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Narasimham V. Jammi

The University of Montana

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Interaction of the 3' end of tRNA with the ribosome:
methods and modifications of the 3' end

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Presented in partial fulfillment of the requirements
for the degree of

Master of Science

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1998

Approved by:

Chairman, Board of Examiners

Dean, Graduate School

7-24-98

Date
ABSTRACT

The goal of this Masters thesis project was to map the neighborhood of the 3' end of tRNA on the ribosome. The first step was to attach a thiol group on to the 3' end of tRNA. This could then be alkylated by iodacetamido, 1-10 orthophenanthroline. Phenanthroline, in the presence of copper and a reducing agent, cleaves nearby nucleic acids via an as yet unknown mechanism. However, the modification process proved to be a very difficult task. Various modification procedures were tried. 5' thiophosphorylation of tRNA proved to be unsuccessful. Ligation of a thiolated nucleotide to the 3' end of tRNA-A was also a failure. Introduction of a thiophosphate at the 3' end of tRNA was yet another procedure that was not able to be accomplished. Finally, annealing of two fragments to form a compact tRNA structure was also not achieved. In short, this manuscript explored various avenues for modification of the 3' end of tRNA. This should serve as a guide for future work in this field.
Acknowledgments

Firstly, I'd like to thank God for blessing me with two wonderful parents without whom I wouldn't be here in the US. I'd like to also thank my grandmother for telling me all those wonderful stories which I believe had a great influence in shaping me and the way I think. My brother, who is also my most trusted friend, has always made me smile. Next I'd like to acknowledge my debt to Prof. Upadhyaya at Osmania University who admitted me into Nizam College despite all odds. I would be failing in my duty if I didn't mention my sister and brother-in-law who created a home away from my home during my days as an undergraduate. My two other sisters know what they mean to me. My friends in the Hill lab have made these last three my most enjoyable, especially Doug Bucklin, Jim Bullard and Michael van Waes. Marty has been very patient with me. To her, I owe a lot. Most important of all, I thank Dr. Walter E. Hill, my mentor, philosopher and guide who taught me to think...perhaps, the greatest gift of all.

I dedicate this work to my grandfather.
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1.0 Introduction

1.1 Ribosome architecture:

The ribosome is a complex ribonucleoprotein that is essential for the process of protein synthesis. Two subunits, three RNA molecules and 53 different proteins are held together to form a complete prokaryotic ribosome. The prokaryotic ribosome is composed of a 50S subunit and a 30S subunit. The 50S subunit is made up of two RNA molecules - 23S (figure 1) (2904 nucleotides in length) and 5S (120 nucleotides in length) as well as 32 different proteins. Each of these, except for one termed L7/L12, is present as a single copy. L7/L12, a duplex protein, is present in two copies. The 30S subunit consists of a single RNA molecule, which has a sedimentation coefficient of 16S (figure 2) (1542 nucleotides in length). In addition to this, there are 21 different proteins in the 30S subunit. The E. Coli 70S ribosome measures about 170 Å X 230Å X 250Å in solution (32). The structure of the ribosome is intricate yet well defined. Proteins and RNA labor in perfect harmony to make protein synthesis an almost flawless process.

1.2 Ribosome: Gross Anatomy

For all practical purposes, the Lake model (43) of the ribosome is widely accepted. The small subunit, the 30S particle, has a head and a base. There is a platform arising from the base of the subunit forming a cleft between the head and the platform (44). The large subunit, the 50S particle is likewise, asymmetric. The striking features of a 50S subunit are its
Figure 1.) Secondary structure of 23S rRNA taken from the ribosomal data base (69)
Figure 2.) Secondary structure of 16S rRNA of the small subunit of the E.coli 70S ribosome (111)
central protuberance and distinct projections on either side of the protuberance at approximately 50 degrees. One of these protrusions is the L7/L12 stalk and the other is the L1 ridge. The two subunits are positioned asymmetrically upon one another to form the monomeric ribosome. All other electron micrographic models of the 50S and 30S subunits (3,10,93,104) are in general agreement with the Lake model, but have definite differences.

There are several methods that have been used to analyze the structure of the ribosome. However, due to the complexity of the ribosome and its inherent asymmetry, very few have yielded high resolution models of the 70S ribosome.

One innovative technique was to measure the distances between proteins in the 30S ribosomal subunit of *E.coli* in order to determine their positions in the ribosome. This was done by neutron scattering of the deuterated proteins whose distance had to be calculated, reconstituted into an otherwise protonated ribosome. The term that was measured was the neutron scattering profile of the deuterated protein in solution. From this, the separation of the centers of a pair of deuterated proteins can be obtained. A series of such distance measurements can be combined by triangulation to give a three dimensional description of the relative positions of all protein in the small subunit of the ribosome (61). The final result of a series of such experiments is shown in figure 3. This describes the relative positions of 19 ribosomal proteins of the 30S subunit.
Figure 3.) Neutron scattering map of 30S proteins.
One of the more successful techniques that has yielded low resolution structures of the 50S, 30S and the 70S particles has been the non-crystallographic image processing and three-dimensional reconstruction techniques (19,21,76,106,107) of negatively stained ribosomal particles prepared with a double-carbon layer method. This reconstruction technique combines a large number of projections (200-500) to form a three dimensional representation of the particle. With this technique, not only have well-established structural landmarks been unambiguously identified, but also new features have been visualized. One of these, is the interface canyon, a deep trough on the interface side of the interface side of the 50S subunit. This is located just underneath the central protuberance and extends from the L1 ridge to the L7/L12 stalk. An interesting feature of this interface canyon is the presence of three pockets; the central one of these is located close to the putative peptidyl transferase center. This pocket actually seems to be a channel which leads to the back of the subunit and which Frank’s group hypothesized as the exit site for the nascent polypeptide chain (77). This is in contrast to Yonath’s idea of a channel running through the middle of the subunit as the site of the nascent polypeptide(114).

A complete reconstruction of the E.coli 70S ribosome in three dimensions has recently been achieved (7,109). This model is in basic agreement with other models. A notable feature of this model is the easy accessibility of the interface canyon, despite the overlap of the two subunits. This seems to indicate that, perhaps tRNA molecules enter and leave from this site on the ribosome (18,20).
Cryo-electron microscopic (cryo-EM) data have revealed new information about the topography of the 70S ribosome (18). Cryo-EM reconstruction data serves as an excellent framework to fit in the data obtained by numerous studies using crosslinking, chemical modification and other techniques. Cryo-electron microscopy of specimens embedded in vitreous ice, maintains the specimen in the fully hydrated form without the help of a heavy metal contrasting agent. Interior features become visible, that can be related to the scattering density distribution and in turn to the electron density distribution. Given sufficient resolution, the spatial distribution of the ribosomal RNA can be visualized because of the high scattering of phosphorous atoms in the RNA backbone. Frank's group has reconstructed the 70S ribosome at 40 Å (see figure 4) resolution. The 40 Å model revealed that the 30S and 50S subunit were fused at the bottom and the head (the head of the 30S and the central protuberance) regions. Other main features of this model were as follows. The 30S subunit lies somewhat obliquely across the 50S subunit. The upper halves of the subunit enclose a space called the intersubunit space. Perhaps, the most striking attribute of this model was the identification of the platform of the 30S subunit as a possible bridge linking the 16S and 23S rRNAs.

In the recent past, the 40 Å map has paved way for a more refined, 25 Å map (figure 5) (45). According to this, the two subunits are not fused at the bottom and are clearly separated. The intersubunit space has an inverted heart shape in cross section.
Figure 4.) Cryo-electron micrographs of the E. coli 70S ribosome at 40 Å resolution. The view on the left is the interface view. The view on the right is related to the one on the left by an axial rotation of 110 degrees. "p" refers to the platform of the 30S subunit, while "h", "sp" and "ch" refer to the head, spur and channel of the 30S subunit. "CP" denotes the central protuberance of the 50S subunit, while "L1" refers to the L1 arm and "St" indicated the L7/L12 stalk. Adapted from Frank et al. (18)
Figure 5.) 25 Å reconstruction of the E. coli 70S ribosome. "ch" denotes the channel and "sp" indicates the spur. Adapted from Frank et al. (20)
The number of bridges linking the two subunits are now six, as opposed to just the one observed in the 40 Å model. The major bridge linking the two subunits, is still the platform of the 30S subunit. The other five may be regions of rRNA-rRNA contact stabilizing the subunit-subunit interaction. Interesting features of the 25 Å model are seen in the 30S subunit. These include a channel penetrating the neck and a "spur" projecting at the subunit's base. The channel widens into a relatively large hole between the neck and platform. It has been postulated that perhaps this forms the passage for the incoming mRNA (45). Other notable characteristics of this model are the two tunnels seen at the back exterior of the 50S subunit. One of these tunnels, is perhaps the exit tunnel that has been established beyond reasonable doubt by biochemical evidence (2,83). This was the putative exit domain of the newly synthesized polypeptide chain (2). The second tunnel observed in the 25 Å model was suggestive of the presence of a second exit tunnel (45). Despite all these new additions to the morphology of the ribosome, there still remains the need for a higher resolution structural map of the ribosome.

1.3 The Translating Ribosome:

There are three important aspects to a translating ribosome: initiation, elongation and termination. Initiation starts with the formation of an "initiation complex" between the 30S subunit, the messenger RNA, and the initiator tRNA; fMet -tRNA\textsuperscript{fMet}. Three proteins involved in this process, the initiation factors (named IF1, IF2 and IF3), are essential for efficient translation on natural mRNA in vitro (79). One of the three, IF2 (a G-protein) complexed with GTP, binds to tRNA\textsuperscript{fMet} forming a ternary complex. Ternary complex
formation is dependent on aminoacylation and formylation of the fmet-tRNA^{fmet} (29). This binary complex then binds to the 30S ribosome to form the 30S initiation complex. The exact order of binding of the 3 initiation factors are yet unknown. IF2 seems to mainly influence the binding of the initiator tRNA to the 30S subunit (105).

The functions of the other two initiation factors are still unclear. IF3 has been the focal point of controversy for some time. It is needed for the dissociation of 70S ribosomes into active subunits (26,89). IF3 has also been implicated in stabilizing the primary binding interaction between the mRNA and the 30S subunit (105).

The site where the initiation complex is formed on the mRNA is protected from nuclease degradation and has been termed the ribosome-binding site (RBS). Most RBSs contain a purine rich sequence, the Shine-Dalgarno sequence (86). This region of the mRNA is complementary to the 3' end of 16S rRNA. This base pairing interaction seems to strongly effect selection of translation initiation sites in vivo (35). IF1 appears to accelerate IF3-dependent 70S dissociation. Apart from this not much is known about the function of IF1. Once the initiation complex is formed, the 30S subunit, somehow gains a high affinity for the 50S subunit, forming a 70S initiation complex with the fMet-tRNA^{fMet} at the peptidyl tRNA binding site of the ribosome, with the anticodon of the tRNA base pairing with the initiation codon of the mRNA. The initiation complex is now ready to receive an incoming aminoacyl tRNA at the aminoacyl site (A site).
Directed by the next codon of the mRNA, the ribosome now accepts the appropriate tRNA in the form of a ternary complex, which consists of an aminoacylated tRNA, GTP and an elongation factor EF-Tu (48). A majority of the tRNAs found in the cytoplasm exist as ternary complexes (48). The ribosome tests for the fidelity of this complex by testing the anticodon-codon interaction at the A site (48).

Once the tRNA is selected, GTP is hydrolyzed on EF-Tu, leaving a cognate aminoacyl-tRNA or sometimes even a non-cognate aminoacyl-tRNA. The near exactness of protein synthesis, however, cannot be explained completely by the codon-anticodon interaction. A second step called the proofreading step was postulated (66). Proofreading may occur in one or several steps (16) after initial tRNA selection. Proofreading seems to be necessary for explaining the accuracy of the ribosomes during protein synthesis. After GTP hydrolysis, the aminoacyl-tRNA has one of two possible courses of action: a) if the tRNA is cognate, the tRNA will remain bound to the ribosome to participate in the next step of peptidyl transfer and b) if it is a noncognate tRNA, it will dissociate from the ribosome (48). If there are a number of noncognate tRNAs selected by the ribosomes, there are going to be an equal number of GTP hydrolysis reactions. The excess dissipation of GTP hydrolysis cycles correspond to the discard of noncognate tRNAs at these proof reading branch points. The driving force for proof reading and tRNA-ribosome stability seems to be GTP hydrolysis. The role of GTP hydrolysis is to ensure that the tRNAs that enter the ribosome at the A-site enter only as ternary complexes and not as free tRNAs. This is done by dovetailing the initial association step with the proofreading step. Proofreading is essential for a high $k_{cat}/K_m$ of
association of cognate tRNA to the ribosome.

Once the correct aminoacyl-tRNA is instituted at the A-site in its proper orientation, peptidyl transfer occurs. Simplistically, the α-amino group of the A-site amino acid (the acceptor site), conducts a nucleophilic attack on the carbonyl of the ester group of the P-site amino acid (the donor site) (52). The amino acid is essentially transferred from the P-site tRNA to the A-site tRNA. This reaction is catalyzed by the peptidyl transferase complex, which has been suggested to be an integral part of the 50S subunit (13,17). The composition of the peptidyl transferase has been the focus of great debate over the last few decades. There exist two schools of thought on this matter. There are investigators who believe that ribosomal proteins primarily serve to aid in function; and those who show RNA to be functional within the ribosome. It has been suggested that both concepts are probably correct.

There are various points of evidence that seem to implicate proteins as the essential core of the peptidyl transferase (PT) center. Many antibiotic inhibitors of the PT reaction have been cross linked to large subunit ribosomal proteins. For example, chloramphenicol crosslinks to L2, L16, L27 and L6 (73). Similarly, puromycin and its derivatives were found to crosslink to L23, L18/L22 and L18 (64,110). This seems to suggest that these proteins and various others (90,100,101) are structural, and perhaps functional, components of the peptidyl transferase center. Other single protein omission reconstitution experiments and minimal set reconstitution experiments are consistent in implication of L2, L3, L4, L15 and L16 as primary nominees for their role in PT activity (85).
Cooperman's recent experiments (9) seem to ascribe protein L2 as an important player in PT activity. The strategy was to replace a highly conserved residue in L2 with a similar amino acid by site directed mutagenesis and observe the effects on PT activity. Histidine 229 was replaced with glutamine. The overall scheme was to simply characterize a mutant 50S subunit by reconstituting 23S rRNA, total protein 50-L2 and H229Q-L2. They found that 50S subunits containing the substitution were dramatically reduced in their capacity to catalyze a peptide bond as compared to their wild-type counterparts. Earlier Nag et al (63) had found that two monoclonal antibodies against L2 epitopes inhibited PT activity. Also, L2 binds to a portion of domain IV of 23S rRNA that is linked to the central loop of domain V(110), a region of the 23S rRNA almost certainly identified as part of the PT center.

To further delineate the environment of the PT center, Wower and Zimmerman used a tRNA, derivatized at its 3' end by photolabile probes, 2-azidoadenosine, 8-azidoadenosine, and 2,6-diazido-9-(β-D-ribofuranosyl)purine (112). When bound to the A site or the P site irradiated with near UV light, these derivatives mainly labeled protein L27. These photo crosslinks are very short in length (2-4 Å), which led them to conclude that L27 was very close to the 3' end of A and P site tRNAs and, by extrapolation, the peptidyl transferase center.

Another hard-to-ignore candidate for PT activity is protein L16, just by the sheer number of publications and the different kinds of experiments performed on it. L16, along with a group of four other proteins, is essential for reconstitution of peptide-bond forming activity
to ribosomal core particles (85). It has also been found that modification of the histidyl residue on L16 inactivates the PT center (1,95). L16, or a proteolytic fragment lacking nine amino acids at its N-terminus can restore activity to ribosomal particles (54) and, when L16 is missing from reconstituted 50S subunits, the particle is inactive for release factor-mediated peptidyl-tRNA hydrolysis (99). All of the above imply that L16 may play a catalytic role in the reactions of the PT center.

Other experiments have shown that ribosomes lacking L16 can form a peptide bond with puromycin, although they cannot with an aminoacyl-tRNA; suggesting that L16 is necessary for correct nestling of the acceptor stem of tRNA on the ribosome (53). Tate et al. demonstrated that the histidine of L16 affects the reconstitution and control of the active center, but is not essential for peptide bond formation and release factor-mediated peptidyl-tRNA hydrolysis (98). Nierhaus et al. have further confirmed this result by showing that L16 was merely a late assembly ribosomal protein and was as such not required for peptide bond formation (94). So, what could be the functional role of L16? It seems that although dispensable for peptide bond formation, L16 along with its elite partners L2, L3 and L4 (40,65) are somehow involved in maintaining an optimal structure at the catalytic core of the ribosome. These are still prime candidates for the elusive peptidyl transferase activity.

On the other hand, there have been recent experiments which strongly suggest that 23S rRNA alone is necessary for PT activity. Noller et al. have showed that 50S subunits of the hyperthermophilic bacterium *Thermus aquaticus* can be treated with proteinase K, sodium
dodecyl sulfate and phenol and still retain 80% of their activity in the fragment reaction (68). This strengthens the view that rRNA is functionally important. This however does not completely exclude the role of proteins in peptide bond formation on the ribosome. Whatever the case, once the peptide bond is formed between the two amino acids at the acceptor and donor sites, there is subsequent movement of the tRNAs; the deacylated tRNA moves from the P-site to the E-site (the exit site), while the tRNA carrying the nascent peptide translocates from the A-site to the P-site. This scheme, is perhaps one of the most poorly understood and maybe the most contentious in all of molecular biology.

**Translocation:**

Translocation concerns not only the peptidyl tRNA and the deacylated tRNA, but also the mRNA, which is moved by 3 nucleotides to expose the next codon in the A-site. This simple explanation, however does no justice to the complexity of the movement of the tRNAs and/or the ribosome. Over the last two decades, there have been 3 different models trying to explain the mechanistic details of translocation: a) the allosteric 3 site model, b) the hybrid states model and c) the recently expounded $\alpha$-$\epsilon$ model.

**1.3.1 The Allosteric 3 Site Model:**

The observation that three tRNAs could be simultaneously bound to the ribosome (81), lead to the postulation of the existence of a third functional site on the ribosome - the exit site or the E-site (82). The E-site seemed to be highly specific for deacylated tRNA (82). This was corroborated by others (25,42,49). Binding to the E-site involves codon-anticodon interaction
as determined by chase experiments (80). Chase experiments also showed the presence of two codon-anticodon interactions simultaneously, before and after translocation (80).

Arbitrarily starting at the pre-translocational state, i.e. with tRNAs at the A and P site, the allosteric model (figure 6) may be described as follows. The alignment of the two tRNAs at the A and P sites leads to peptide bond formation between the amino acids at the acceptor and donor sites. Once peptidyl transfer occurs, there is translocation of the two tRNAs in concert with EF-G-dependent GTP hydrolysis. The peptidyl tRNA is now at the P-site and the deacylated tRNA is at the E-site. Binding of the deacylated tRNA to the E-site somehow reduces the affinity of the A-site to charged tRNA and concomitantly maintains the ribosome with a high affinity E-site. An important effect of this reduction in A-site affinity is the accuracy of translation.

According to Nierhaus’ model, the rationale for this is as follows. The free energy for A-site binding has two components. The first one is the codon-anticodon interaction between the mRNA and the tRNA. The second component consists of the interaction between tRNA and the ribosome. It is this second component that is affected by a reduced A-site affinity. Among a pool of 60 or so tRNAs, the ribosome is able to almost always select the correct tRNA. This can happen only if there is a distinguishing factor at the A-site for cognate and non-cognate tRNAs. This factor is the reduced A-site affinity for non-cognate tRNAs. When a successful codon-anticodon interaction is established, there is a trigger which causes the low affinity A-site to now become a high affinity A-site for a cognate aminoacylated tRNA.
Figure 6.) The allosteric 3-site model of elongation as explained by Nierhaus et al. (82)
Concomitantly, there is decreased affinity for the deacylated tRNA at the E-site, following which there is a release of the deacylated tRNA from the ribosome. This allosteric bidirectionality in the affinity of the A and E sites for their respective tRNAs forms the basic tenet of the allosteric three site model.

Wintermeyer’s model challenges this stable E-site binding of tRNA. According to Wintermeyer, following EF-G binding to the pre-translocational state, the 3' terminus of the deacylated tRNA moves into the E-site, leaving the body of the tRNA firmly planted in the P-site. This E-site-tRNA interaction is believed to involve a Watson-Crick base pair between A76 of the tRNA and U2111 of the 23S rRNA (50). The residual tRNA binding at the P-site is now weakened and the deacylated tRNA wholly shifts into the E-site. Codon-anticodon interactions at the E-site are now greatly weakened and the tRNA leaves the ribosome. The ribosome is now ready for a new cycle of elongation.

1.3.2 The Hybrid States Model:

It is essential to describe the three sites on the ribosome as defined by Noller. His model is derived from a conceptually different approach, which focuses on tRNA-rRNA interactions. The operational sites on the ribosome have been redefined in terms of ribosomal RNA footprints. Using chemical probing techniques, Noller’s group has shown that characteristic sets of ribosomal RNA bases are protected when tRNA is bound to the ribosome (see table 1). Almost all the protected bases are phylogenetically conserved and have been implicated in ribosomal function. In their simplest experiment, they looked for footprints of P-site
bound tRNA immediately after peptidyl transfer using the puromycin reaction and compared those to the footprints before peptidyl transfer. Surprisingly, they found that the 23S rRNA P-site footprints disappeared after the puromycin reaction, while the 16S rRNA footprints persisted. Concomitantly, there was an appearance of E-site footprints on the 23S rRNA. They decided that they could best explain these results by a movement of the tRNA on the 50S subunit, resulting in an E-site footprint, but by staying at a constant site on the 30S subunit, effectively not changing the P-site footprint. They called this the P/E state (the first letter denotes the position of tRNA with respect to the 30S subunit and the second letter indicates the position of the tRNA on the 50S subunit). Additional experiments with N-Acetyl-tRNA-Phe and the ternary complex (57,58), showed the disappearance and appearance of protections which can be accounted for by a simple model of translocation (see figure 7). Starting with a tRNA at the P/P site, a modest, stepwise explanation of this would first entail tRNA being delivered to the ribosome as a ternary complex at the A/T site. The T-site is the location of the newly bound aminoacyl-tRNA when it is still held by EF-Tu (59). The 3’ end of the aminoacyl tRNA is now shielded from peptidyl transferase. After GTP hydrolysis by EF-Tu and release of EF-Tu-GDP, the aminoacyl tRNA moves to the A/A site. After or during the process of peptide bond formation, the two tRNAs move with respect to the 50S subunit, but remaining as such with respect to the 30S subunit. The deacylated tRNA is now in the P/E state while the peptidyl tRNA is in the A/P state. This implies that the nascent peptide itself remains stationary with respect to the ribosome. After GTP hydrolysis by EF-G, the two tRNAs are again realigned with respect to the 30S subunit, i.e. the deacylated tRNA is now at the E-site (there is no corresponding site on the 30S subunit
according to the hybrid states model) and the peptidyl tRNA is at the P/P state. The ribosome is now ready for a new cycle of elongation.
Figure 7.) The hybrid states model for elongation as propounded by Noller (59)
# Table 1

Protection of specific bases in E. coli 23S rRNAs by tRNA*  

<table>
<thead>
<tr>
<th></th>
<th>16S rRNA</th>
<th>23S rRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A site protections</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strong</td>
<td>G530, A1492</td>
<td>C2254, A2439, A2451,</td>
</tr>
<tr>
<td></td>
<td>A1493</td>
<td>G2553, U2555, A2602,</td>
</tr>
<tr>
<td>Weak</td>
<td>A1408, G1494</td>
<td>G1068, G1071, U2609</td>
</tr>
<tr>
<td><strong>P site protections</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strong</td>
<td>G693, A794, C795</td>
<td>G2252, G2253, A2439,</td>
</tr>
<tr>
<td></td>
<td>G926, G1401</td>
<td>A2451, U2506, U2584, U2585</td>
</tr>
<tr>
<td>Weak</td>
<td>A532, G1338, A1339</td>
<td>A1916, A1918, U1926,</td>
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<td>C2394</td>
</tr>
<tr>
<td>Weak</td>
<td>----</td>
<td>G2112, G2116</td>
</tr>
</tbody>
</table>

* adapted from (59)
1.3.3 The $\alpha$-$\varepsilon$ model (11):

This is a departure from the earlier allosteric three site model. According to this model, both tRNAs present on the ribosomes are transferred \textit{in toto} by two ribosomal domains to which both tRNAs maintain their points of contact before and after translocation. The contact patterns were those determined by cleavage of phosphorothioate residues by molecular iodine. This model (figure 8) requires the definition of two new sites; the $\alpha$ and the $\varepsilon$ sites. These are two movable domains on the ribosomes which interact with tRNA. The A, P and E "sites" are now termed A, P and E "positions". In addition to these two new sites, there exists a low affinity region for tRNA, called the decoding center, "$\delta$". This is an immovable domain on the ribosome which is physically different from the $\alpha$-$\varepsilon$ domains in the post-translocational state but coincides with the $\alpha$ site during the pre-translocational state.

 Arbitrarily starting at the post-translocational state, with a deacylated tRNA in the E$\varepsilon$ site and a peptidyl tRNA in the P$\alpha$ state, the A position is empty with the $\delta$ domain having a low affinity for an aminoacylated tRNA. According to this scheme, the occupation of the A position occurs in two steps. The first step is the decoding step, where the tRNA in the ternary complex, interacts with the $\delta$ domain. At this point there are three tRNAs bound to the ribosome. When the decoding process has been satisfactorily accomplished, the $\alpha$ and the $\varepsilon$ sites slide back from the P and E positions respectively to the A and P positions respectively. This causes the E position tRNA to be expelled from the ribosome, because the tRNA binding site (i.e. the $\varepsilon$ site) is now at the P position. This explains the reciprocal binding effect as explained by the allosteric three site model, only there is no allostery
Figure 8.) The $\alpha$-$\epsilon$ model of elongation; adapted from Dabrowski et al. (11)
involved. At this point the ribosome is at the pre-translocational state. At this point peptide bond formation takes place, after which GTP hydrolysis by EF-G causes the translocation of the two tRNAs along with their movable α and ε sites. The ribosome is now ready for a new cycle of elongation.

All three of the above models have their merits, but no one model has been universally accepted as the working model.

1.4 tRNA-rRNA interactions

It is apparent from the very outset that in order to understand the working of the ribosome, one has to be able to understand the interaction of the various ligands with which ribosome associates. Over the course of the last few decades, innovative and powerful techniques have been developed to study interactions of the ribosomal RNA with its ligands, especially tRNA. It is apparent that ribosomal RNA plays a pivotal role in the function of the ribosome. It follows intuitively that knowledge of the structure of the RNA will provide an insight into its function as well. To study this functional role, various physical methods have been used, all with a certain degree of success.

Crosslinking:

This technique involves photolabile chemical moieties that, when exposed to near UV light, form covalent bonds to nitrogens and carbons of both RNA and proteins. The strategy involves attaching these photoreactive molecules to specific sites on molecules that interact
with the ribosome (23,113). These modified ligands are then bound to the ribosome and irradiated with UV light. Crosslinks to proteins are then identified by two dimensional gel electrophoresis (112), while the exact site of crosslinking to RNA is determined by RNA sequencing (34).

Site Directed Mutagenesis:
In order to determine the role of one particular base in an RNA molecule or that of a single amino acid residue in a protein, there is no better alternative than site-specific mutagenesis. Using a single-stranded vector containing a portion of the appropriate gene, a mismatch oligonucleotide is hybridized and extended. The extension product is then cloned back into a plasmid vector containing either the rRNA operon or the gene for the protein. This plasmid can then be used to transform a suitable strain to generate ribosomes containing the mutant rRNA or in the case of a protein, reconstituted back into the ribosome (9,12).

cDNA probing:
Complementary DNA (cDNA) probing involves binding a short piece of DNA (typically hexamers) to highly conserved single stranded regions of rRNA in the ribosome. The specificity of probe binding is tested using RNase H assays. RNase H cleaves the RNA involved in the RNA-DNA heteroduplex. The cleaved RNA can then be assayed to determine binding specificity. The idea is to perturb function and to elucidate subtle intermolecular interactions, as seen by increase or decrease in ligand binding to the ribosome (33,36,70,71).
Chemical Modification:

Perhaps one of the more successful techniques to study rRNA-tRNA interactions, chemical modification involves the modification of the nitrogen of bases, specifically N1 of purines and N3 of pyrimidines, by different chemical probes. These modified bases are detected as premature termination sites in reverse transcriptase catalyzed primer extension assays, using the modified RNA as template and short DNA oligomers as primers. When tRNA is bound to the ribosome at various sites, there is a change in the accessibility of different bases to the chemical probes. The protection or enhancement of the modification is interpreted as either a direct or indirect interaction between the ribosome and tRNA (57-60).

Chemical Nucleases:

Although crosslinking and chemical modification have been successfully used in probing RNA structure and function, both have limitations. Efficient crosslinking depends on favorable chemical and stereochemical circumstances, while interpretation of footprinting results as a result of chemical modification is subject to the ambiguity of whether protection of rRNA by tRNA is direct or indirect. Enzymatic nucleases have played a key role in elucidating the secondary structure of RNA. Sequence specific cleavage of RNA by ribonucleases involves the scission of the phosphodiester bond between adjacent nucleotides via an in-line S_N2 mechanism giving the cleavage products; 3' and 5' phosphates and 5-methylene-2,3-dione (87).

Chemical nucleases are a new class of coordination complexes that essentially act by
accomplishing strand scission of nucleic acids in the presence of oxygen or reducing agents (87). These include the tetrahedral 1,10-phenanthroline-cuprous ion, ferrous-EDTA and metalloporphyrin derivatives (15,28,87). Recently, peroxonitrous acid has been used by Ehresmann’s group in France to study the structure of RNA (24).

Fe-EDTA was first used by Dervan and his colleagues for the controlled scission of DNA. Methidiumpropyl-EDTA, a simple bifunctional molecule, contains the DNA intercalator methidium covalently bound by a short hydrocarbon tether to the metal chelator EDTA. In the presence of ferrous ion and oxygen this reagent efficiently generates single strand breaks and some double strand breaks in double helical DNA (31). It was observed that superoxide dismutase inhibited the MPE-Fe\(^{2+}\) cleavage of DNA, indicating the importance of \(O_2^-\). Catalase, an enzyme that converts hydrogen peroxide into water and oxygen also inhibits the MPE-Fe\(^{2+}\) DNA cleavage reaction, suggesting the apparent importance of hydrogen peroxide as an important intermediate in strand scission. These led Dervan and colleagues to suggest that the strand scission proceeded via free oxygen radicals or by iron-bound oxygen species (31). The free oxygen radicals were assumed to be generated by hydrogen peroxide in a known Fenton reaction (31). Tullius and co-workers have used untethered Fe2+-EDTA as a general cleavage reagent directed towards the nucleic acid backbone, to probe for altered DNA structure of kinetoplast DNA (103). They have also utilized the same approach to localize protein-DNA contacts in the lambda repressor and Cro protein interaction with bacteriophage lambda DNA (5). Cech and co-workers first described the use of untethered Fe2+-EDTA to probe for higher order structure of an RNA molecule (46). They used free
Fe²⁺-EDTA to investigate the structure of the *Tetrahymena* ribozyme. Cleavage patterns generated by the nuclease activity of Fe-EDTA greatly enabled them to impose major constraints for modeling the three dimensional structure of the *Tetrahymena* ribozyme. More recently, a tethered version of EDTA, named BABE [1-(p-bromoacetamidobenzyl)-EDTA] (78) has been used by Noller to map the neighborhood of the acceptor end of tRNA on the ribosome (38). Cleavage of the ribosomal RNA was induced by OH⁻ radicals generated by the complexed Fe²⁺ by initiation of the Fenton reaction (38).

Another versatile chemical nuclease is the tetrahedral phenanthroline-copper (OP-Cu) complex (figure 9). It was the first chemical nuclease discovered that cleaved DNA (88). It binds DNA in the minor groove where it is oxidized to form a copper-oxo species that may be responsible for oxidation of the deoxyribose moiety (102). The chemistry of scission has been extensively studied and the reaction pathway in figure 12 seems to account for 70-90% of the reaction depending on the primary sequence. Since the above reaction mechanism involves initial attack on the C-1 H of the deoxyribose that is nooded in the floor of the minor groove of double stranded DNA, the phenanthroline-copper complex has been surmised to bind to this general domain of DNA. Further evidence for this comes from the fact that single stranded DNA is not an efficient substrate for OP-Cu DNA scission (55). B-DNA is preferred over A-DNA and Z-DNA. The secondary structure preference of OP-Cu indicates that, perhaps the minor groove provides a binding site for the OP-Cu complex. Since its discovery, OP-Cu has been used in a wide variety of ways.
Figure 9.) Iodoacetamido, 1-10, orthoPhenanthroline-$\text{Cu}^{2+}$ complex.
Figure 10.) Proposed reaction pathway for the cleavage of RNA by phenanthroline-Cu (87)
Sigman first used the nucleolytic activity of OP-Cu to investigate the conformation of the *lac* operon (91). The *E.coli* Trp repressor has been converted into an operator specific nuclease by alkylating cysteine-49 with 5-iodoacetamido-1,10-phenanthroline. The patterns of scission obtained from this study have helped confirm the orientation of the repressor relative to the operator as predicted by X-ray studies (96). This provides a novel way to analyze high molecular weight DNAs.

Conformation and ligand-binding properties of RNAs are central to their function (67). Fe-EDTA and other chemical probes such as dimethyl sulfate and diethyl pyrocarbonate have been used to study structure-function aspects of RNA (67). Fe-EDTA is ribose-directed in its reactivity and has a potential of cleaving all nucleotides. It generates a diffusible hydroxyl radical which does not distinguish between riboses linked to bases within loops or double stranded stems. As a result, it cannot be used as a probe for secondary structure. However, an RNA's reactivity with Fe-EDTA can be used to distinguish nucleotides whose riboses are solvent exposed from those that are solvent inaccessible (8). In contrast, phenanthroline-copper with hydrogen peroxide as a co-reactant does exhibit secondary structure specificity (87).

Developments in DNA footprinting and in mapping sites on RNA molecules exposed to chemical probes, suggested that perhaps a similar procedure could be used to footprint RNA. Nierlich used OP-Cu as a footprinting agent to monitor the binding of ribosomes to *E.coli*
lacZ mRNA (62). In these studies, the reactivity of the Shine-Dalgarno sequence, located in the single stranded bulge of the RNA, was protected upon association with ribosome. In another study, Murakawa et al., observed that scission of RNA by Op-Cu displayed a specificity to single stranded loop regions of lac mRNA relative to the double stranded A-helix found in RNAs (62). However, it is important to note that not all single-stranded regions are cleaved nor are all reactive sites in single-stranded regions (87). More recently phenanthroline has been covalently attached to tRNA. The tRNA-phenanthroline conjugate has been used to map the tRNA neighborhood of the ribosome at the P/E site (4). The mechanism of RNA cleavage by phenanthroline, however, is not well understood.

1.5 Interaction of the 3'end of tRNA with the ribosome:

It has been shown that certain regions of the ribosomal RNA are involved in interactions of tRNA at the ribosomal A, P and E sites (56,58,60). Chemical protection studies have shown that the 16S rRNA interacts with features of the anticodon stem-loop structure alone (56). Similarly, protection of the 23S rRNA by tRNA is progressively abolished by the deletion of the aminoacyl group, 3' adenylate and the 3'cytidynyladenylate (58,59). These results suggest that the -CCA end of tRNA and the aminoacyl moiety are involved in interactions between tRNA and 23S rRNA. It has earlier been shown that protection of bases of 23S rRNA from chemical probing of P-site bound Ac-Phe-tRNA\(^{\text{phe}}\) is nearly indistinguishable from its RNase T\(_1\) product, CACCA-Ac-Phe [bound to the ribosome in the presence of sparsomycin] (58). For example, bases G2252, G2253, A2439, A2451, U2506, U2584, U2585 and A2602, are all protected by P site bound Ac-Phe-tRNA\(^{\text{phe}}\) and the 3'
terminal nuclease digested fragment. The only base that is protected by the intact tRNA and not by the fragment is G2505. This seems to indicate that nearly all, if not all of the interactions between tRNA and the 23S rRNA involves the 3'-CCA terminus of tRNA.

Wintermeyer's group has shown that deacylated tRNA, when in the E-site interacts with 23S rRNA (50). They believe that this interaction involves a Watson-Crick base pair between A76 of tRNA and U2111 of the 23S rRNA (50). In a related crosslinking experiment, Zimmerman's group has shown that tRNA containing a photoreactive residue (2-azidoadenosine) at position 76, crosslinked to G1945 in domain IV of the 23S rRNA (112). Domain IV has previously been shown, by UV-crosslinking experiments, to be tightly linked to domain V of the 23S rRNA (92). All of the Noller protections of 23S rRNA by the 3'-terminal fragment of tRNA and the intact tRNA are located in domain V of the 23S rRNA. Domain V has been implicated as an integral part of the peptidyl transferase center (14,27,108) and is also the site of action of several antibiotics (74). All of the known mutations conferring resistance to chloramphenicol and anisomycin are found in the central loop of domain V (74,75). One of the fragment-protected bases in Noller's experiment, A2451, is also protected by chloramphenicol and carbomycin (39). These data, taken together, seem to suggest that a) peptidyl transferase activity is perhaps a function of domain V of the 23S rRNA and b) the 3' end of tRNA is involved in the interaction between tRNA and 23S rRNA. To further test this hypothesis, interaction of the conserved 3' terminus of tRNA with 23S rRNA in the P site of the ribosome was studied using in vitro genetics (84). Mutations at G2252 been shown to have a dominant lethal phenotype (47), while those at
G2253 have been shown to have a recessive slow growth phenotype. Ribosomes containing mutations at these two positions have been shown to have impaired peptidyl transferase activity (47). When mutations were introduced at positions 2252 of the 23S rRNA, it was found that the oligonucleotide fragment binding was observed only when position 2252 of 23S rRNA and the position that corresponded to base 74 on an intact tRNA were complementary to each other (84). Mutations that corresponded to position 75 of tRNA failed to bind to ribosomes that contained mutations at 2253. These results provide evidence that binding of the CCA oligonucleotide to the P site requires a Watson-Crick base pair between G2252 of 23S rRNA and C74 of tRNA.

The role of this interaction is to probably position the acceptor end of tRNA in the peptidyl transferase site. No such effect was observed between G2253 and C75 of tRNA. This cannot be explained because C75 of tRNA is required for protection of G2253 from kethoxal modification (58,60). However, it is possible that C75 forms a hydrogen bond with the sugar moiety of the nucleotide. This, however, remains to be tested. Although these results suggest the involvement of both Cs of the universally conserved -CCA terminus of tRNA, the other conserved nucleotide A76 of tRNA has yet to be implicated in base-pairing of any kind. The protections that arise as a result of A76 are U2506 and U2585. It is interesting to note that both the protected bases are capable of Watson-Crick base pairing.

In an attempt to further delineate the environment of the acceptor end of tRNA on the ribosome, we decided to modify the 3' end of tRNA by attaching phenanthroline via a thiol
group. The phenanthroline conjugated tRNA would then be used to cleave the ribosomal RNA by initiating a cleavage reaction. As will become evident, it is not easy to modify successfully the 3' end of tRNA.
2.0 Materials and Methods

2.1 Isolation of ribosomes:

Isolation of ribosomes was essentially a modification of the protocol developed by Tam et al (67).

E. coli MRE 600 cells were grown to their early log phase in a Microferm Fermentor. The cells were then concentrated using a Millipore Pellicon Cassette system. Subsequently, the bacteria were pelleted at 4500 rpm in a Sorvall GSA-1799 rotor. The bacteria were then stored at -80 °C until required. At a desired point in the time-space continuum, the cells were thawed and burst upon to release their contents. This was achieved by grinding the cells in a mortar containing an abrasive substance, in this case, alumina. The grinding was vigorous and lasted for an hour, with alumina being added whenever necessary. After grinding, enough 1X buffer (10 mM Tris-Cl [pH 7.5], 0.5 M NH₄Cl, 15 mM MgCl₂) was added to insure ease in pouring of the grindate into 30 mL polycarbonate clinical centrifuge tubes. Cell debris, intact cells and alumina were removed from the preparation by centrifugation in an SS-34 rotor at 10,000 rpm for 10 minutes. The supernatant was poured into high-speed polycarbonate tubes and subject to a low speed spin at 28,000 rpm. The supernatant was collected and crude ribosomes were pelleted at 60,000 rpm for 2.5 hours. Tight-couple 70S ribosomal particles were obtained by resuspending the crude pellet in 1X TC70 buffer (10mM Tris.Cl [pH 7.0], 60mM KCl, 6 mM MgCl₂). Pure TC-70s were obtained by repeating the low and high speed spins. Final purification was attained by spinning the ribosomes through a 10-38% sucrose gradient in 1X TC70 buffer. The ribosomes were collected by taking absorbance readings at 260 nm. The appropriate fractions were pooled.
and centrifuged at 60,000 rpm for 12 hours. The ribosome pellets were resuspended in 0.5 mL of 1X TC70 buffer and dialyzed against the same buffer for 12 hours. The ribosomes were aliquoted into eppendorf tubes and stored in a -80 °C freezer until required for further experimentation. To test the quality of ribosomes, the samples were analyzed by polyacrylamide gel electrophoresis, analytical centrifugation and tRNA binding assays.

2.2 tRNA preparation:

2.2.1 Modification of tRNA at the 5' end:

760 picomoles of yeast tRNA^phe were reacted with 75 units of shrimp alkaline phosphatase (SAP) at 37 °C for 3 hours in 20 mM Tris.Cl [pH 8.0] and 10 mM MgCl₂. The enzyme was then inactivated at 65 °C for 15 minutes. tRNA was purified by phenol and chloroform extractions and concentrated by ethanol precipitation. The dephosphorylated tRNA was then resuspended in water and 50 mM Tris.Cl [pH 7.6], 10 mM MgCl₂, and 10 mM 2-mercaptoethanol. 100 units of T4 polynucleotide kinase (PNK) and 1 μL of 10 mM γ-S-ATP were added to the reaction. The reaction was incubated overnight at 37 °C. The enzyme was inactivated at 65 °C for 5 minutes. tRNA was then purified by phenol and chloroform extractions and concentrated by ethanol precipitation.

2.2.2 3' Modification of tRNA:

Modification of the 3' Adenine:

2-thioAdenosine was purchased from Sigma and pyrophosphoryl chloride was procured from Aldrich Chemicals. 16.3 milligrams of nucleoside was added to 67 μL of
pyrophosphoryl chloride. The reaction was vortexed for 4.5 hours in a microfuge tube at -10 °C. The reaction was quenched by the rapid addition of ice, quickly followed by the addition of 0.5M TEAB [pH 8.0]. This was then evaporated to dryness in a vacuum concentrator. The nucleotide (assumedly a mixture of bisphosphates, monophosphates and higher phosphorylated compounds) was then dissolved in HPLC grade methanol and evaporated to dryness several times in a rotary evaporator. The dried nucleotide was then resuspended in ethanol and the ethanol was then evaporated again to dryness. The pure nucleotide was then resuspended in 0.05M TEAB [pH 8.0]. This was then loaded on to a DEAE-cellulose column (DE-52) and washed with 0.05M TEAB [pH 8.0]. The bisphosphorylated nucleotide was then eluted by running a gradient of 0.05M - 0.4M TEAB [pH8.0] for approximately 10 hours.

2.2.3 Ligation of 2-thioAdenosine bisphosphate to 3' end of tRNA:

Yeast tRNA<sup>phe</sup> was oxidized at the 3' end with sodium periodate. This was done to break the 2'-3' C-C bond to generate a dialdehyde. This was achieved by reacting 1000 picomoles of tRNA in a volume of 75 μL containing 83.3 mM sodium periodate. The reaction was 16.3 mM in NaOAc [pH 4.5], 3.3 mM in MgCl<sub>2</sub> and 3.3 mM in NaCl. The reaction was incubated in the dark and on ice for 1 hour, following which the reaction was quenched by the addition of 1.39μL of 1.793M ethylene glycol. The excess salts were then removed from the reaction by passing it through a Biospin-30 column. After ethanol precipitation of desalted nucleic acids, the dry pellet was resuspended in 0.3M aniline [pH 5.0] and incubated
in a reducing environment (this was done by maintaining a positive pressure of nitrogen) for 2 hours. This effectively eliminates the nucleotide and the sugar moieties of the terminal nucleotide. The tRNA lacking the 3' -A was then purified by chloroform extraction and
2.2.5 Thiophosphorylation of 3' end of E.coli tRNA<sup>pro</sup>:

The overall strategy of this experiment was to generate a 5' tRNA<sup>pro</sup> fragment consisting of the first 58 bases and annealing this to the 3' portion of the tRNA<sup>pro</sup> containing a phenanthroline at the 3' terminus. The 5' fragment was generated by in vitro transcription of a pUC19 plasmid containing the gene for tRNA<sup>pro</sup> with a TaqI restriction site (51). The 58mer was purified by running the transcription reaction on a 10% denaturing polyacrylamide gel. The integrity of the transcription product was verified by enzymatic sequencing of the RNA. The 3' fragment, from base 59 through base 76, was synthesized chemically. An extra nucleotide was added at the very end, i.e. there were a total of 19 nucleotides consisting the 3' fragment. The internucleotidic phosphate linkage between bases 76 and 77 was designed to be a thiophosphate. The 3' fragment containing the internal thiophosphate was periodate oxidized and treated with aniline as described before. This was done in order to expose the thiophosphate at the 3' end.

2.2.6 Modification of tRNA<sup>pro</sup> at position 67:

The approach in this experiment as essentially the same as that described in the previous section. The only difference was the position of attachment of phenanthroline. The 5' fragment was generated as before. The 3' portion of the tRNA was chemically synthesized with position 67 carrying a thiouridine in place of uridine. The two fragments were annealed together in 10mM MgCl<sub>2</sub> and 50mM HEPES [pH 8.0] at 65 °C for 3 minutes, after which they were slow cooled to room temperature. The annealed product was then electrophoresed on a non-denaturing gel consisting of a 5% stacking gel and 12% resolving gel.
2.2.7 OP-labeling reaction:

tRNA-AMBH (section 2.2.4) was incubated for 15 hours at room temperature and in the dark, in a reaction volume of 100 µL. The reaction contained 1500 picomoles of tRNA-AMBH in 1mM DTT, 100mM TEAA [pH 7.0] and 10mM IOP. After the incubation, the tRNA-AMBH-OP was purified by extracting five times with butanol:chloroform in a ratio of 1:1. The nucleic acid-phenanthroline complex was then ethanol precipitated and centrifuged at 13,000 rpm for half an hour. The supernatant was carefully aspirated and dried down while the pellet, which contained the free phenanthroline was discarded. The "mock" tRNA was prepared in essentially the same way, the only difference being that the tRNA was not coupled to AMBH.

Alternatively, tRNA-AMBH was reacted with 1mM IOP in a reaction volume of 50 µL. The reaction contained 20mM Tris.Cl [pH6.8]. After incubating for 2 hours at 37 °C, the reaction was ethanol precipitated with 0.3M NaOAc [pH 6.5]. The resulting pellet was dried and resuspended in water.

2.2.8 Cleavage Reaction:

Ribosomes were taken out of the -70 °C freezer and diluted in 1X TC70 buffer to µM, after which they were incubated for half an hour, at 37 °C. While the ribosomes were being "run off", appropriate ligands were aliquoted as follows: a) the cleavage reaction contained 22.5 picomoles of tRNA-AMBH-OP, 0.5 µL of 6.25 µg/µL poly U, 40mM Tris.Cl [pH 7.5], 6mM MgCl₂, 60mM KCl and 0.1mM CuSO₄. The "mock" reaction contained tRNA from the mock
labeling reaction (section 2.2.7) and the same salt conditions. The competition reaction contained tRNA-AMBH-OP and yeast tRNA^{phe} at a ratio of 1:4 respectively. The salt conditions in the competition reaction were identical to the mock and cleavage reactions. After the ribosomes were "run off", exactly 1.5\( \mu \text{L}\) of ribosomes were added to each tube, thereby making the ratio of ligands to ribosomes 1.5:1. The tubes were incubated for half an hour at 37 °C to allow binding of the various ligands to the ribosome. At the end of the incubation, 1\( \mu \text{L}\) of 40mM MPA was added to each tube. This initiates cleavage. The cleavage reaction was allowed to proceed for 2 hours at 37 °C, at the end of which 5 \( \mu \text{L}\) of 5mM neocuproine was added to quench the reaction. The neocuproine chelates all of the copper thus preventing the continuation of the cleavage reaction. At this point, all the tubes are equilibrated with various ligands so that each reaction now contains exactly the same amount of each kind of tRNA and mRNA used. The reaction is then ethanol precipitated with two volumes of ethanol and incubated on ice for 30 minutes. After centrifugation at 13,000 rpm for half an hour and drying the samples, the nucleic acids were purified by phenol extracting five times with acid-phenol and chloroform extracting two times. The nucleic acids were then recovered by ethanol precipitation and resuspended after drying to a final concentration of 0.2\( \mu \text{M}\).

2.2.9 Primer extension analysis of cleavage products:

The template for primer extension by avian myeloblastoid viral reverse transcriptase was annealed to a 17mer primer which was designed essentially according to Moazed and Noller (58). The primer is complementary to a certain region of the 23S rRNA. The primer is designated as the first base incorporated in the extension product; for example, primer #2890
indicates that the first base incorporated in the extension reaction is the base complementary to base #2890 of the 23S rRNA. The first step of the primer extension reaction was the annealing reaction in which 2μL of a primer solution (the primer solution is a diluted solution made from a primer stock of 1μg/50 μL) was annealed to 2.5μL of template. This was done by heating the primer-template solution for two minutes at 90 °C and slow-cooling to 45 °C. At this point, the extension reaction was started by adding reverse transcriptase, α-32 TTP and deoxynucleotides -TTP. The reaction was incubated at 40 °C for half an hour, at the end of which incomplete extension products were chased by adding an excess of dideoxynucleotides at a concentration of 67 μM. The reaction was then precipitated by adding 66% ethanol, 100mM NaOAc [pH 6.5] and 5mM EDTA. The precipitated products were then centrifuged for 30 minutes at 13,000 rpm and dried in vacuum. The nucleic acid was then resuspended in 10 μL of tracking dye and run on a 6% denaturing polyacrylamide sequencing gel for 3 hours at 55 watts. The gel was then peeled, dried and exposed to an X-ray film for autoradiography.
3.0 Results:

3.1 5' thiophosphorylation of Yeast tRNA\textsuperscript{phe}:

The polynucleotide kinase catalyzed transfer of a thiophosphate from ATP-\(\gamma\)-S to the dephosphorylated 5' end of yeast tRNA\textsuperscript{phe} was analyzed on a denaturing polyacrylamide gel containing APM. APM retards nucleic acids containing free sulfhydryls relative to non-thiolated nucleic acids (37). APM gels indicated that the kinase reaction did not proceed efficiently, if at all.

3.2 3' modification of tRNA:

3.2.1 Ligation of p-^A-p to 3' end of aniline treated tRNA:

Reaction of 2-thioadenosine with pyrophosphoryl chloride yielded a mixture of mono, bis and higher phosphorylated nucleotides. This mixture was separated on a DEAE-cellulose column. \(A_{260}\) readings of the eluant fractions were taken. Two peaks were detected - a) from fractions 13-25 and b) from fractions 67-75. The two different eluant peaks were pooled, dried down and resuspended in water. Analysis of the products was carried out by thin layer chromatography (TLC) on a silica-gel TLC plate. The solvent contained n-butanol:acetone: acetic acid: 5% ammonium hydroxide: water at a ratio of 45:15:10:10:20. From the TLC migration pattern, it was diagnosed that the first peak was a monophosphorylated product and the second, later peak was the required bis-phosphorylated adenosine. A bisphosphorylated adenosine was the desired product so that ligation of this nucleotide would prevent concatenated ligation products. tRNA was periodate oxidized and treated with aniline to remove the 3' base and sugar moieties (72). This occurs through a process of beta
elimination in which the primary amine conducts a nucleophilic attack on the ribose sugar that has been oxidized to a dialdehyde by periodic acid generated by sodium periodate (72). The attack leads to the degradation of the ribose sugar generating a free base (in this case, the terminal adenine of tRNA), ribose degradation products and the remainder of the tRNA with a 3' phosphate. The 3' phosphate was removed by treatment with shrimp alkaline phosphatase (72). Whether aniline treated tRNA was susceptible to a ligase reaction or not, was tested by ligating [5'-P³²]pCp to the above tRNA. It was found that [5'-P³²]pCp was indeed readily ligated to tRNA, as detected by an autoradiogram. The ligation of p-⁵A-p to aniline treated tRNA was analyzed on a denaturing polyacrylamide gel containing APM. The positive control used for this experiment was tRNA that was labeled at the 5' end by ATP-γ-P³². It was observed that p-⁵A-p was not a good substrate for the ligase reaction (figure 11).

3.2.2 tRNA-AMBH-OP:

Yeast tRNA was periodate oxidized to generate a dialdehyde at the 2' and 3' carbons. The dialdehyde was then reacted with 2-acetamido, 4-mercaptobutyric acid hydrazide. This was done by a simple coupling reaction in which the hydrazide readily reacts with the aldehyde to form a morpholine derivative of the tRNA. About 10% of the periodate oxidized tRNA coupled efficiently with AMBH to yield the tRNA-AMBH complex (figure 12). The tRNA-AMBH complex was then conjugated to phenanthroline via an acetamido tether. The tRNA-AMBH-OP complex was then used as a ligand for the
Figure 11.) Ligation of p-$^5$Ap to the 3' end of tRNA-A.
lane 1) p-$^5$Ap ligated to tRNA-A
lane 2) control: 5' end labeled tRNA
Figure 12.) Denaturing polyacrylamide gel containing APM.

Lane 1) Control: native tRNA
Lanes 2 and 3) tRNA-AMBH
Lane 4) tRNA-AMBH-OP
cleavage reaction in which the ligand was bound to the ribosomes at a ratio of 1.5:1 (tRNA-AMBH-OP: ribosomes). Primer extension analysis of the cleavage reactions showed that there were cleavage events in domain V of the 23S rRNA. The nucleotides corresponding to this region were 2058-2064 (figure 13), 2583-2587 and 2554-2555 (figure 14). Cleavage events were also detected in the 100 region of the 23S rRNA, corresponding to nucleotides 64-66 and 90-93 (figure 15). However, cleavages were seen in the control lanes as well, which led to the conclusion that perhaps these cleavage events were a result of free phenanthroline that “leaked” through the purification procedure. To confirm this, tRNA-AMBH-OP was purified by ethanol precipitation (this supposedly removes excess phenanthroline by selectively precipitating tRNA-AMBH-OP alone). The purified tRNA-AMBH-OP was then used as a ligand for cleavage reactions. Primer extension analysis of the cleavage products yielded the same results.

3.2.3 Modification of tRNA at the 3’ end by thiophosphorylation:

In vitro transcription of the TaqI digested pUC19 plasmid (a kind gift of Dr. Musier-Forsyth) yielded a fragment of tRNApro consisting of nucleotides 2-58. This was a ΔC1 mutant which did not contain the first nucleotide of tRNApro (which happens to be a C). The requirement of a G as the first base to be incorporated by T7 RNA polymerase in a transcript, necessitated removal of the first nucleotide. The ΔC1 mutant, when annealed to its complementary 3′ portion was demonstrated to form a compact tertiary structure and to be fully functional in aminoacylation assays (51). Enzymatic sequencing of the transcript showed that the transcript was indeed 57 nucleotides long (data not shown).
Figure 13.) 2058-2064 clips. Primer Extension analyses of cleavage events emanating from tRNA-AMBH-OP. Lanes G and A: Sequencing lanes that utilized ddCTP and ddTTP. Other lanes are extension products of 23S rRNA from reaction of TC70s with 1) unmodified tRNA, 2) tRNA-AMBH-OP, 3) tRNA-AMBH-NOP 4) tRNA-AMBH-OP in the presence of poly U, 5) tRNA-AMBH-OP in the presence of 5 fold excess of unmodified tRNA and 6) free OP and free tRNA, i.e. OP that was untethered to tRNA. All concentrations and conditions are as explained in the Materials and Methods sections 2.2.8 and 2.2.9. Arrows indicate regions where cleavage occurred.
Figure 14.) 2583-2587 and 2554-2555 clips. Primer Extension analyses of cleavage events emanating from tRNA-AMBH-OP. Lanes G and A: Sequencing lanes that utilized ddCTP and ddTTP. Other lanes are extension products of 23S rRNA from reaction of TC70s with 1) unmodified tRNA, 2) tRNA-AMBH-OP, 3) tRNA-AMBH-NOP 4) tRNA-AMBH-OP in the presence of poly U, 5) tRNA-AMBH-OP in the presence of 5 fold excess of unmodified tRNA and 6) free OP and free tRNA, i.e. OP that was untethered to tRNA. All concentrations and conditions are as explained in the Materials and Methods sections 2.2.8 and 2.2.9. Arrows indicate regions where cleavage occurred.
Figure 15.) 100 pseudoknot clips. Primer Extension analyses of cleavage events emanating from tRNA-AMBH-OP. Lanes G and A: Sequencing lanes that utilized ddCTP and ddTTP. Other lanes are extension products of 23S rRNA from reaction of TC70s with 1) unmodified tRNA, 2) tRNA-AMBH-OP, 3) tRNA-AMBH-NOP 4) tRNA-AMBH-OP in the presence of poly U, 5) tRNA-AMBH-OP in the presence of 5 fold excess of unmodified tRNA and 6) free OP and free tRNA, i.e. OP that was untethered to tRNA. All concentrations and conditions are as explained in the Materials and Methods sections 2.2.8 and 2.2.9. Arrows indicate regions where cleavage occurred.
The 3' portion of the tRNA was chemically synthesized and was designed to carry a thiophosphate between nucleotides 76 and 77. The 3' fragment was periodate oxidized to break the 2'-3' C-C bond of the terminal ribose; following which it was aniline treated to remove the base and sugar degradation products. This was done in order to expose the thiophosphate for subsequent OP-attachment. When electrophoresed on a denaturing, APM-containing polyacrylamide gel, the fragment was not retarded relative to the control fragment (which was not periodate oxidized nor aniline treated). This indicated that there was no available thiol for conjugated of OP.

3.2.4 Modification of tRNA at position 67:

Modification of E.coli tRNA* at position 67 entailed a protocol similar to section 3.2.3, with the difference being the position of modification. 5' and 3' fragments were synthesized essentially as above. The 3' fragment contained 4-thiouridine at position 67 instead of uridine and did not carry a thiophosphate at the 3' end. The fragments were annealed together in 50mM HEPES [pH 8.0] and 10mM MgCl₂. The product of the annealing reaction was run on a 12% non-denaturing polyacrylamide gel with a 5% stacking gel. Upon staining, it was observed that there were two distinct bands corresponding to the 5' and 3' fragments, which were run as controls in adjacent lanes.
4.0 Discussion

The basic goal of this study was to map the interaction of the 3' end of tRNA with the ribosome. Five different approaches were taken toward this end. The first method was to try and attach a phenanthroline to the 5' end of tRNA via a thiophosphate. This proved unsuccessful due to what seemed to be a low efficiency in the kinase reaction in which ATP-γ-S was the donor of the thiophosphate. The second approach was to ligate 2-thioadenosine-bisphosphate to the 3' end of tRNA lacking the terminal adenosine nucleotide. This too failed, due to what we think was an inefficient ligation procedure. The next strategy was to attach a tether (AMBH) to the 3' end of tRNA and conjugate phenanthroline to the sulfhydryl group on the tether. Despite inefficient attachment of IOP to tRNA-AMBH, cleavage data were generated. Cleavage events occurred at the peptidyl transferase center and the 100 pseudoknot region of the 23S rRNA. However, these data were considered ambiguous due to spurious cleavage events occurring in control reactions as well. A promising approach to tackling his problem was to generate a tRNA with an intact tertiary structure by annealing two fragments of E coli tRNA pro. One of the fragments (the 3' fragment) contained a thiophosphate between the two terminal bases. The thiophosphate had to be exposed in order to be attached to phenanthroline. Periodate oxidation of the terminal sugar moiety, followed by aniline treatment revealed the absence of a sulfhydryl group. The fifth approach was essentially the same as the previous method, the only difference being in the position of the thiol group. The sulfhydryl was introduced in the form of a 4-thiouridine in the 3' fragment, corresponding to position 67 of the wild-type tRNA pro. We failed to detect annealing of the
two fragments (data not shown) as seen in a non-denaturing polyacrylamide gel.

In the past, there have been several studies focusing on the acceptor stem of tRNA and its position on the ribosome. Most have been able to identify particular bases on the ribosomal RNA that interact with specific bases on the tRNA. For example, as described earlier, bases G2252, G2253, A2439, A2451, U2506, U2584, U2585 and A2602 are all protected by P site bound Ac-Phe-tRNA^{phe} and its RNase T_{1} product, CACCA-Ac-Phe [bound to the ribosome in the presence of sparsomycin] (58). Wintermeyer's group has shown that deacylated tRNA, when in the E site interacts with 23S rRNA. This interaction involves a Watson-Crick base pair between U2111 of domain V of the 23S ribosomal and A76 of tRNA (50). In a complementary study Zimmerman's group has shown that A76 of yeast tRNA^{phe} forms a crosslink with G1945 in domain IV of the 23S rRNA (112). Significant as these results are, what is needed is a more comprehensive mapping of the neighborhood of the 3' terminus of the tRNA. This is important because very little is still known about the players in the transpeptidation reaction that the ribosome so efficiently catalyzes. Do the the proteins play a key role or do they just form a scaffold for the RNA to do the catalysis? The 3' end of tRNA, especially A76, needs to be mapped on the ribosome to determine which nuclotides are closest to the 3' end and by extrapolation which nucleotides are important for peptide bond formation. When this is accomplished, more insights can be gained about the peptidyl transferase activity.

To this end, Noller's group attached a cleavage agent, BABE, to the 5' end of tRNA^{phe} and mapped its environment on the ribosome (38). They found that in the A site, cleavages were
found in the region 2555-2573: around bases previously shown to be protected by A site tRNA and in the α-sarcin region. P site cleavages were localized to the 2250 loop. G2252, which is found in this loop, forms a Watson-Crick base pair with C74 of tRNA (84). E site cleavages occurred in the 2390-2440 region. Unexpectedly, no cleavages were detected in the central loop of domain V of 23 S rRNA, which is the putative peptidyl transferase center.

We thought an ideal complement to this study would be to map the 3' end of tRNA on the ribosome. Although Zimmerman found a single site on the 23S rRNA that crosslinked to A76 of a P site bound tRNA, this could hardly suffice to provide a clear picture of all the components involved in the interaction. What was needed was a study designed to analyze more than a single nucleotide. Previous studies with phenanthroline-copper seemed to indicate that it would be an ideal tool for the above (91, 62). This study was an ambitious one because little was known about the position of the 3' end of tRNA on the ribosome and very few studies had been undertaken in trying to modify the 3' adenine of tRNA. What follows is a discussion of the various modification strategies and they may have failed.

We first decided to modify the 5' end of tRNA for the simple reason that it seemed easier to manipulate. The fact that the 3' end was universally conserved, served as a deterrent for modification. The 5' end of tRNA was dephosphorylated with SAP and was subsequently subject to phosphorylation with a thiophosphate. The thiophosphorylation of yeast tRNAphe was a highly inefficient process. The inefficient phosphorylation may be accounted for by poor substrate recognition by the enzyme polynucleotide kinase. Possibly, polynucleotide kinase has a high $K_M$ for ATP-γ-S and the amount of ATP-γ-S used was insufficient.
Another possible explanation for low product yield is the length of the incubation time of the reaction. Igloi demonstrated that, to get a reasonable yield of 5'-thiophosphorylated tRNA, the PNK reaction had to be incubated at 37 °C for 2 days (37). When the same conditions were used in our lab, it was found that the tRNA was greatly degraded, showing that the process, in our hands, was not very efficient. Whatever the case, 5'-thiophosphorylation by polynucleotide kinase was not efficacious. In hindsight, perhaps the best way to have introduced a thiophosphate at the 5' terminus was to transcribe the tRNA gene in the presence of a five-fold molar excess of 5'-guanosine-α-phosphorothioate over each NTP (38); so that the first nucleotide incorporated in the tRNA would be GMP-α-S. The sulfur could have then been reduced and conjugated to IOP.

Although the 3' end of tRNA is universally conserved and seemed likely to be resistant to modification, Wower's results with crosslinking agents attached to the 3' adenine of tRNA\textsuperscript{phe} (112) spurred us to initiate modification studies on the 3' end of tRNA. The strategy was to replace the 3' adenine with a sulfur containing adenine base, 2-thioadenine. To convert the commercially available nucleoside, 2-thioadenosine to a nucleotide, the nucleoside was treated with pyrophosphoryl chloride as described in section 2.2.3. Pyrophosphoryl chloride-treated 2-thioadenosine was purified on a DEAE-cellulose column. Two peaks were collected, dried and analyzed on a TLC plate. Products from the two peaks were run against non-thiolated controls to check for migrational differences and similarities. The monophosphorylated nucleotide that eluted in the first peak comigrated with AMP on the TLC plate. The second peak that contained the putative 2-thioadenosine bisphosphate
comigrated with ADP. This led us to believe that the product, 3',5'-2-thioadenosine bisphosphate, eluted in the second peak. The nucleotide bisphosphate was then used in a ligase reaction with aniline-treated tRNA. The tRNA was 5'-end labeled prior to the ligase reaction. The ligase reaction was analyzed on a denaturing polyacrylamide gel containing APM. tRNA containing the thioadenosine at the 3' end would have migrated slower than the control tRNA due to retardation by APM. However, both control and reaction lanes showed that there were faint bands just below the well. This meant that the retarded bands were not due to incorporation of the 2-thioadenosine bisphosphate.

We hypothesize that the ligase was complexed to tRNA and two phenol extractions were insufficient to purify tRNA away from the ligase and the retarded bands near the well of the gel are just tRNA-ligase complexes. There are a few other points that may be well worth considering at this stage. We may not have obtained the desired product. TLC analysis, although helpful, is not sufficient to prove the product’s integrity. An NMR analysis of the fraction may have resolved this, but due to insufficient material, this was experiment was not performed. Assuming we had the right product i.e. 3',5' 2-thioadenosine bisphosphate, perhaps the ligase reaction did not work due to improper purification of the nucleotide (the nucleotide solution contained 0.4 M triethylamine). Excess triethylamine may have inhibited the ligase reaction. Even if the purification was achieved to reasonable standards, the ligase reaction may have failed due to a low affinity of ligase for the bisphosphorylated thioadenosine.
Since modification of the 3' adenine of tRNA proved unsuccessful in our hands, we decided to simply attach a tether (AMBH) to the 3' end of tRNA and modify the tether with phenanthroline. This tRNA-AMBH-OP conjugate would then be used as our ligand for the ribosome. tRNA-AMBH-OP directed cleavage occurred at two highly conserved regions of the 23S rRNA. One of the sites was the putative peptidyl transferase center and the second site the 100 pseudoknot of the 23S rRNA of which very little is known. Although cleavages at the peptidyl transferase center were expected, the data couldn't be taken at face value because of poor controls. The "mock" lane that contained tRNA-AMBH that had been treated with nitrophenanthroline showed the exact same clips. This meant that the clips were not due to site-specific phenanthroline cleavage events but due to excess phenanthroline that "leaked" through the purification process.

One has to be careful in noting that the AMBH coupling reaction to periodate oxidized tRNA proceeded at about 10% efficiency. This value was determined quantitatively. Nevertheless, the fact remains that there was a considerable amount of uncoupled AMBH in the reaction. Even though phenol extraction was carried to extract uncoupled AMBH, it is very unlikely that all of the AMBH was extracted out from the aqueous phase containing the tRNA-AMBH complex. Now, the unreacted AMBH could potentially be alkylated by IOP to give untethered AMBH-OP conjugates that could be carried through to the final cleavage reaction. This could explain the presence of cleavage bands in the control lanes. This experiment was lacking in two aspects. Firstly, there was no good assay to determine the absence or presence of unreacted AMBH. This led to the great ambiguity in analysis of
the data in the sequencing gels. Secondly, the alkylating reaction in which phenanthroline was covalently attached to the thiol group of AMBH was inefficient as well. Only 10% of tRNA-AMBH was modified by phenanthroline. This effectively made the concentration of the tRNA-AMBH-OP species to be only 1% of the total tRNA. The cleavage emanating from this tRNA would not be much greater than the background cleavage seen in the control lanes. This could perhaps explain why cleavages of nearly the same intensity were seen in both lanes.

As an aside, it is interesting to note that other experiments performed in this lab with different ribosomal ligands have yielded the same 100 pseudoknot clips (unpublished data). This would seem to indicate that this region is situated in a rather solvent-accessible portion of the ribosome. Whether this is true or not remains to be tested.

tRNA-AMBH-OP experiments yielded ambiguous data. The main problem in the above experiment was insufficient yield of the cleaving species. We then thought that modification of a phosphate of the tRNA instead of the base could conceivably be more fruitful. So far all known tRNAs have been known to fold into a general L-shaped tertiary structure (22). The crystal structure of tRNA^phe shows that nine conserved tertiary interactions make up the central core of the tRNA molecule and are responsible for its three dimensional structure (41). Experiments by Musier-Forsyth and colleagues have shown that two separate fragments of tRNA^pro can be annealed to form a fully functional tRNA molecule (51). This tRNA molecule lacks a phosphodiester bond between the two fragments. The annealed tRNA
comigrates with native tRNA pro on a native polyacrylamide gel. This demonstrates the compactness of the tertiary structure that two fragments can form upon annealing.

The overall strategy of our experiment was to generate a 5' fragment of E.coli tRNA pro and anneal this fragment to a chemically synthesized 3' segment containing a thiophosphate at the extreme 3' end. To achieve this, an oligoribonucleotide whose sequence matched the 3' segment of tRNA pro (from positions 59-76) was synthesized. It also had an extra adenine at position 77. The inter-nucleotidic phosphodiester bond between position 76 and 77 was designed to contain a sulfur at one of the non-bridging positions. The idea was to expose the sulfur by removing adenosine at position 77 and the corresponding ribose by periodate oxidation and aniline treatment. After reducing the exposed sulfur, IOP could be conveniently conjugated to the sulfhydryl group. The IOP containing 3' fragment could be annealed to the 5' segment to form a fully functional tRNA. Results showed that the 3' fragment, after aniline treatment and reduction with DTT, comigrated with a non-treated 3' fragment in a denaturing, polyacrylamide gel containing APM. If the sulfur was indeed reduced, then the aniline treated fragment should have migrated slower in comparison to the non-treated control. Variations of the experimental conditions and freshly prepared reagents failed to yield different results.

Periodate is an extremely potent oxidizing agent. During the oxidation process prior to aniline treatment of the fragment, periodate may have oxidized the non-bridging position of the internucleotidic phosphate moiety from a sulfur to an oxygen. This may very well explain
the non-retardation of the aniline treated fragment in an APM gel. The next experiment we carried out was similar to the above experiment, with the difference being, we had a 3' fragment with a 4-thiouridine at position 67 of the tRNA instead of a a thiophosphate at the end. Although this doesn't represent the 3' end of the tRNA, the modification was still in the acceptor stem.

We had assumed that substituting a thiouridine at position 67 would not make any difference to the annealing reaction (10 mM MgCl₂ and 50mM HEPES [ph 8.0]; incubate at 65°C for 3 minutes and slow cool to room temperature) in terms of the kinetics and effectiveness. This could have been a rather naive assumption on our part. The uridine in the wild-type tRNA is part of the acceptor stem and forms a Watson-Crick base pair with A6 of the acceptor stem. Introduction of a thiouridine at this position may possibly affect this base pair formation and prevent annealing. Another important effect the substitution might have had is the disruption of a stacking interaction between U67 and C66. A combination of this disruption of stacking and H-bond interactions could have a deleterious effect on the formation of an annealed helix. This could very well provide an explanation for the two fragments seen on the non-denaturing polyacrylamide gel. This hypothesis, seems to be the only explanation likely enough to interpret the two bands on the native gel.

The experiments described in this thesis are by no means complete. More work needs to be done on the 3' end of tRNA which has proven to be elusive to modification yet again. I think the project that holds the most promise of success among the above is the p-5A-p project. If
this project had been subjected to more rigorous experimentation and detailed analysis, it could have potentially provided some insights into the neighborhood of the 3' end of tRNA on the ribosome.
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