evidence that A/P state formation is required for Sps-dependent translocation, perhaps because A/P site binding allows Sps to bind the 50S A site \((43)\). We hypothesized that each mutation would render ribosomes defective in Sps-dependent translocation, as P/E state formation is a prerequisite for movement into the A/P site. In order to determine the effects of C2394 substitutions on A/P state formation, we monitored the rate of Sps-dependent translocation by toeprinting \((87)\). First, a PRE complex was formed by sequentially adding tRNA\(^{\text{Tyr2}}\) to bind the P site, and \(N\)-Acetyl-Phe-tRNA\(^{\text{Phe}}\) to bind the A site of m297-programmed control ribosomes or ribosomes harboring each substitution of C2394. Next, Sps (500 \(\mu\)M) was added to the complexes, and portions were removed at the indicated times to pre-warmed extension cocktails containing viomycin. Viomycin inhibits forward translocation, and acts as a quench while the reactions are being extended by reverse transcriptase. Following extension the products were resolved on a sequencing gel and the fraction of POST complex remaining was quantified and plotted versus time. For each experiment, the rate was determined by fitting to a single-exponential function.
In control ribosomes, the rate of Sps-dependent translocation was 0.47 s\(^{-1}\), and the extent of translocation was 75%, which is similar to what was previously measured for this complex \((87)\). The mutations C2394A and C2394G conferred a decrease of \(~4\)-fold in the rate of translocation, and slightly reduced the extent of translocation. In contrast, C2394U conferred less than a 1.5-fold decrease in rate, and the same extent of translocation.

**Figure 3.11. Substitutions of C2394 decrease the apparent rate of sparsomycin-catalyzed translocation.** Apparent rates are listed and were derived by plotting the fraction of POST remaining versus time and fitting the data to a single exponential term.
These data provide additional evidence that ribosomes harboring C2394A or C2394G are defective in hybrid state formation. In ribosomes harboring C2394U, it is possible that another event is rate-limiting, so that slight defects in P/E state formation do not confer as large of a defect in the rate of sparsomycin-dependent translocation. Although the order of defect conferred by the substitutions in this assay still follows the same trend that was observed for growth rate and P/E state attributed mRNA repositioning (A>G>U), the effects of the substitutions are less pronounced. The C2394U-containing ribosomes behave more like the control, while the C2394G-containing ribosomes behave more like the C2394A-containing ribosomes. The basis for this difference in the magnitude of defect is unclear at this time.
3.4. Discussion

Here, we show that the stability of codon-anticodon interaction in the P site depends on the binding state of the tRNA. When peptidyl-tRNA in the POST complex is deacetylated by treatment with puromycin, the P-site codon-anticodon pairing is destabilized, allowing RT-dependent repositioning. We provide several lines of evidence that destabilization of the codon-anticodon helix results from movement of tRNA into P/E site (Fig. 3.12).

![Figure 3.12. Summary of data to support the model that movement of tRNA into the P/E state destabilizes the codon-anticodon helix.](image)
Truncation of the 3' end of tRNA, increased concentrations of MgCl₂, and E-site mutation C2394A are each predicted to inhibit binding to the P/E site, and each experimental condition increases frame retention in our assay. Moreover, P-site mutation G2252U is predicted to destabilize binding to the P/P site and thereby increase the relative affinity of tRNA for the P/E site, and G2252U confers decreased frame retention. These data support a model in which movement of tRNA from the P/P to P/E site destabilizes the P-site codon-anticodon helix. Accordingly, the importance of the peptidyl group for reading frame retention stems from its ability to restrict tRNA to the P/P site.

Cryo-EM studies indicate that the transition from the P/P to the P/E site involves large-scale displacement of both the elbow and acceptor end of tRNA, without net movement of the anticodon region (27, 28). The difference in position between tRNA in the P/P and P/E sites can be explained by a ~35° rotation about the pivot-point anticodon. We propose that this ~35° rotation of the anticodon region during P/E site binding distorts the codon-anticodon helix, which results in the mRNA repositioning that we observe. Our interpretation is consistent with the identification of a UV-induced photoproduct specifically observed after deacylation of tRNA in the P site (120). This photoproduct was determined to be a trimer between cmo₅U₃₄ of tRNAᵱᵥᵱ and C1400 and m⁵C967 of 16S rRNA and has been attributed to an altered conformation of the anticodon of tRNA in the P/E site.

The presence of a peptidyl group on P-site tRNA has also been shown to influence the conformation of the ribosome and its interaction with translation factors (27, 67). Binding of RF₃•GDPNP or EF-G•GDPNP to the ribosome is strongly inhibited.
when peptidyl-tRNA occupies the P site, and in either case, inhibition is relieved by deacylation of the tRNA. Furthermore, the ribosome-dependent GTPase activity of either factor is specifically inhibited by the presence of a peptidyl group on the P-site tRNA (67). Cryo-EM analysis of these ribosomal complexes (containing either peptidyl-tRNA or deacetylated tRNA bound to the P site) reveals a difference in their propensity to adopt a "unratcheted" or "ratcheted" conformation (27). In the presence of EF-G•GDPNP or EF-G•GDP•fusidic acid, only ribosomes containing deacetylated tRNA are observed in the ratcheted conformation. In the ratcheted conformation, the 30S subunit is rotated relative to the 50S subunit, the L1 stalk is positioned closer to the 50S E site, and the tRNA is bound in the P/E site. Accordingly, it was suggested that the unratcheted and ratcheted conformations correspond to the classical and hybrid-state complexes, respectively, and that the ribosome toggles between these two conformations during elongation. Here, we describe an additional functional distinction between the unratcheted and ratcheted states of the ribosome. The P-site codon-anticodon helix is stable in the unratcheted state and labile in the ratcheted state.

During translation elongation, tRNA moves into the P/E site after its peptidyl group is transferred to the α-amino group of aminoacyl-tRNA in the A site. Consequently, the P/E site is only occupied when an additional tRNA, peptidyl-tRNA, is paired to the A codon and presumably bound in the A/P site. We suggest that, in the hybrid-state complex, codon-anticodon interaction in the A site compensates for the decreased stability of codon-anticodon interaction in the P site. In other words, peptidyl-tRNA, whether bound in the P/P or A/P site, is critical for reading frame maintenance.
during translation elongation. Accordingly, we do not observe mRNA repositioning in complexes which contain deacylated tRNA in the P site when another tRNA substrate is present in the A site.

In this study, we have also observed mRNA repositioning after translocation of aminoacyl-tRNA in control ribosomes and after translocation of aminoacyl- or N-acetyl-aminoacyl-tRNA in G2252U ribosomes. In each case tested, mRNA repositioning was suppressed by substitutions of C2394, indicating involvement of the 50S E site. Because the E site is specific for deacylated tRNA (121-123), we considered the hypothesis that, in these cases, mRNA repositioning was due to deacylation of tRNA prior to P/E-site binding. According to this hypothesis, it followed that the increased mRNA repositioning observed in G2252U ribosomes resulted from an increase in tRNA deacylation conferred by the mutation. To test this hypothesis, we determined the extent of deacylation after translocation of Tyr-tRNA^Tyr2 or Val-tRNA^Val1 in control and G2252U ribosomes (Fig. 3.6). In the case of Tyr-tRNA^Tyr2, 23% of the tRNA was deacylated after translocation in control ribosomes, while 37% of the tRNA was deacylated in G2252U ribosomes. These data suggest that deacylation contributes to but cannot fully account for the mRNA slippage observed. When Val-tRNA^Val1 was translocated, low and comparable levels of deacylation were detected in both control and G2252U ribosomes. These data suggest that, under these conditions, deacylation is not an absolute requirement for mRNA slippage, and lend the possibility that certain acylated forms of tRNA can also occupy the P/E site. Incidentally, a recent study demonstrated that bis-aminoacylated tRNAs (tRNAs aminoacylated on both the 2’ and 3’ hydroxyl groups) could participate in cell-free
protein synthesis (124). This indicates that mono-aminoacylated tRNA species remained in the P site following peptide bond formation. It is likely that these aminoacyl tRNAs were then translocated through the P/E site before exiting the ribosome.

In this study, we provide several lines of evidence that movement of tRNA into the P/E state destabilizes its interaction with mRNA. Deacylation of tRNA in the posttranslocation complex by addition of puromycin results in repositioning of mRNA, similar to that conferred by translocation of deacylated tRNA. This mRNA repositioning is suppressed by 3'-truncation of tRNA, increased MgCl$_2$ concentration, and E-site mutations C2394A, C2394G, and C2394U and each of these conditions is predicted to inhibit occupation of the P/E state. Furthermore, P-site mutation G2252U, predicted to increase the relative affinity of tRNA for the P/E state, increases mRNA repositioning. The fact that the stability of the codon-anticodon helix is compromised by occupation of the P/E site has important implications for translation.
CHAPTER 4

ROLE OF HYBRID-TRNA BINDING STATES IN RIBOSOMAL TRANSLOCATION

4.1. Introduction

Movement of tRNAs through the ribosome is believed to occur in a stepwise manner (6). Chemical protection experiments showed that, after peptidyl transfer, the acceptor ends of the tRNAs can move spontaneously with respect to the 50S subunit to form the hybrid state. In the hybrid state, the deacylated tRNA occupies the 30S P site and 50S E site (P/E) while the peptidyl-tRNA occupies the 30S A site and 50S P site (A/P). It was proposed that hybrid state formation precedes codon-anticodon movement within the 30S subunit, and only the latter event requires catalysis by elongation factor G (EF-G).

Several models have been proposed to describe translocation in kinetic terms. Early experiments showed that single-turnover translocation is catalyzed by EF-G in the presence of a non-hydrolyzable GTP analog (Reviewed in (58)). These data indicated that GTP hydrolysis is not strictly required for translocation, and it was proposed that hydrolysis is necessary for dissociation of EF-G from the posttranslocation (POST)
complex instead. However, more recent pre-steady-state studies showed that GTP hydrolysis precedes and accelerates codon-anticodon movement, suggesting that the energy of hydrolysis is used to drive translocation (62, 66). Experiments in which the rates of tRNA movement and phosphate (Pi) release were monitored suggest that GTP hydrolysis is followed by a conformational rearrangement, termed unlocking, that limits both codon-anticodon movement and Pi release (63). Deletion of domains 4 and 5 of EF-G slowed codon-anticodon movement and Pi release identically, supporting the existence of a rate-limiting rearrangement that precedes both events. Effects of antibiotics and ribosomal mutations indicated that, although both events are limited by the unlocking step, codon-anticodon movement and Pi release are independent of each other and probably occur in random order (63-65). Finally, ribosomal rearrangements must occur to "relock" the tRNAs in their new sites, followed by release of EF-G•GDP from the POST complex, although the kinetics of these events have yet to be fully characterized.

A number of studies suggest an important role for hybrid state formation in the mechanism of EF-G-dependent translocation (43, 49, 50, 66). However, it is still not fully clear how the P/P-to-P/E and A/A-to-A/P transitions relate to the kinetically defined events of translocation. Here, we address this question by analyzing mutations in rRNA and substitutions in the translocated tRNA that inhibit movement of tRNA into the P/E and A/P sites, respectively. Based on our findings, we present a kinetic model for EF-G-dependent translocation that incorporates movement of tRNA within both subunits of the ribosome.
4.2. Materials and Methods

4.2.1. Strains and plasmids

Strains expressing homogeneous populations of ribosomes harboring each substitution of C2394 were constructed by transforming plasmid p278MS2 (Amp\textsuperscript{R}) and its derivatives pKM1 (C2394A), pKM2 (C2394U), and pKM6 (C2394G) (45, 112) into the Δ7prrn strain SQZ10 (kindly provided by C. Squires, Tufts University, Boston, MA). Transformants were grown in liquid media and spread onto LB plates containing Amp (100 µg/mL) and sucrose (5%) to select for the p278MS2 plasmid, and against pHKrrnC-sacB (Kan\textsuperscript{R}), the resident plasmid of SQZ10. Replacement of the pHKrrnC-sacB plasmid with each p278MS2 derivative yielded isolates that were sensitive to Kan, but resistant to both sucrose and Amp. Plasmid replacement was confirmed by purification of plasmid DNA from each strain and sequencing the relevant region of the 23S rRNA gene.

4.2.2. Growth Rate Determination

Growth rates were determined by inoculating 25 mL of LB Amp in 250 mL flasks with 50 µL of saturated overnight cultures of each Δ7 prrn strain, and then incubating in a shaking water bath at 37°C. OD\textsubscript{600} readings were taken every 30 minutes until the cultures reached stationary phase. Data from the logarithmic phase of growth were plotted in Kaleidagraph (Synergy Software) and fit to the equation OD\textsubscript{600} = C (2\textsuperscript{k\textsubscript{t}}), where C is a constant, \(k\) is growth rate (in doublings per hour), and \(t\) is time (in hr).
4.2.3. Biochemical reagents

Tight-couple ribosomes, acylated tRNAs, message m293, and EF-G were prepared as described (85, 87, 113). Messages m432 (5’-AAGGAAUAUAAUGUUUACUUUGUU-3’) and m433 (5’-AAGGAAUAACAUUACGUAGCU-3’) contained a 2’-amino-pyrene modification on the 3’-terminal uridine, and were purchased (Dharmacon) and de-protected as recommended by the supplier.

4.2.4. Translocation Experiments

To form PRE complexes, control or mutant ribosomes (1.5 µM) were first incubated with mRNA (m432 or m433; 1.25 µM) and tRNA\textsuperscript{\text{Tyr}} (1.5 µM) in buffer 1 [50 mM Tris (pH 7.6), 100 mM NH\textsubscript{4}Cl, 15 mM MgCl\textsubscript{2}] for 10 minutes at 37°C to fill the P site. Then, Ac-Val-tRNA\textsuperscript{\text{Val}}, Ac-Phe-tRNA\textsuperscript{\text{Phe}}, Val-tRNA\textsuperscript{\text{Val}}, or Phe-tRNA\textsuperscript{\text{Phe}} (1.5 µM) was added (as indicated) and the reactions were incubated for 10 more minutes to bind the A site. PRE complexes were then diluted 5-fold (to 0.25 µM) with buffer 1 containing GTP (1 mM) just before performing an experiment. To form EF-G•GTP, EF-G (at various concentrations as indicated) was incubated with GTP (1 mM) in buffer 2 [50 mM Tris (pH 7.6), 30 mM NH\textsubscript{4}Cl, 70 mM KCl, 5 mM MgCl\textsubscript{2}, 6 mM β-mercaptoethanol] for 5 minutes at 37°C. To measure the rate of mRNA movement, equal volumes of PRE complex (0.25 µM) and EF-G•GTP (as indicated), were rapidly mixed at 37°C in an SX18-MV stopped-flow spectrometer (Applied Photophysics) essentially as described (41). The excitation wavelength was set at 343 nm and fluorescence emission was
detected after passage through a 360 nm cutoff filter. Typically, traces from 6 or more shots were averaged, and data were fit to a single or double exponential curve using the SX18-MV software. Apparent rates were then plotted versus EF-G concentration and fit to the equation $k_{\text{app}} = ([\text{EF-G}] \cdot k_{\text{trans}}) / ([\text{EF-G}] + K_{1/2})$.

4.2.5. GTPase Experiments

The rate of single-turnover GTP hydrolysis was determined essentially as described (125). To form PRE complexes, control or mutant ribosomes (2 µM) were first incubated with message m404 (4 µM; see reference (126)) and tRNA$^{\text{Yr2}}$ (3 µM) in buffer 3 [50 mM Tris (pH 7.6), 30 mM NH$_4$Cl, 70 mM KCl, 7 mM MgCl$_2$, 1 mM DTT] for 10 minutes at 37°C to fill the P site. Then, Ac-Val-tRNA$^{\text{Val}}$ (2.5 µM) was added to bind the A site and the complexes were placed on ice. To form EF-G•GTP, EF-G (3) was incubated with $[^{\gamma-32}\text{P}]-\text{GTP}$ (Perkin Elmer; 50 µM, ~1000 dpm/pmol) in buffer 3 for 2 minutes at 37°C and then placed on ice. Equal volumes of PRE complex (1 µM final) and EF-G•GTP (1.5 µM final) were then rapidly mixed at 25°C in a quench flow apparatus (Kintek) for indicated times before quenching with 0.6 M HClO$_4$ containing 1.8 mM KH$_2$PO$_4$, as described (125). Data were fit to the equation $y = A \cdot \exp(-k_{\text{app1}} \cdot t) + k_{\text{app2}} \cdot t + C$; where $A$ is the burst amplitude, $k_{\text{app1}}$ is the apparent burst rate, $t$ is time, $k_{\text{app2}}$ is the apparent turnover rate, and $C$ is a constant.
4.3. Results

4.3.1. Mutations of C2394 confer moderate defects in cell growth

To investigate the role of the P/E hybrid state in translocation, we mutagenized C2394, a key nucleotide of the 50S E site that interacts with the 3’-terminal adenosine (A76) of tRNA (6, 15, 22, 24, 46, 52). Plasmids encoding rrnB without or with mutation C2394A, C2394G, or C2394U were moved into an E. coli strain lacking all chromosomal rrn operons (Δ7 prrn), replacing the resident plasmid containing rrnC. Although C2394 is highly conserved, ribosomes with any base substitution at this position supported cell growth with only moderate defects in growth rate (control = 1.7 ± 0.08, C2394A = 1.1 ± 0.06, C2394G = 1.2 ± 0.05, and C2394U = 1.4 ± 0.14 doublings per hour). Similar data regarding mutation C2394G have been previously reported (54).

4.3.2. Mutations of C2394 confer defects in EF-G-catalyzed translocation

To study the effects of the E-site mutations on translocation, we used a single-turnover assay that monitors the rate of codon-anticodon movement in the ribosome by fluorescence stopped-flow (41). This assay employs an mRNA labeled at its 3' end with pyrene, a fluorophore sensitive to local environment. In the pretranslocation (PRE) complex, the pyrene is highly fluorescent, but when the mRNA translocates by 3 nucleotides toward or into the mRNA channel, the fluorescence is quenched (Fig 4.1).
Fig. 4.1. Schematic of the stopped-flow assay used to monitor codon-anticodon movement in the ribosome. Pretranslocation (PRE) complexes were formed by incubating ribosomes with pyrene-labeled message m433 and tRNA\textsuperscript{Tyr2} to bind the P site, and then adding Ac-Val-tRNA\textsuperscript{Val} to bind the A site. Experiments were performed by rapidly mixing PRE complex (0.25 μM) with a mixture of EF-G and GTP in a stopped-flow spectrometer. Translocation of mRNA results in quenching of fluorescence as the pyrene moves toward or into the mRNA channel of the ribosome.

Ribosomes were purified from each Δ7 prrn strain. There were no obvious differences between control and mutant 70S ribosomes based on sucrose gradient sedimentation and SDS-PAGE analyses (data not shown). Moreover, the efficiency of tRNA binding to the P and A sites and the extent of translocation were comparable among the ribosome preparations (Fig. 4.2).
Figure 4.2. Ribosomes harboring substitutions of 23S rRNA nucleotide C2394 bind tRNA and undergo complete translocation. The position of mRNA within ribosomal complexes was mapped following each of several additions. A PRE complex was formed by incubating ribosomes harboring each substitution at position 2394 of the 23S rRNA (as indicated) with m297 and tRNA\textsuperscript{Tyr2} to fill the P site (P lanes). N-acetyl-Val-tRNA\textsuperscript{Val} was then added to bind the A site (A lanes), after which complexes were diluted 5-fold with buffer 1. Portions of the diluted complexes were then added to equal volumes of buffer in the presence of GTP (- lanes) or EF-G plus GTP (+ lanes). At each stage of the experiment, the position of mRNA was mapped by toeprinting.

Control and mutant ribosomes were used to assemble PRE complexes containing pyrene-labeled mRNA (m433), tRNA\textsuperscript{Tyr2} in the P site, and N-acetyl-Val-tRNA\textsuperscript{Val} (Ac-Val-tRNA\textsuperscript{Val}) in the A site. These complexes were rapidly mixed with EF-G in the presence of GTP, and decreased pyrene fluorescence was observed at different rates depending on the mutation (Fig 4.3). In each case, the decrease in fluorescence intensity over time did not fit a single-exponential function, but instead fit a double-exponential function (Fig. 4.4). This phenomenon was also seen using ribosomes isolated from \textit{E. coli} MRE600 (data not shown) and has been observed previously by others (64)(S. Joseph,
personal communication). Presumably, the two phases reflect two populations of ribosomes undergoing translocation at different rates, although the basis for this putative population heterogeneity remains unclear. The fraction amplitude corresponding to the fast \((A_1 \approx 0.5)\) and slow \((A_2 \approx 0.5)\) processes remained constant regardless of whether the ribosomes harbored an E-site mutation. Thus, this putative PRE complex heterogeneity seems unrelated to the ability of the ribosomes to adopt the hybrid-state conformation. In fact, the fast and slow processes were similarly affected by the mutations (see below), suggesting that both processes involve movement of tRNA through the P/E site.

Fig. 4.3. Effects of 50S E-site mutations on EF-G-dependent translocation. Examples of fluorescence traces for ribosomes without and with mutations at position 2394 of 23S rRNA.
Figure 4.4. Monitoring translocation of pyrene-labeled mRNA paired to $N$-acetyl-Val-tRNA$^{Val}$ in control (wild-type) ribosomes. In this experiment, syringe 1 was loaded with PRE complexes programmed with m433 and containing tRNA$^{Tyr}$ in the P site and $N$-acetyl-Val-tRNA$^{Val}$ in the A site. Syringe 2 contained EF-G (8 mM) and GTP (1 mM). Upon rapid mixing, decreased fluorescence intensity (open blue circles) was observed as a function of time. These data were fit to either a single (orange trace) or double (yellow trace) exponential function. Based on inspection of the residual plots (bottom panels), two exponential terms were necessary to provide a reasonable fit to the data. No fluorescence change was observed in experiments when EF-G was omitted from syringe 2 under otherwise identical conditions (data not shown).

4.3.3. Mutations of C2394 decrease $k_{trans}$ and increase $K_{1/2}$

Apparent rates were measured at several concentrations of EF-G to derive $k_{trans}$, the maximal rate of translocation, and $K_{1/2}$, the concentration of EF-G at which half-maximal rate was observed (Fig. 4.5, Table 4.1). In control ribosomes, when Ac-Val-
tRNA\textsuperscript{Val} was translocated to the P site, $k_{\text{trans}}$ for the fast process was found to be 19 s\(^{-1}\), with a $K_{1/2}$ of 0.36 µM, consistent with previously reported values (63, 66).

Table 4.1. Kinetic parameters for EF-G-dependent translocation in control and mutant ribosomes

<table>
<thead>
<tr>
<th>Ribosomes</th>
<th>tRNA translocated to the P site</th>
<th>Fast process</th>
<th>Slow process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Ac-Val-tRNA\textsuperscript{Val}</td>
<td>$19$ s(^{-1})</td>
<td>$5.8$ s(^{-1})</td>
</tr>
<tr>
<td>C2394A</td>
<td>Ac-Val-tRNA\textsuperscript{Val}</td>
<td>$2.4$ s(^{-1})</td>
<td>$0.42$ s(^{-1})</td>
</tr>
<tr>
<td>C2394G</td>
<td>Ac-Val-tRNA\textsuperscript{Val}</td>
<td>$4.7$ s(^{-1})</td>
<td>ND</td>
</tr>
<tr>
<td>C2394U</td>
<td>Ac-Val-tRNA\textsuperscript{Val}</td>
<td>$8.0$ s(^{-1})</td>
<td>$2.1$ s(^{-1})</td>
</tr>
<tr>
<td>Control</td>
<td>Val-tRNA\textsuperscript{Val}</td>
<td>$4.3$ s(^{-1})</td>
<td>$1.1$ s(^{-1})</td>
</tr>
<tr>
<td>Control</td>
<td>Ac-Phe-tRNA\textsuperscript{Phe}</td>
<td>$39$ s(^{-1})</td>
<td>NA</td>
</tr>
<tr>
<td>Control</td>
<td>Phe-tRNA\textsuperscript{Phe}</td>
<td>$0.21$ s(^{-1})</td>
<td>$0.05$ s(^{-1})</td>
</tr>
</tbody>
</table>

$K_{1/2}$, not determined; in these cases, a reliable fit to the data was not obtained. NA, not applicable. At each concentration of EF-G, translocation of m432 with N-acetyl-Phe-tRNA\textsuperscript{Phe} fit a single-exponential function.

Mutation C2394A increased $K_{1/2}$ by 23-fold and decreased $k_{\text{trans}}$ by 8-fold; C2394G increased $K_{1/2}$ by 13-fold and decreased $k_{\text{trans}}$ by 4-fold; and C2394U increased $K_{1/2}$ by 4-fold and decreased $k_{\text{trans}}$ by 2-fold. Kinetic parameters for the slow process were also derived, and these parameters were similarly influenced by substitution of C2394 (Table 4.1). The degree to which these E-site mutations affected each kinetic parameter followed the trend A > G > U, the same trend that was observed when growth rate was measured (see above).
Figure 4.5. Effects of mutations of C2394 on $k_{\text{trans}}$ and $K_{1/2}$. The apparent rate of translocation was measured in ribosomes harboring each substitution at 2394 at several concentrations of EF-G. Apparent rate constants ($k_{\text{app}}$) for the fast (●) and slow (○) processes were plotted versus EF-G concentration for control and mutant ribosomes (as indicated). Resultant plots were fit to the equation $k_{\text{app}} = ([\text{EF-G}] \cdot k_{\text{trans}}) / ([\text{EF-G}] + K_{1/2})$ to yield $k_{\text{trans}}$, the maximal rate of translocation, and $K_{1/2}$, the concentration of EF-G at which half-maximal rate was observed (see Table 4.1).

4.3.4. Replacing the $N$-acetyl-aminoacyl group with an aminoacyl group decreases $k_{\text{trans}}$ but does not increase $K_{1/2}$

Chemical protection and single-molecule FRET experiments provided evidence that aminoacyl-tRNA is defective in A/P state formation (6, 46-48). The aminoacyl group decreases the apparent rate and extent of translocation, suggesting an important role for the A/P state in translocation (42, 45, 85). Mutation G2553U, predicted to destabilize tRNA in the A/A site, suppressed the inhibitory effect of the aminoacyl group, further suggesting that this group inhibits the A/A-to-A/P transition (45). To investigate the role of the A/P state in translocation, we analyzed the kinetics of mRNA movement when aminoacyl-tRNA was translocated to the P site (Fig. 4.6). When Val-tRNA$^{\text{Val}}$ was translocated, $k_{\text{trans}}$ was reduced by 4-fold compared to the control (Ac-Val-tRNA$^{\text{Val}}$), while $K_{1/2}$ of EF-G for the PRE complex was unaffected (Fig. 4.6, Table 4.1). We repeated the
experiment in a different context, comparing translocation of Ac-Phe-tRNA\textsuperscript{Phe} to that of Phe-tRNA\textsuperscript{phe}. Replacing Ac-Phe with Phe decreased $k_{\text{trans}}$ by 190-fold, while no increase in $K_{1/2}$ was observed (Fig 4.6, Table 4.1).

**Figure 4.6. Substitutions predicted to inhibit A/P state formation decrease $k_{\text{trans}}$ but do not increase $K_{1/2}$**. PRE complexes were formed by incubating control ribosomes with pyrene-labeled mRNA and tRNA\textsubscript{Tyr2} to bind the P site, followed by addition of Ac-Val-tRNA\textsubscript{Val}, Val-tRNA\textsubscript{Val}, Ac-Phe-tRNA\textsubscript{Phe}, or Phe-tRNA\textsubscript{Phe} to bind the A site. Apparent rates of translocation were measured at several concentrations of EF-G to obtain $K_{1/2}$ and $k_{\text{trans}}$ for each A-site tRNA species translocated: Ac-Val-tRNA\textsubscript{Val} (A, ●), Val-tRNA\textsubscript{Val} (A, ○), Ac-Phe-tRNA\textsubscript{Phe} (B, ■), and Phe-tRNA\textsubscript{Phe} (B, □). Plots include only the apparent rate constants for the fast process.
If anything, the Phe substitution seemed to enhance the apparent affinity of EF-G for the PRE complex. However, analysis of the slow process did not indicate a decrease in $K_{1/2}$ (Table 4.1), suggesting that the $K_{1/2}$ value for the fast process may be an underestimate. Nevertheless, these data are similar to the Val-tRNA$^{Val}$ case, except that the rate decrease conferred by the Phe substitution was much larger.

4.3.5. Mutation C2394A does not decrease the rate of EF-G-dependent GTP hydrolysis

Next, we tested whether mutation C2394A affected the GTP hydrolysis step of EF-G-dependent translocation. To measure the rate of single-turnover GTP hydrolysis, we employed chemical quench flow as described previously (125). PRE complexes were assembled with control or C2394A ribosomes and then mixed with EF-G•[γ-$^{32}$P]-GTP for various periods of time before quenching with 0.6 M perchloric acid. The amount of GTP hydrolyzed at each time point was then determined by extraction and quantification of $^{32}$Pi. Under the conditions employed (25 µM GTP, 1.5 µM EF-G, 1 µM PRE complex), a burst phase corresponding to single-turnover GTP hydrolysis was observed, followed by a linear phase corresponding to multiple-turnover hydrolysis. The apparent single-turnover (burst) rate was similar in control (25 s$^{-1}$) and C2394A (44 s$^{-1}$) ribosomes. The burst amplitude was also similar, although somewhat (15%) lower in the mutant ribosomes. Multiple-turnover GTP hydrolysis was noticeably inhibited by C2394A, which may result from substantially slower translocation in these ribosomes (Fig. 4.7).
Figure 4.7. Mutation C2394A does not affect the apparent rate of GTP hydrolysis. PRE complexes containing tRNA$^{Tyr}$ in the P site and Ac-Val-tRNA$^{Val}$ in the A site were rapidly mixed with EF-G•[$\gamma^{32}$P]-GTP for indicated amounts of time before quenching with 0.6 M perchloric acid and quantifying the amount of $^{32}$Pi produced. For complexes without (●) or with mutation C2394A (■), the reaction exhibited burst kinetics with exponential and linear phases corresponding to single- and multiple-turnover GTP hydrolysis, respectively. Burst rates (control = 25 s$^{-1}$; mutant = 44 s$^{-1}$) and amplitudes (control = 0.46; mutant = 0.39) deduced from curve-fitting were similar for both complexes. Substantially less GTP hydrolysis was evident in the absence of ribosomes (○). Both panels represent the same experiment; the bottom panel shows a narrower time window.
The fact that C2394A did not decrease the rate of single-turnover GTP hydrolysis suggests that the mutation affects neither the initial binding of EF-G nor the GTP hydrolysis step. Rather, C2394A inhibition of overall translocation must be due to inhibition of a step following GTP hydrolysis, presumably that of P/E state formation. Our findings are consistent with an earlier study showing that the acylation state of the P-site tRNA (which strongly influences the P/P-to-P/E transition) does not affect the rate of single-turnover GTP hydrolysis (62).

4.4. Discussion

Here, we study the contribution of a key E-site nucleotide, C2394, to EF-G-dependent translocation. Each substitution of C2394 decreases $k_{\text{trans}}$ and increases $K_{1/2}$, and the magnitude of the defects follows the trend A > G > U. The same trend is observed when growth rate in the Δ7 prrn background is measured, suggesting that translocation limits growth rate in those strains expressing mutant ribosomes. The same trend (A > G > U > WT) is also observed when the stability of mRNA in complexes containing P-site deacylated tRNA is assessed in an mRNA repositioning assay (S. E. W., K. G. McGarry, and K. F., unpublished, section 3.3.7 of this document). In this assay, mRNA repositioning correlates with the ability of tRNA to bind the P/E site (45, 118). Thus, effects of these E-site mutations on growth, translocation, and P/E-binding all correlate. The fact that purine substitutions at position 2394 are more deleterious can be rationalized in structural terms. In the 50S E site, the 3'-terminal adenosine of tRNA (A76) intercalates between 23S rRNA nucleotides G2421 and A2422 and forms
hydrogen bonds with C2394 (15, 22). Replacement of C2394 with either purine should not only disrupt H-bonding to A76 but also sterically inhibit stacking of A76 between G2421 and A2422.

In contrast to the effects of substitutions of C2394 on both $k_{\text{trans}}$ and $K_{1/2}$, substituting Val-tRNA$^\text{Val}$ for Ac-Val-tRNA$^\text{Val}$ in the PRE complex decreases $k_{\text{trans}}$ with little effect on $K_{1/2}$ (Table 4.1). The model presented in Figure 4.8 to describe translocation provides a qualitative explanation of these results. In the model, EF-G•GTP binding and rapid, reversible GTP hydrolysis (steps 1 and 2)(62, 66) are followed first by movement of deacylated tRNA to give the P/E state (step 3), and then by movement of peptidyl-tRNA to give the A/P state (step 4), which is probably equivalent to the INT complex described earlier (66). The A/P state is then converted to the POST complex by codon-anticodon movement, which coincides with 30S unlocking and Pi release (step 5)(63), and is the rate-determining step for the process. Steps 4 and 5 may be conformationally linked: thus, EF-G-dependent translocation is accelerated by either mutation of the 50S A site or lengthening of the peptidyl group (43), and each of these changes promotes the A/A-to-A/P transition (48).

**Figure 4.8. Model for EF-G-dependent translocation that incorporates tRNA movements with respect to both subunits.** See text for details.
Substitution at C2394 should have the greatest effect on the rate constant for step 3, whereas substitution of Val-tRNA\textsuperscript{Val} for Ac-Val-tRNA\textsuperscript{Val} should principally affect the rate constant for step 4. Kinetic modeling of the model presented in Figure 4.8 was performed by S. Shoji and B.S. Cooperman and suggests that, under certain assumptions and in accord with the results presented in Table 1, reductions in the value of \( k_3 \) can lead to larger effects on \( K_{1/2} \) than on \( k_\text{trans} \) while reductions in \( k_4 \) can be confined largely to \( k_\text{trans} \).

Independent formation of the P/E and A/P states is consistent with results of recent kinetic and single-molecule FRET experiments (48, 66). In the work of Pan et al. (2007), which examined translocation of a PRE complex containing tRNA\textsuperscript{fMet} in the P site and fMetPhe-tRNA\textsuperscript{Phe} in the A site, the P/E state could only be demonstrated in the presence of viomycin, leading to the conclusion that, in the absence of antibiotic, \( k_4 >> k_3 \). The large reduction in \( k_4 \) that should result from substituting Val-tRNA\textsuperscript{Val} for Ac-Val-tRNA\textsuperscript{Val} might also lead to some accumulation of the P/E state.

In the presence of GTP, EF-G interacts only transiently with the ribosome. However, binding of EF-G can be stabilized in the presence of non-hydrolyzable GTP analogs (e.g., GDPNP) or antibiotics like fusidic acid, which trap EF-G•GDP on the ribosome after GTP hydrolysis. A number of groups have analyzed structural differences between ribosomal complexes with EF-G trapped in the GTP form (e.g., GDPNP) versus the GDP form in an effort to understand the role of GTP hydrolysis in the mechanism of EF-G-dependent translocation (27-29, 68, 118, 127). A particularly intriguing observation from these studies was that bound EF-G promotes an inter-subunit rotation
(termed *ratchet subunit rotation* or RSR) in complexes containing P-site deacylated tRNA. This RSR correlates with P/E-bound tRNA and movement of the L1 stalk toward the 50S E site. In a complementary study, it was shown that deacylation of P-site tRNA enhances EF-G binding, suggesting that the RSR conformation (with P/E-tRNA) stabilizes EF-G (67). Based on these studies, it was recently proposed that initial binding of EF-G•GTP promotes hybrid state formation, which is followed by GTP hydrolysis (128). Here the timing of hybrid state formation versus GTP hydrolysis is largely based on results obtained with EF-G•GDPNP, which is taken as a model for EF-G•GTP. However, recent kinetic studies (66), along with our present results, indicate that EF-G catalyzes GTP hydrolysis prior to hybrid state formation. This apparent disagreement is readily resolved if it is assumed that, during translocation, ribosome-bound EF-G•GDPNP can be a model for bound EF-G•GDP•Pi, as suggested elsewhere (66), with hybrid state formation preceding Pi release. Support for this assumption is provided by the recent structural characterization of an archael initiation factor aIF2•GDP•Pi complex, in which the position of the switch 2 region is similar to that seen in the aIF2•GDPNP complex (129).

Integral to our model is that step 2, GTP hydrolysis, is reversible. This is consistent with studies showing that, within the active sites of ATPases and GTPases, including eukaryotic initiation factor eIF2 (130), the free energy of NTP hydrolysis approaches zero, in sharp contrast to the thermodynamics in solution (131-135). Lorsch and colleagues have provided compelling evidence for an internal equilibrium between GTP and GDP•Pi within the nucleotide-binding pocket of eIF2 with an estimated
equilibrium constant of 0.5 \((130)\). When eIF2\(^{\text{GTP\cdotMet-tRNA}}\) interacts with a model pre-initiation complex, hydrolysis of GTP occurs rapidly regardless of whether a start codon is present or absent. However, in the latter case, the amplitude of GTP hydrolysis is reduced and the subsequent step of Pi release is blocked. The authors propose that prior to start codon recognition an internal equilibrium between GTP and GDP•Pi is established. Subsequent recognition of the start codon allows Pi release to occur, making hydrolysis of GTP irreversible. The mechanism of EF-G-dependent translocation is similar in that Pi release occurs much more slowly than GTP hydrolysis \((63, 125)\) and is limited by a conformational rearrangement \((63, 136)\). Further experiments will be required to determine the internal equilibrium position of GTP and GDP•Pi within ribosome-bound EF-G.
CHAPTER 5

CONCLUSIONS AND PERSPECTIVES

Studies on translation elongation contribute to our understanding of the large scale conformational dynamics allowed within an RNA-based machine. The work presented here focuses on events which are crucial to cell viability (frame maintenance and rapid translocation).

Mutant tRNAs containing an extra nucleotide in the anticodon loop are known to suppress +1 frameshift mutations, but in no case has the molecular mechanism been clarified. It has been proposed that the expanded anticodon pairs with a complementary mRNA sequence (the frameshift sequence) in the A site, and this quadruplet 'codon-anticodon' helix is translocated to the P site to restore the correct reading frame. In Chapter 2, the ability of tRNA analogs containing expanded anticodons to recognize and position mRNA in ribosomal complexes was analyzed in vitro. In all cases tested, 8-nt anticodon loops position the 3' three-quarters of the frameshift sequence in the P site, indicating that the 5' bases of the expanded anticodon (nucleotides 33.5, 34, and 35) pair with mRNA in the P site. Evidence was also provided that suggested four base pairs can
form between the P-site tRNA and mRNA, and the fourth base pair involves nucleotide 36 of the tRNA and lies toward (or in) the 30S E site. In the A site, tRNA analogs with the expanded anticodon ACCG are able to recognize either CGG (the 5' three-quarters of the frameshift sequence) or GGU (the 3' three-quarters of the frameshift sequence). These data imply a flexibility of the expanded anticodon in the A site. Recognition of the 5' three-quarters of the frameshift sequence in the A site and subsequent translocation of the expanded anticodon to the P site results in movement of mRNA by 4 nucleotides, explaining how these tRNAs can change the mRNA register in the ribosome to restore the correct reading frame.

In Chapter 2, the recognition and positioning of mRNA by suf+1 type tRNAs was analyzed. Recognition of either the 5' 3/4 or the 3’ 3/4 of the frameshift site by A-site expanded anticodon loop tRNAs suggests a flexibility in the positioning of the anticodon sequence in the P site. Recent structures of expanded anticodon ASLs bound to the ribosome support this idea (103). A question which remains is how do expanded anticodon loop tRNAs get decoded? Under normal circumstances, cognate codon-anticodon pairing results in a local rearrangement of the decoding center and GTP hydrolysis by EF-Tu. To determine how tRNAs with expanded loops decode mRNA, single-turnover binding, GTPase activation and hydrolysis, peptidyl transfer, and Pi release will need to be measured for tRNAs with either the 5’ 3/4 or 3’ 3/4 of the frameshift sequence positioned immediately 3’ of the P site.

From studies with expanded anticodon loop tRNAs, we also concluded that in the P site, positioning of the base immediately 3’ of U33 is strictly maintained. This suggests
that rRNA bases around U33 are important for the positioning in the P site. In particular, C1400 normally stacks with nt 34 in the P site, essentially continuing the anticodon stack. An interesting question that stems from this work is how does mutation of C1400 affect the positioning of mRNA in the P site? Repeating the toeprinting described in chapter 2 with ribosomes harboring mutations of C1400 may shed light on the mechanism by which the ribosome positions all tRNAs, and may hold relevance for translation initiation, as the initiator is the only tRNA to recognize its codon in the P site. Similar experiments could be performed with a collection of ribosomes harboring P-site mutations (137), including substitutions of base m2G966, which contacts the backbone of P-tRNA and is likely to contribute to positioning.

Retention of the reading frame in ribosomal complexes after single-round translocation depends on the acylation state of the tRNA. When tRNA lacking a peptidyl group is translocated to the P site, the mRNA is destabilized. The data presented in Chapter 3 suggest that this ribosomal activity results from movement of tRNA into the P/E site. Repositioning of mRNA was suppressed by 3’-truncation of the translocated tRNA, increased MgCl₂ concentration, and mutation C2394A of the 50S E site, and each of these conditions inhibits P/E-state formation. Mutation G2252U of the 50S P site stimulates mRNA slippage, suggesting that decreased affinity of tRNA for the P/P state also destabilizes mRNA in the complex. The effects of G2252U are suppressed by C2394A, further implicating P/E-site binding in mRNA destabilization. This work uncovers a functional attribute of the P/E state crucial for understanding translation.
In Chapter 3, several antibiotics were shown to affect toeprint distributions when ribosomes are bound to mRNAs which yield toeprints corresponding to both cognate and near-cognate codons. These near-cognate codons are likely the result of mRNA repositioning that occurs when reverse transcriptase encounters a complex containing a tRNA in the P/E site. These data reiterated our conclusion that binding of tRNA to the P/E site destabilizes the codon-anticodon helix. It will be of future interest to further explore the possibility that these antibiotics alter the P/P to P/E equilibrium. How do streptomycin and spectinomycin destabilize the codon-anticodon interaction? Are streptomycin and spectinomycin stabilizing tRNA in the P/E conformation, or is the mRNA destabilized by some other means? Likewise, how do antibiotics such as paromomycin stabilize the codon-anticodon interaction? It is possible that these antibiotics induce binding of a second tRNA to the complex in the A site, or prevent movement of the mRNA by some other means. Measuring FRET between a fluorophore on L1 of the E site and the 3’ end of P-tRNA would allow P/E-site binding to be measured easily with each antibiotic. An alternative method to monitor binding would be footprinting the rRNA with chemical probes.

During translation, tRNAs must move rapidly to their adjacent sites in the ribosome while maintaining precise pairing with mRNA. This movement (translocation) occurs in a stepwise manner with hybrid-state intermediates, but it is unclear how these hybrid states relate to kinetically defined events of translocation. In Chapter 4, mutations at position 2394 of 23S rRNA were analyzed in a pre-steady-state kinetic analysis of translocation. These mutations target the 50S E site and are predicted to inhibit P/E state
formation. Each mutation decreases growth rate, the maximal rate of translocation ($k_{\text{trans}}$), and the apparent affinity of EF-G for the pretranslocation complex (i.e., increases $K_{1/2}$). The magnitude of these defects follows the trend A > G > U. Since the C2394A mutation did not decrease the rate of single-turnover GTP hydrolysis, the > 20-fold increase in $K_{1/2}$ conferred by C2394A can be attributed to neither the initial binding of EF-G nor the subsequent GTP hydrolysis step. We propose that C2394A inhibits a later step, P/E state formation, to confer its effects on translocation. Substitution of the peptidyl group for an aminoacyl group, which is predicted to inhibit A/P state formation, decreases $k_{\text{trans}}$ without increasing $K_{1/2}$. These data suggest that movement of tRNA into the P/E and A/P sites are separable events. This mutational study allows tRNA movements with respect to both subunits to be integrated into a kinetic model for translocation.

In chapter 4, a new model for translocation was proposed based on stopped-flow and quench flow data obtained with ribosomes harboring mutations or with tRNA substitutions that specifically inhibited A/P or P/E site binding. This model indicates that GTP hydrolysis precedes P/E state formation, and is followed by sequential movements of the two tRNAs within the 50S subunit. We mention a similar model in which two intermediates (the P/E and INT complexes) were observed during translocation of fluorescent tRNAs. It may be of future interest to measure the rates of INT complex formation with ribosomes harboring each substitution of C2394. This will help to determine whether the INT complex corresponds to the hybrid state conformation of the ribosome. Measuring INT formation when aa-tRNA is bound to the A site of the PRE complex, a complex defective in A/P formation may also be of interest. In the event that
INT formation does correspond to hybrid state formation, an accumulation of the P/E complex should be observed, followed by slow translocation.

Another interesting observation from the results of chapter 4 was that mRNA movement was a double exponential process. It will be of considerable interest to further investigate the nature of this process. It is possible that there are two intermediates or heterogeneity. To determine what the second phase corresponds to, experiments should be performed with similar mRNA movement assays, and with antibiotics known to inhibit events that occur following mRNA movement. The second process may correspond to a second movement of the fluorophore that occurs either as a result of EF-G or ribosome-related events, or due to the physical nature of the probe. This may allow further studies to be conducted to characterize later events in translocation, of which little is currently known.
APPENDIX A

PREPARATION AND EVALUATION OF ACYLATED tRNAs

In the cell, the activity of tRNA is governed by its acylation state. Interactions with the ribosome, translation factors, and regulatory elements are strongly influenced by the acyl group, and presumably other cellular components that interact with tRNA also use the acyl group as a specificity determinant. Thus, those using biochemical approaches to study any aspect of tRNA biology should be familiar with effective methods to prepare and evaluate acylated tRNA reagents. Here, methods to prepare aminoacyl-tRNA, N-acetyl-aminoacyl-tRNA, and fMet-tRNA\textsuperscript{fMet} and to assess their homogeneity are described. Using these methods, acylated tRNAs of high homogeneity can be reliably obtained.

A.1. Introduction

In the cell, tRNA exists in three forms: aminoacyl-tRNA, peptidyl-tRNA, and deacylated tRNA. These forms are distinguished by their acylation state (i.e., whether an amino acid, peptide, or no group is attached to the 3' end of tRNA). As one might expect,
the acylation state is a critical specificity determinant for interactions with the ribosome (6, 42, 45, 46, 48, 59, 85, 90, 121), translation factors (138-143), regulatory elements (144, 145), and other cellular components that interact with tRNA (reviewed in (146)).

The amino acid (or peptide) is linked to the ribose of the 3' terminal adenosine (A76) of tRNA via an ester bond. This ester bond is sensitive to alkaline hydrolysis, which is generally true of esters with a neighboring hydroxyl group (147, 148). In solutions of near-neutral pH (7 to 9), aminoacyl-tRNAs can spontaneously deacylate with half-lives that range from minutes to tens of minutes. Compared to aminoacyl-tRNA, peptidyl-tRNA is considerably more stable (147, 149). This can be explained by the fact that a free α-amino group, largely protonated at neutral pH, has higher propensity to withdraw electrons from the carbonyl carbon than a substituted amino group. The nature of the amino acid also influences the stability of the ester bond (147, 150), which presumably reflects the contribution of the side chain to inductive (electron-withdrawing) effects. Among the least stable aminoacyl-tRNAs are Arg-tRNA, Lys-tRNA, Pro-tRNA, and Ala-tRNA.

Because the acylation state of tRNA governs its biological activities and the ester linkage can be quite susceptible to spontaneous hydrolysis, those interested in studying tRNA need to know how to prepare and evaluate acylated tRNAs. In this article, we describe methods to prepare acylated tRNA reagents and assess their homogeneity. Approaches to purify particular isoacceptors from bulk tRNA are not presented here but have been discussed elsewhere (151-153). Currently, there are at least three commercial sources for purified tRNAs (Sigma, Chemical Block, and MP Biomedicals).
A.2. Preparation of aminoacyl-tRNA

It is important to keep in mind that the ester bond of aminoacyl-tRNA is susceptible to alkaline hydrolysis. Therefore, after the charging reaction, the aminoacyl-tRNA product should be extracted, precipitated, de-salted, and stored under acidic conditions (e.g., pH 5.2).

It is highly recommended that the efficiency of aminoacylation be tested in analytical experiments (~40 pmol tRNA) before preparative scale reactions (~4000 pmol tRNA; see details below) are set up. In analytical experiments, there is no need to extract aminoacyl-tRNA from the charging reaction. Simply add sodium acetate (NaOAc; pH 5.2) to 300 mM, ethanol precipitate, dissolve the pellet in acid gel loading buffer, and subject the sample to acid gel electrophoresis (see Section A.3). Based on our experience, the extent of aminoacylation of purified tRNAs from various sources (e.g., Subriden, Sigma, Chemical Block) should exceed 90%.

Whenever working with RNA, gloves should be worn to prevent contamination by RNases, which are a natural component of healthy human skin. As an additional precaution, all solutions should be made using nanopure water and baked glassware or new plasticware.
A.2.1. Materials required

- purified tRNA (Note: The extinction coefficient at 260 nm for a particular species of pure tRNA can be estimated based on its amino acid acceptor activity, which is typically provided by the supplier.)
- amino acid (Use of $^{14}$C-labeled amino acid allows a more accurate quantification of the aminoacyl-tRNA product. If the product will be acetylated, $^{14}$C-labeled amino acid is recommended in order to assess the extent of acetylation.)
- 1 M HEPES-KOH (pH 7.6)
- 100 mM ATP (pH 7)
- 100 mM DTT
- 3 M KCl
- 1 M MgCl$_2$
- aminoacyl-tRNA synthetase [AARS; Although S-100 extracts from *E. coli* often give high-level charging, we typically use purified or partially-purified AARS. Over-expression constructs encoding tagged-versions of AARSs are available from several laboratories and are also available from the ASKA collection (National Institute of Genetics, Japan).]
- 3 M NaOAc (pH 5.2)
- phenol saturated with 300 mM NaOAc (pH 5.2)
- CHCl$_3$ and isoamyl alcohol (24:1 mixture)
- ethanol
- Sephadex G-25 (Amersham Biosciences). To prepare, combine dry resin (~ x g) and ~ 70 mL 2 mM NaOAc (pH 5.2) in a 125 mL screw-cap glass bottle, autoclave for 10 minutes with the cap loose, and allow to cool. The volume of buffer above the soaked resin should be about half of the total volume. If it is
more than half after cooling, remove the excess. Store the equilibrated resin at 4°C.

- Disposable Bio-Spins columns (Bio-Rad).
- Falcon 6 mL, 12 x 75 mm, disposable test tubes (Becton Dickinson) or other appropriately-sized tubes

**A.2.2. Protocol**

1. In a reaction volume of 500 µL, combine the following components:
   
   tRNA (4000 pmol; ~ 3 A_{260} units)
   
   amino acid (100 µM; 14C-labeled is recommended)
   
   HEPES-KOH (pH 7.6; 100 mM)
   
   ATP (10 mM)
   
   DTT (1 mM)
   
   KCl (10 mM)
   
   MgCl₂ (20 mM)
   
   AARS (~ 1 µM; The amount added should be sufficient to ensure complete aminoacylation as judged by preliminary analytical experiments.)

2. Incubate the reaction in a circulating water bath at 37°C for 20 minutes.

3. Add 50 µL 3 M NaOAc (pH 5.2).

4. Extract with phenol for 10 minutes using a vortex-mixer in the cold room.

5. Separate the phases by spinning in a microfuge for 5 minutes at top speed (~ 13,000 rpm). Move the aqueous phase to a new microfuge tube.
6. Back extract the phenol phase with 300 mM NaOAc (pH 5.2), spin, remove the aqueous phase and combine it with the aqueous fraction saved from step 5.

7. Repeat steps 5 and 6. (Note: The volume of the aqueous phase keeps increasing, so additional microfuge tubes will be needed for subsequent extractions. Always extract 500 µL or less per 1.5 mL tube).

8. Extract the aqueous phase twice with a mixture of CHCl₃ and isoamyl alcohol (24:1).

9. Add 2.5 volumes of 100% ethanol to precipitate the aminoacyl-tRNA. The solution will become cloudy as the ATP co-precipitates.

10. Pellet the precipitate by centrifugation in the cold room (e.g., 15 minutes in a microfuge at 13K rpm). Remove supernatant.

11. Wash pellets with 70% ethanol.

12. Dissolve pellets in a small volume of 2 mM NaOAc (pH 5.2). For example, if microfuge tubes were used in step 9, dissolve each pellet in 20 µL and then combine.

13. Prepare Sephadex G-25 spin columns. Add 2.5 mL of G-25 slurry (in 2 mM NaOAc, pH 5.2) to each column, place in an appropriately-sized collection tube (e.g., Falcon 6 mL disposable test tube), and use a clinical centrifuge to pack the column. The volume of the packed resin should be about 1 mL.

14. Pass the aminoacyl-tRNA through spin columns. Load < 50 µL on each ~ 1 mL column. For collection, stack two top-less microfuge tubes in a 15 mL plastic tube (e.g., Falcon screw-capped), and then place the (loaded) spin column into the top microfuge tube prior to centrifugation. This step removes the remaining small molecules.

15. Combine, aliquot, and store at -70°C.
16. Determine counts per minute of the $[^{14}\text{C}]$-aminoacyl-tRNA preparation and of the stock $[^{14}\text{C}]$-amino acid by scintillation counting. Calculate the concentration of aminoacyl-tRNA based on the specific activity of the amino acid stock. If radiolabel is not employed, estimate the concentration of aminoacyl-tRNA based on $A_{260}$. Determine the extent of aminoacylation using acid gel electrophoresis (see below). Generally, the yield is $\sim 75\%$ and the extent of charging is $> 90\%$.

**A.3. Use of acid gel electrophoresis to determine the extent of aminoacylation**

A key technical advance came when RajBhandary and coworkers showed that aminoacyl-tRNA can be separated from deacylated tRNA using polyacrylamide gel electrophoresis under acidic conditions (154). This technique allows direct assessment of the extent of aminoacylation and is clearly superior to earlier methods that involved estimating the relative amount of tRNA and amino acid in the preparation based on $A_{260}$ and $^{14}\text{C}$ measurements, respectively. Aminoacyl-tRNA migrates more slowly through acid gels than deacylated tRNA (Fig. A.1). This difference in mobility is lessened when the free amino group is acetylated. Thus, when preparing $N$-acetyl-aminoacyl-tRNA, an aliquot of aminoacyl-tRNA should be removed for acid gel electrophoresis before the remainder is acetylated.

**A.3.1. Materials required**

- electrophoresis rig
- power supply
• glass plates (~ 30 cm length by ~ 20 cm width)
• spacers and comb (0.75 mm thick)
• 40% acrylamide solution (19:1; mono:bis)
• 3 M NaOAc (pH 5.2)
• Urea
• 10% ammonium persulfate (APS)
• N,N,N’,N’-Tetramethylethylenediamine (TEMED)
• bromophenol blue
• methylene blue
• Pyrex dish with dimensions larger than the gel
• rotator

A.3.2. Protocol

1. Pour a 6.5% polyacrylamide gel containing 100 mM NaOAc (pH 5.2) and 8 M urea. The length of the plates should be about 30 cm and the spacer thickness should be 0.75 mm. To polymerize the gel, add 600 µL 10% APS and 60 µL TEMED per 100 mL of acrylamide solution.

2. Once polymerized, set up the gel in the cold room. Use 100 mM NaOAc (pH 5.2) as running buffer.

3. Add loading dye (100 mM NaOAc (pH 5.2), 7 M urea, 0.05% bromophenol blue) to the tRNA samples. Load approximately 40 pmol tRNA per lane. Do not heat samples before loading.
4. Electrophoresis for 12 hours at 10 Watts.

5. Remove the spacers and then separate the plates. Carefully transfer the gel into ~ 500 mL of staining solution (500 mM NaOAc (pH 5.2), 0.06% methylene blue) contained in a large Pyrex dish. Place the dish on a rotator set at its lowest speed and allow the gel to stain thoroughly (~ 30 minutes).

6. Transfer the staining solution (which can be reused) into a large glass bottle using a funnel. This can be done by holding the gel against the bottom of the dish with one gloved hand while supporting the weight of the dish with the other.

7. Destain the gel with water. Several changes of water will be necessary for complete destaining. It works well to siphon the water from a corner of the dish.

It is wise to load the gel with some asymmetry, so that the lane orientation is unambiguous. This can be accomplished by loading a dilution series of each sample (Fig. A.1), which also serves to compensate for poor estimations of concentrations prior to loading and to better assess the fraction of tRNA charged.
Fig. A.1. Acid gel electrophoresis resolves aminoacyl-tRNA from deacylated tRNA, allowing the extent of aminoacylation to be determined. (a) Various amounts (as indicated) of tRNA\textsuperscript{Val}, Val-tRNA\textsuperscript{Val}, and N-acetyl-Val-tRNA\textsuperscript{Val} were subjected to acid gel electrophoresis, the gel was stained with methylene blue (top panel), dried, exposed to a phosphorimager screen, and scanned (bottom panel). (b) Various amounts of tRNA\textsuperscript{fMet} and fMet-tRNA\textsuperscript{fMet} were resolved on an acid gel, which was then stained with methylene blue.
A.4. N-acetylation of aminoacyl-tRNA

The peptidyl-tRNA analog N-acetyl-aminoacyl-tRNA can be readily made by chemical acetylation of aminoacyl-tRNA using acetic anhydride. This reaction is highly efficient and thus homogeneous preparations of N-acetyl-aminoacyl-tRNA are typically obtained.

A.4.1. Materials required

- aminoacyl-tRNA
- 3 M NaOAc (pH 5.2)
- acetic anhydride
- ethanol

A.4.2. Protocol

1. Dilute aminoacyl-tRNA to 1.6 µM in microfuge tubes containing 250 µL cold 200 mM NaOAc (pH 5.2).
2. Add 4 µL acetic anhydride to each tube, mix, and incubate on ice for one hour.
3. Add another 4 µL acetic anhydride, mix, and incubate on ice for another hour.
4. Raise the NaOAc (pH 5.2) concentration to 300 mM.
5. Ethanol precipitate.
6. Wash pellets with 70% ethanol.
7. Dissolve each pellet in 20 µL 2 mM NaOAc (pH 5.2), pool samples, aliquot, and store at -70° C.
A.5. Use of thin layer chromatography (TLC) to quantify the extent of N-acetylation

A.5.1. Materials required

- N-acetyl-[\textsuperscript{14}C]-aminoacyl-tRNA
- [\textsuperscript{14}C]-amino acid
- acetic anhydride
- ammonium hydroxide (NH\textsubscript{4}OH)
- silica gel thin-layer chromatography plates (e.g., Analtech silica gel HLF)
- butanol
- acetic acid
- plastic film (e.g., Saran Wrap)
- phosphorimager

A.5.2. Protocol

1. To prepare markers for the TLC, treat the appropriate [\textsuperscript{14}C]-amino acid with acetic anhydride as described in Protocol 4.2 (steps 1-3). Since this acetylation reaction is less efficient that that of the aminoacyl-tRNA, the resulting sample is expected to contain both the acetylated and unacetylated amino acid.

2. To a small aliquot of N-acetyl-[\textsuperscript{14}C]-aminoacyl-tRNA (~50 pmol), add NH\textsubscript{4}OH to 3% and incubate at 55° C for 20 minutes. This step deacylates the tRNA.
3. Spot 1 µL onto a small silica TLC plate with the appropriate controls (i.e., the [14C]-amino acid and the N-acetylated amino acid prepared as described above). Allow the spots to dry.

4. Develop the TLC with a mixture of butanol, water, and acetic acid (4:1:1).

5. Air dry the TLC plate completely. Cover with plastic film and expose to a phosphorimager screen. Care should be taken to prevent contamination of the screen with 14C.

Fig. A.2. The extent of acetylation or formylation can be determined using TLC. N-acetyl-[14C]-Tyr-tRNA Tyr (panel a), N-acetyl-[14C]-Val-tRNA Val (panel b), and N-acetyl-[14C]-Phe-tRNA Phe (panel c) were deacylated by treatment with NH4OH and spotted onto a silica-gel TLC plate (lane 3) along with the corresponding [14C]-amino acid (lane 1) and acetylated [14C]-amino acid (lane 2). The TLC plate was developed with a mixture of butanol, water, and acetic acid (4:1:1), dried, wrapped with plastic film, and exposed to a phosphorimager screen. (d) TLC analysis comparing deacylated formyl-[35S]-Met-tRNA fMet (lane 2) with a partially oxidized sample of [35S]-methionine (lane 1). Assignments of spots are based on separate experiments in which hydrogen peroxide was used to promote oxidation.
It is worth noting that extent of acetylation of unlabeled aminoacyl-tRNA can be qualitatively assessed by acid gel electrophoresis (Fig. A.1.A). However, the difference in migration between the aminoacyl-tRNA and \(N\)-acetyl-aminoacyl-tRNA is typically too small to allow accurate quantification.

A.6. Preparation of fMet-tRNA\(\text{fMet}\)

Translation initiation involves a specific tRNA, the initiator tRNA, which reads the start codon in the ribosomal P site. In bacteria, the initiator tRNA is fMet-tRNA\(\text{fMet}\), which has a number of unique features that specify its role in initiation (139).

fMet-tRNA\(\text{fMet}\) can be generated in a reaction containing methionyl-tRNA synthetase (MetRS) to charge tRNA\(\text{fMet}\) and methionyl-tRNA formyltransferase (MTF) to formylate Met-tRNA\(\text{fMet}\). The electrophoretic mobility of both Met-tRNA\(\text{fMet}\) and fMet-tRNA\(\text{fMet}\) is sufficiently different from that of tRNA\(\text{fMet}\), allowing the extent of aminoacylation to be assessed after both reactions have occurred (Fig. A.1.B).

Methionine is susceptible to oxidation by air, which can result in sulfoxide formation. If this poses a problem, methionine oxidation can be reversed enzymatically using MsrA or chemically using a reducing agent. Typically, about 30% of fMet-tRNA\(\text{fMet}\) is oxidized when prepared as described below (Fig. A.2.D).
A.6.1. Materials required

- $N_5,N^{10}$-methenyltetrahydrofolic acid [To make, dissolve 25 mg folinic acid (Ca$^{2+}$ salt; available from Sigma) in 2 mL 50 mM β-mercaptoethanol, add 220 µL 1M HCl, and incubate at room temperature for 3 hours (Solution should turn yellow; if a precipitate forms, heat at 50°C to dissolve). Dilute with 1 mL of 100 mM HCl and store in 200 µL aliquots at -20°C (155).]
- 1M KOH
- 1M Tris-HCl (pH 7.9)
- pH test strips
- purified tRNA$^{\text{Met}}$
- methionine (Use of radiolabel is recommended in order to assess the product.)
- 1 M HEPES-KOH (pH 7.6)
- 100 mM ATP (pH 7)
- 100 mM DTT
- 3 M KCl
- 1 M MgCl$_2$
- S-100 extract from *E. coli* or purified methionyl-tRNA synthetase (MetRS) and methionyl-tRNA formyltransferase (MTF) [Over-expression constructs encoding tagged-versions of these enzymes are available from laboratories that study these enzymes and can also be obtained from the ASKA collection (National Institute of Genetics, Japan).]
- 3 M NaOAc (pH 5.2)
• 20% SDS
• phenol saturated with 300 mM NaOAc (pH 5.2)
• CHCl₃ and isoamyl alcohol (24:1 mixture)
• ethanol
• Sephadex G-25 (Amersham Biosciences; To prepare, see Section 2.1 above.)
• Disposable Bio-Spins columns (Bio-Rad)
• Falcon 6 mL, 12 × 75 mm, disposable test tubes (Becton Dickinson) or other appropriately-sized tubes

A.6.2. Protocol

1. Neutralize a 200 µL aliquot of $N^5,N^{10}$-methenyl-tetrahydrofolic acid to form $N^{10}$-formyl-tetrahydrofolate by adding 10 µL 1M Tris-HCl (pH 7.9) and 20 µL 1M KOH and incubating at room temperature for 15 minutes. Solution should turn clear. Check the pH, which should be ~ 7.5.

2. In a reaction volume of 500 µL, combine the following components:
   - tRNA (4000 pmol; ~ 3 A₂₆₀ units)
   - methionine (100 µM; use of radiolabel is recommended)
   - HEPES-KOH (pH 7.6; 100 mM)
   - ATP (10 mM)
   - DTT (1 mM)
   - KCl (10 mM)
   - MgCl₂ (20 mM)
*N*<sup>10</sup>-formyl-tetrahydrofolate, neutralized in step 1 (300 µM)

S-100 extract (25 µL) or MetRS and MTF (~ 1 µM each)

Note: The amount of S100 or enzyme necessary for complete aminoacylation and formylation can be determined in analytical experiments before preparative scale reactions are set up. The neutralized *N*<sup>10</sup>-formyl-tetrahydrofolate left over is unstable and should be discarded - not reused.

3. Incubate the reaction in a circulating water bath at 37° C for 20 minutes.

4. Add 50 µL 3 M NaOAc (pH 5.2) and 14 µL 20% SDS.

5. Extract with phenol.

6. Back extract the phenol phase with 300 mM NaOAc (pH 5.2).

7. Repeat steps 5 and 6.

8. Extract the aqueous phase twice with a mixture of CHCl<sub>3</sub> and isoamyl alcohol (24:1).


10. Wash pellets with 70% ethanol.

11. Dissolve pellets in 20 µL of 2 mM NaOAc (pH 5.2) and combine.

12. Pass the fMet-tRNA through spin columns as described in section A.2.1.

13. Combine, aliquot, and store at -70°C.

The extent of aminoacylation and formylation should be assessed by acid gel electrophoresis and TLC as described above (*sections A.3 and A.5*). β-mercaptoethanol (50 mM) is often included in the electrophoresis buffer and developing solvent to help
prevent oxidation of the methionine. To generate markers for the TLC analysis, hydrogen peroxide (100 mM) can be used to convert methionine and formyl-methionine to their sulfoxide and sulfone forms.

A.7. Concluding Remarks

Here we describe methods to prepare aminoacyl-tRNA, N-acetyl-aminoacyl-tRNA, and fMet-tRNA\textsuperscript{fMet} and to assess their homogeneity. These protocols seem generally applicable, regardless of tRNA species, and thus should be useful for a number of laboratories. Throughout the text, we have tried to emphasize the importance of evaluating the acylated tRNA preparations. The techniques involved are easy and the data unambiguous, allowing the investigator to interpret subsequent experiments with a higher degree of confidence. In our hands, the extent of acylation is generally high (>90%), obviating the need for subsequent purification steps (156).
APPENDIX B

FUNCTIONAL EVIDENCE FOR OCCLUSION OF THE RIBOSOMAL A SITE
BY INITIATION FACTORS

B.1. Introduction

The timing and manner by which protein and tRNA ligands interact with the ribosome are highly coordinated. The binding of aa-tRNA to the ribosomal A site must not take place before a start codon is paired with the initiatior tRNA, or translation could take place from the wrong starting point. It is thought that initiation factors IF-1 and IF-2 sterically occlude binding to the A site during initiation, although there is no functional evidence to support this hypothesis. Here the first functional evidence (to my knowledge) for occlusion of the A site until complete 70S initiation complex formation is presented. From this preliminary result, further experiments are suggested which would address the timing of factor binding and release during the transition from initiation to elongation.

Ribosomes, tRNAs, and mRNA were prepared as described (85, 87, 113). Recombinant His-tagged IF2 and IF3 were purified as described by Ni-NTA affinity chromatography (157). EF-Tu:His6-EF-Ts was purified as a 1:2 complex by metal ion affinity chromatography as described (90).

Recombinant initiation factor IF1 was purified by ion exchange chromatography from BL21(DE3/pET24b-IF1) cells after a 2-hour IPTG (1 mM) induction at mid-log phase growth. First, clarified lysate was bound in batch to S resin (Bio-Rad) equilibrated in Buffer A [20mM Tris (pH 7.6), 20 mM NaCl, 10 mM MgCl$_2$, 6 mM β-mercaptoethanol, 0.5 mM EDTA], by rotating at 4°C for 1 hour. After washing 4 times, bound protein was eluted with 1M NaCl in Buffer A and dialyzed overnight against 1L Buffer A to remove excess salt. Next, the batch-purified fraction was applied to a 6mL resource S column (Amersham GE Healthcare) equilibrated in Buffer A, and eluted by a linear gradient over 20 column volumes with Buffer B [20 mM Tris (pH 7.6), 500 mM NaCl, 10 mM MgCl$_2$, 6mM β-mercaptoethanol, 0.5 mM EDTA] by FPLC. IF1 eluted at ~125 mM NaCl. Fractions that contained protein were identified by Bradford and analyzed by SDS-PAGE. Those fractions containing pure protein were combined, dialyzed against 1L Polymix buffer [5 mM potassium phosphate (pH 7.3), 95 mM KCl, 5 mM NH$_4$Cl, 0.5 mM CaCl$_2$, 5 mM magnesium acetate, 8 mM putrescine, 1 mM spermidine, and 1 mM DTT] (88) containing 10% glycerol, and stored in small flash-frozen aliquots at –70°C.
EF-Tu was activated as described (90) with the following modifications. 20 µM EF-Tu:EF-Ts was incubated with 3 mM GTP in EF-Tu storage buffer [50 mM HEPES (pH 7), 30 mM KCl, 70 mM NH₄Cl, 10 mM MgCl₂, 0.1 mM EDTA, 10% glycerol] for 15 min. at 37°C. Activated EF-Tu (10 µM) was further incubated with Tyr-tRNA^{Tyr2} (5 µM) on ice for at least 5 minutes to form the EF-Tu•GTP•Tyr-tRNA^{Tyr2} ternary complex.

Toeprinting was performed as described (85). The P site was bound in the presence or absence of initiation factors, and the position of mRNA with respect to the ribosome mapped after each addition. First tRNA^{fMet} (1 µM, lane 1) or fMet-tRNA^{fMet} (1 µM, lane 8) was added to bind the P site of m299-programmed ribosomes (0.7 µM) and incubated for 10 minutes at 37°C in polymix buffer in the presence or absence of initiation factors (1 µM) and GTP (1mM). A portion of each complex was removed and stored on ice for toeprinting. Next, each reaction was further incubated with Tyr-tRNA^{Tyr2} (1 µM) or EF-Tu•GTP•Tyr-tRNA^{Tyr2} (1 µM) for 5 minutes at 37°C before removing an aliquot to ice for toeprinting. Finally, a portion of each reaction was further incubated for 5 minutes after addition of GTP (300µM) or EF-G (1 µM final) and GTP. Each aliquot on ice was then toeprinted in parallel at 37°C for 10 minutes, and analyzed on a sequencing gel.

B.3. Results and Discussion.

In order to assess the effects of initiation and elongation factors on tRNA binding and translocation, the toeprinting assay was used to map mRNA after each step in a series of incubations. First, tRNA^{fMet} or fMet-tRNA^{fMet} was bound to the P site of m299-
programmed ribosomes in the presence or absence of initiation factors (IFs; Fig. B1, P lanes). In both cases, a single toeprint was observed at +16, indicating the AUG codon was positioned in the P site. In the presence of IFs (lane 8), the +16 toeprint was more pronounced, demonstrating that the factors enhanced the level of tRNA binding.

Figure B.1. Enzymatic binding of the P site prevents non-enzymatic A-site binding. The position of mRNA was mapped by toeprinting after each of several additions. First, tRNA^{fMet} or fMet-tRNA^{fMet} was bound to the P site either non-enzymatically or enzymatically (P lanes). Next Tyr-tRNA^{Tyr2} was added nonenzymatically or as part of a ternary complex with EF-Tu, as indicated, to each reaction (A lanes). Finally, complexes were further incubated with buffer and GTP in the presence or absence of EF-G (-G, +G lanes).
Next, Tyr-tRNA$^{\text{Tyr2}}$ was added to bind the A site (A lanes), either non-enzymatically (lanes 2 and 9) or as part of a ternary complex with EF-Tu•GTP (lanes 5 and 12). For those complexes assembled in the absence of IFs and EF-Tu (lane 2), the toeprint position shifted by one nucleotide. This A-site shift is indicative of binding to the A site, and is thought to be the result of a conformational change and drawing in of the mRNA which yields a shorter cDNA product (96). In the presence of IFs, when Tyr-tRNA$^{\text{Tyr2}}$ was bound directly to the P site, the toeprint pattern was unexpected. First of all, a faint toeprint appeared at +19, corresponding to UAC positioning in the P site. This is likely the result of dissociation and de novo assembly of a complex containing Tyr-tRNA$^{\text{Tyr2}}$ paired to UAC in the P site. Because IF3 is known to prevent subunit association and stimulate the dissociation of tRNAs from the P site, it is possible that on this timescale (5 minutes), the initiation complexes are being disassembled and reassembled, allowing Tyr-tRNA$^{\text{Tyr2}}$ to pair with UAC in the P site in a portion of the ribosomes. Another possibility is that there was a small amount of EF-G in one of the initiation factor preparations which catalyzed translocation. The second, and more intriguing observation was that in the presence of IFs, when tRNA was bound non-enzymatically to the A site, this shift was no longer observed. This suggests that either 1) A-site binding is inhibited by one or more of the IFs, or 2) that the presence of one or more of the factors prevents the ribosome from undergoing the conformational change upon A-site tRNA binding. IF1 and IF2 bind over the 30S and 50S A sites, and bind cooperatively. Either factor is a likely candidate for inhibiting A-site binding. In the
absence of IFs, addition of Tyr-tRNA^{Tyr2} resulted in the appearance of a strong toeprint at +17.

While aa-tRNA was unable to cause a toeprint shift when added to the enzymatically-formed initiation complex, when EF-Tu\text{•}GTP\text{•}Tyr-tRNA^{Tyr2} was added to the enzymatically-formed initiation complex, a shift was observed. This indicates that EF-Tu compensated for the inhibition of binding or conformational change. It is possible that EF-Tu displaces one or more factors that prevent the A-site shift.

To further investigate these results, it will first be necessary to ascertain what is required to prevent the A-site shift, by using similar toeprinting experiments and systematically testing each component. Next, it will be of use to assess the nature by which the A-site shift is inhibited. Are the initiation factors inhibiting the conformational change that takes place upon binding, or are they sterically blocking A-site binding? Filter binding to determine whether the A site is bound will readily distinguish between these two possibilities. Finally, determination of which factors remain bound may shed light on the timing and means by which EF-Tu promotes the shift. Further investigation of these results may provide clues into the roles of initiation and elongation factors in translational fidelity.
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