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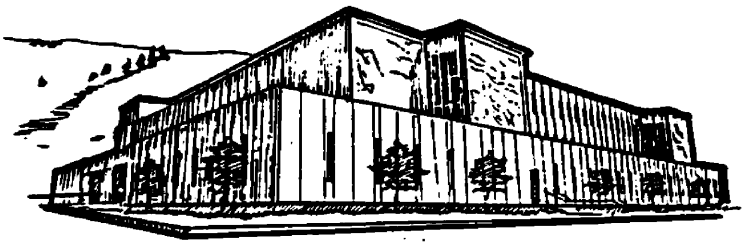

Probing the Exit Site of the *Escherichia coli* Ribosome Using Short Complementary DNA Oligomers

By

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Probing the Exit Site of the Escherichia coli Ribosome Using Short Complementary DNA Oligomers (95 pp.)

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The exit (E) site of the Escherichia coli ribosome was investigated using oligodeoxriboynucleotides complementary to single-stranded regions of ribosomal RNA thought to be involved in tRNA binding in the E site. Radiolabeled DNA oligomers (probes) were hybridized in situ to the ribosomal RNA of ribosomes or ribosomal subunits, and the effects of simultaneous tRNA or antibiotic binding on probe binding were measured using a nitrocellulose filter binding assay. Site specificity of probe binding was assured using ribonuclease H to cleave the ribosomal RNA at the site of probe binding.

When 50S ribosomal subunits were hybridized with a probe spanning bases 2109-2119 and deacylated tRNA was added incrementally, probe binding decreased, indicating this region is involved in tRNA binding either directly or indirectly. On the other hand, when probes 2109-2119 or 2165-2171 were hybridized to intact 70S ribosomes and deacylated tRNA was added, probe binding actually increased at lower concentrations of tRNA. Probe 2109-2119 binding was attenuated a higher tRNA concentrations, but probe 2165-2171 binding remained at the enhanced level even at high tRNA concentrations. A probe spanning bases 2382-2394, which is another area thought to be involved in tRNA binding in the E site, did not hybridize well, suggesting that this region is sterically constrained or possesses some secondary or tertiary structure not conducive to DNA probe hybridization.

The data presented here provide evidence for tRNA/rRNA interaction in the exit site of the Escherichia coli ribosome and provide evidence for a conformational change in the E site upon tRNA binding in the P site. The data suggest a model wherein a deacylated tRNA in the P site serves as a translocational trigger causing the E site to change conformations, making it more available for tRNA (and probe) binding, therefore promoting translocation.

Acknowledgements

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Chapter I

Introduction

The ribosome is an extremely complex ribonucleoprotein particle present in the cells of all life forms. Its principle duty is protein synthesis, but it may have other roles in controlling metabolism. In the following pages, the ribosome and its workings will be described in general terms, then more specifically in dealing with the ribosome's interaction with other cellular components, namely tRNAs. A brief history of related research will be presented, followed by a description of some key issues and current research in the field of ribosome/tRNA research.

In this study, evidence for an allosteric interaction between the peptidyl (P) and exit (E) sites on the Escherichia coli ribosome is presented. This is an exciting discovery, as it is suggestive of a previously undescribed control mechanism in the elongation cycle of protein synthesis.

Protein synthesis and the "central dogma"

In the same way that an architect's blueprint is not a house, but rather the plans for a house, so is the genetic material of a particular organism a plan for the organism. Contained within the DNA (or RNA) genome is all the

information the organism will generally need to respond to the environment and to reproduce. But the information is not the organism, it is the "virtual" organism. The ribosome is the sub-cellular factory responsible for transforming the information present in the genetic material into the proteins that make the organism.

The "central dogma" proposed by Crick (Crick, 1958) shows the general flow of information in living systems from the genomic DNA to a "disposable" transcript of the genetic information, and finally to a functional protein molecule that serves some purpose in the cell. The scheme can be summarized as follows:

DNA----------> mRNA----------> protein
transcription translation

The disposable transcript is called messenger RNA (mRNA) and generally has a short half life in the cell, which provides a mechanism for controlling the production of the protein. The ribosome then reads the code on this mRNA transcript and translates it into the specific protein described by the gene. The protein is the functional product described by the genetic material. It may serve a structural role, a catalytic (enzymatic) role, a contractile or motility function, as a means of intercellular or intracellular communication, or as a component of the organism's defense system. Therefore, according to the central dogma, the ribosome provides the link between the plans for a protein (in the form of mRNA) and the protein itself.

The *Escherichia coli* ribosome

The *E. coli* ribosome is composed of two unequal subunits and sediments at about 70S (see figure 1 (Oakes *et al.*, 1990)). The individual subunits sediment at about 30S and 50S. The 70S ribosome measures about 170Å X 230Å X 250Å in solution (Hill *et al.*, 1969) and its cellular copy number is 15,000-20,000 in mid-log phase *E. coli* cells. The small subunit is composed of one ribosomal RNA (rRNA) molecule containing 1542 nucleotides (nts) that sediments at 16S, and 21 integral proteins designated S1-S21, numbered roughly in decreasing order of molecular weight. The large subunit contains two

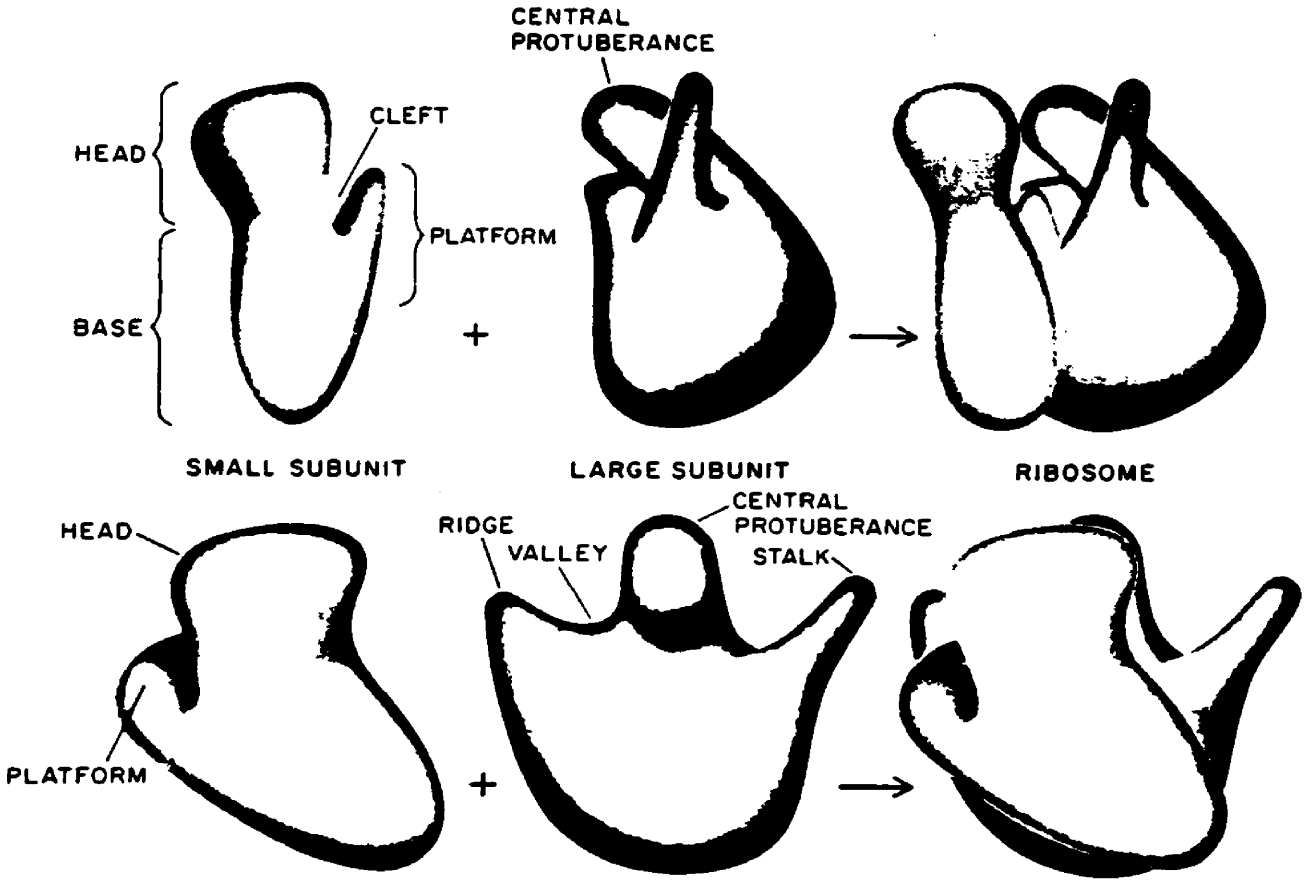

Figure 1: General morphology of the *Escherichia coli* ribosome, side and front views, derived from electron microscopy studies. Models reproduced from Oakes *et al.* (1990).

rRNA molecules, a 5S rRNA (120 nts) and a 23S rRNA (2904 nts). The large subunit also contains 32 proteins designated L1-L32. Many of the ribosomal proteins have been located at least approximately on the subunits. Figure 2 shows the approximate locations of some of the proteins on the 30S and 50S subunits (Oakes et al., 1990).

The role of ribosomal RNA

Up until the last decade or so, it was generally accepted that the rRNA served a "scaffolding" function, to hold the

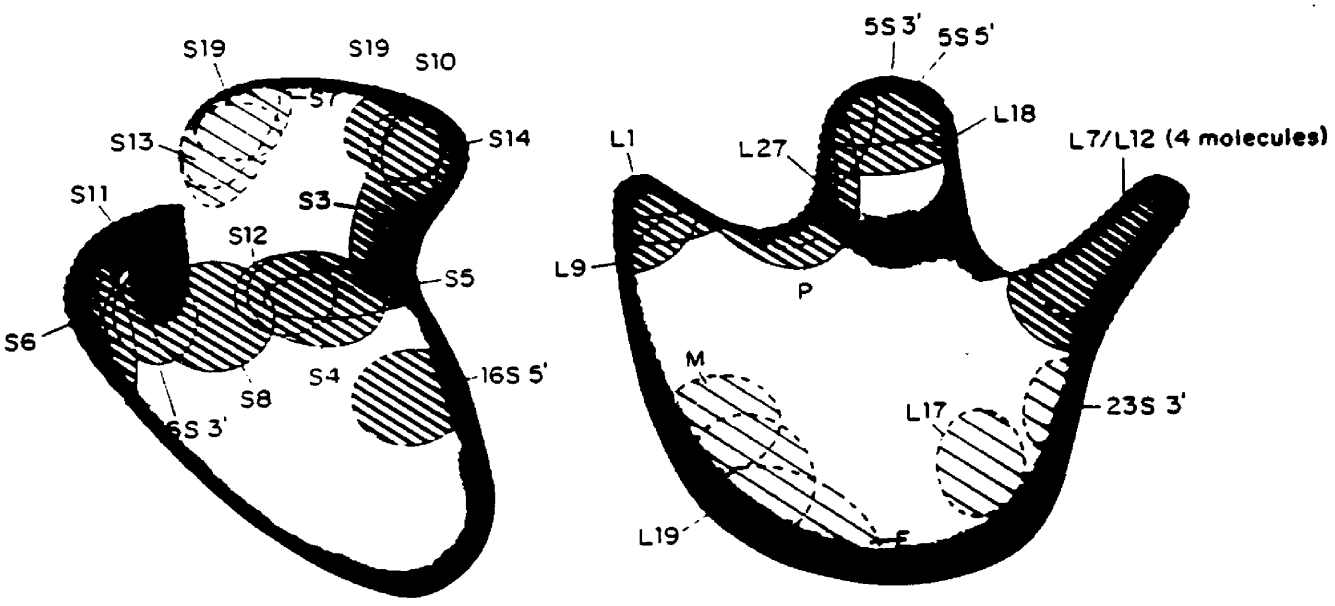

Figure 2: Approximate locations of some of the ribosomal proteins superimposed upon a consensus model of the ribosome. Figure reproduced from Oakes et al. (1990).

ribosomal proteins in position so that they could carry out the process of polymerizing amino acids into proteins. The emerging view now is that the rRNA plays a more active role in translation than previously thought, and may even be the primary agent responsible for catalysis of peptide bond formation. In this view, the ribosomal proteins are the "helper" molecules, rather than the catalytic agents. They may be required to keep the rRNA in the proper conformation or they may interact with other factors required for protein synthesis. The "truth" about protein biosynthesis probably lies between these two extreme viewpoints, as no single ribosomal protein or piece of rRNA alone has been shown to catalyze the formation of a peptide bond.

The overall secondary structures of ribosomal RNAs are highly conserved phylogenetically. The secondary structure maps of 16S and 23S rRNA are shown in figures 3 and 4 (Gutell et al., 1985; Egebjerg et al., 1990). The secondary structures of rRNAs have been subdivided into smaller portions termed domains, numbered I through III and the 3' minor domain in 16S rRNA, and domains I-VI in 23S rRNA. The majority of the research presented here pertains to domains IV and V of 23S rRNA. Figure 5 shows an enlarged portion of domains IV and V and some of the bases thought to be important in tRNA binding, based on a chemical modification footprinting method (Moazed & Noller, 1989).

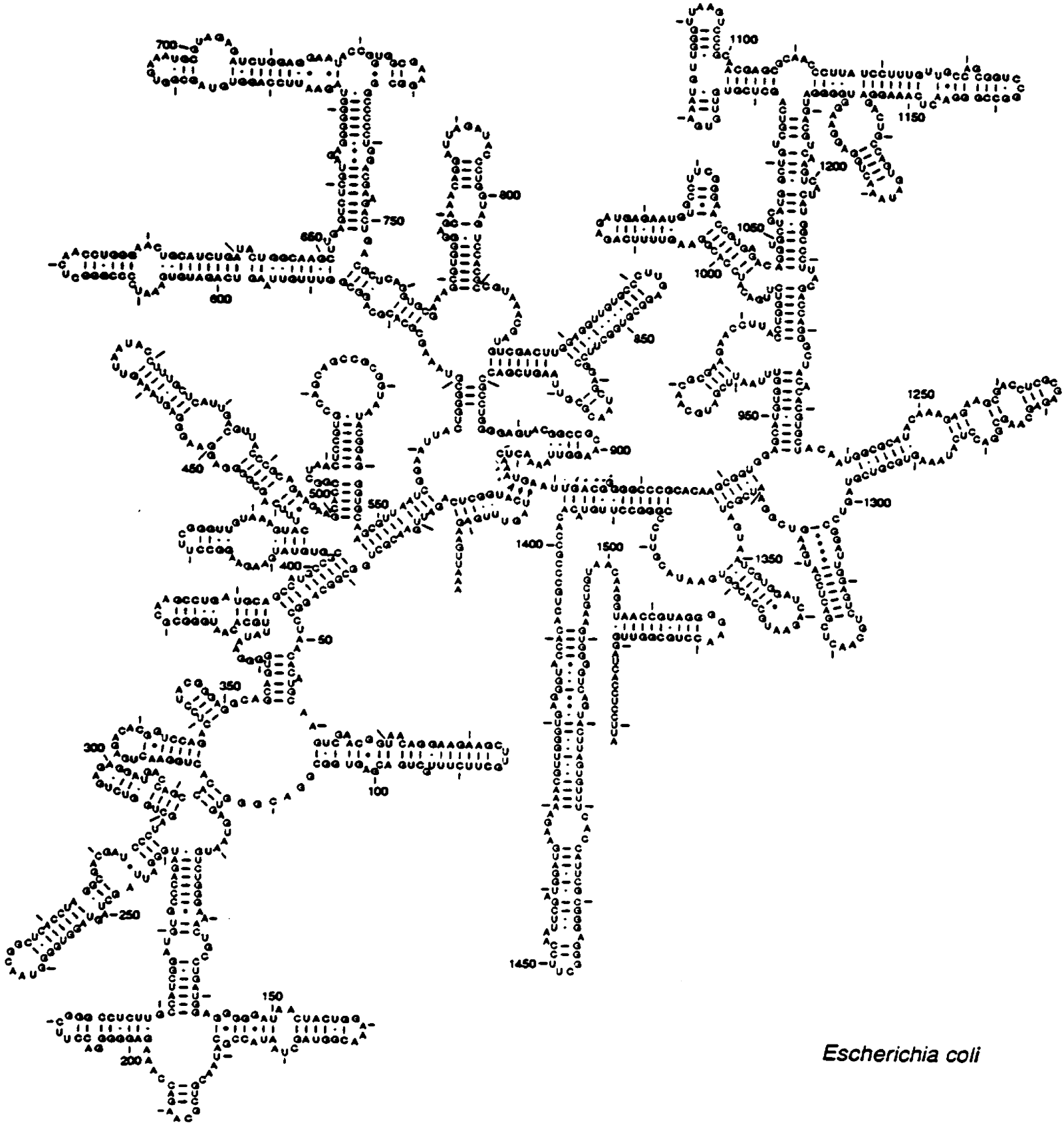

Figure 3: Secondary structure map of 16S rRNA from Escherichia coli. Figure taken from Gutell et al. (1985).

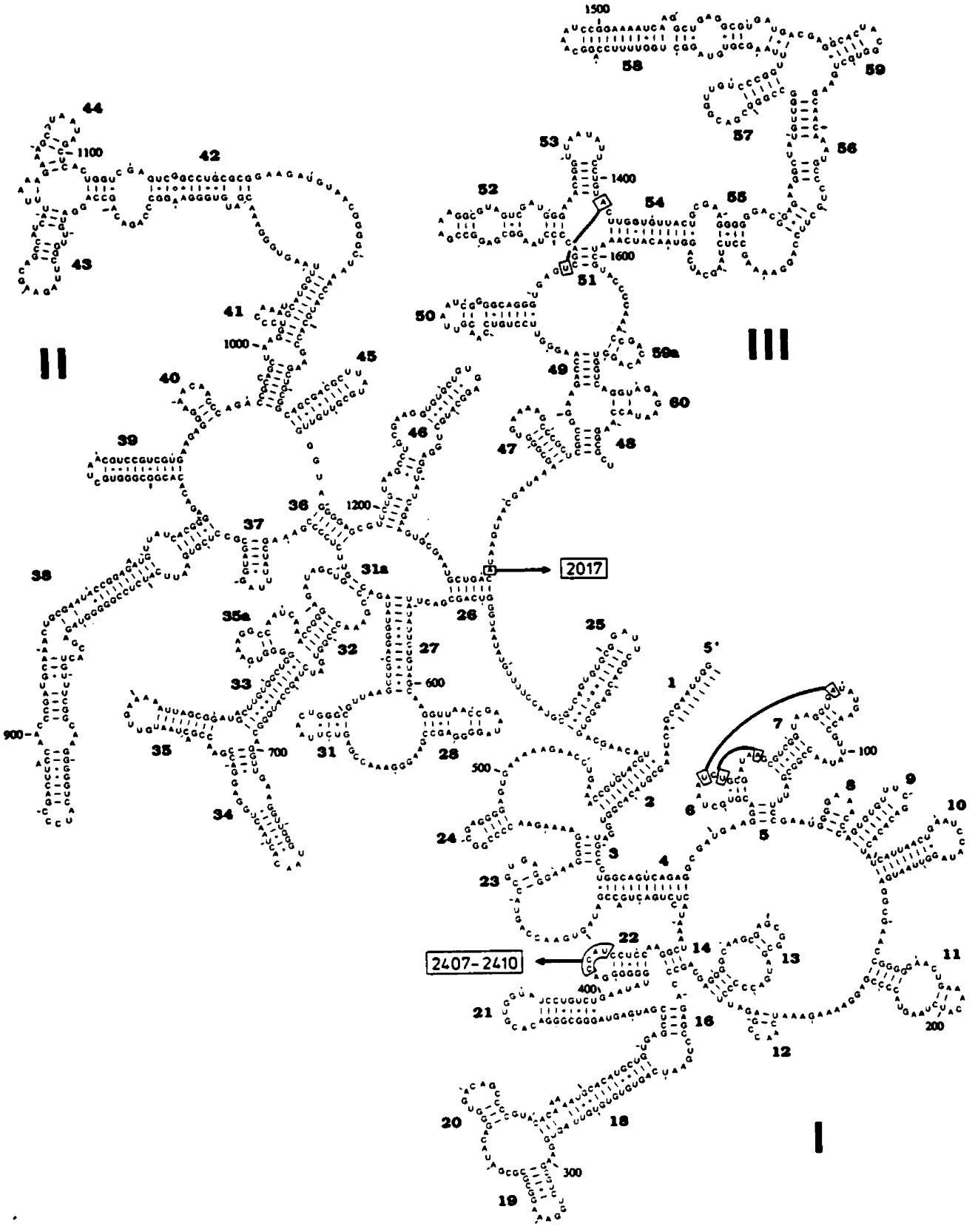

Figure 4a: Secondary structure map of 23S rRNA from Escherichia coli, left half. Domains are indicated in roman numerals, helices are numbered in bold type, and nucleotide numbers are marked by slashes every 10 nucleotides. Figure taken from Egebjerg et al. (1990).

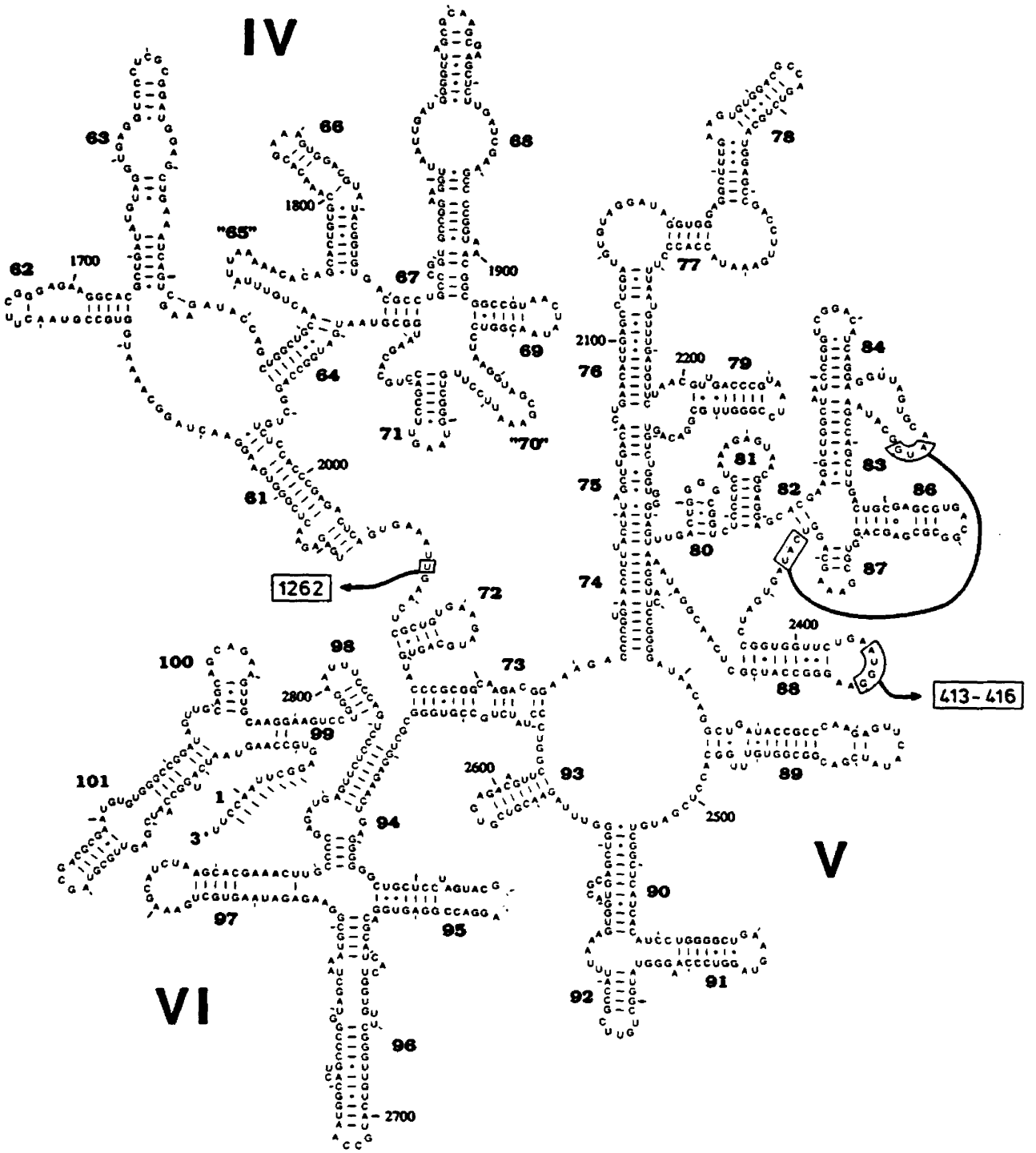

Figure 4b: Secondary structure map of 23S rRNA from Escherichia coli, right half. Figure taken from Egebjerg et al. (1990).

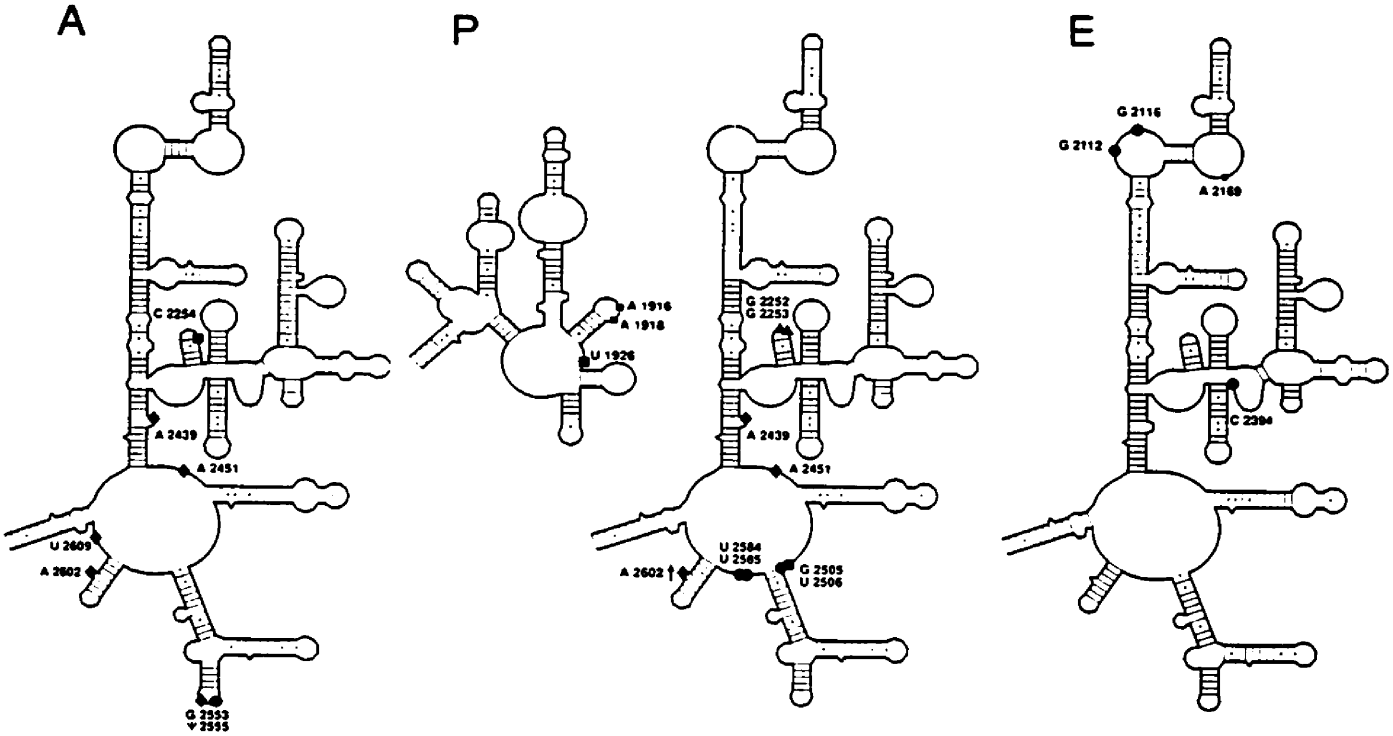

Figure 5: Enlarged view of domains IV and V of 23S rRNA showing specific bases shown by Moazed and Noller to be involved in tRNA binding in the A, P, and E sites (Moazed & Noller, 1989).

Initiation of protein synthesis

The initiation of protein synthesis involves bringing together a messenger RNA, an initiator formylmethionine-tRNA (fMet-tRNA), the 30S subunit, and three initiation factors (IFs). The first step is the formation of a 30S/IF-1/IF-3 complex. The IFs prevent the 30S subunit from reassociating with the 50S subunit and they assist in mRNA positioning on the ribosome. A conserved purine-rich region on the mRNA, usually called the Shine-Dalgarno region, interacts with a pyrimidine-rich region of rRNA on the 30S subunit to help align the message on the subunit (Shine & Dalgarno, 1974). A ternary complex of fMet-tRNA/IF-2/GTP then binds to the 30S subunit with the concomitant release of IF-3. As the 50S

subunit binds, IF-1 and IF-2 dissociate, and the GTP bound to IF-2 is hydrolyzed. In the complete initiation complex, the anticodon of the fMet-tRNA base-pairs with the AUG initiation codon on the mRNA in the peptidyl (P) tRNA binding site and the aminoacyl (A) site is empty (the functions of these sites are described below). Elongation of the peptide chain then occurs in a repetative series of reactions denoted the elongation cycle.

The classic model of the elongation cycle

In 1964, James Watson proposed a model for the peptide elongation cycle which, over the years, gained widespread acceptance since it proved useful in explaining a wide range of experimental data (Watson, 1964). Most textbooks of general biochemistry still describe protein biosynthesis using the Watson model. As shown in figure 6, the model describes the peptide elongation cycle in terms of a ribosome containing two tRNA binding sites, termed the peptidyl (P) and aminoacyl (A) sites. Upon initiation, a tRNA with a formyl-methionine attached to its 3' end, resides in the P site, and the A site is empty. An elongation factor Tu (EF-Tu) coupled with a GTP molecule brings the aminoacylated tRNA called for in the next codon of the message to the ribosome in the form of an EF-Tu/GTP/aminoacyl-tRNA ternary complex. The ternary complex binds the A site and, if the codon/anticodon match is correct, the aminoacyl-tRNA stays in the A site, the GTP is hydrolyzed

to GDP + P_i, and the EF-Tu dissociates from the ribosome. Peptidyl transfer ensues, wherein the carboxy terminus of the fMet on the P site bound tRNA is transferred to the amino group of the amino acid on the A site bound tRNA and a peptide bond is formed between the two amino acids. According to the classic model, the deacylated P site bound tRNA then dissociates from the ribosome and the A site bound tRNA with the growing peptide chain attached moves to the P site in a process called translocation. This process is catalyzed by another elongation factor, G (EF-G), and is also accompanied by the hydrolysis of one molecule of GTP. Some groups believed that EF-G caused release of deacylated tRNA from the P site, then the peptidyl tRNA in the A site diffused over to

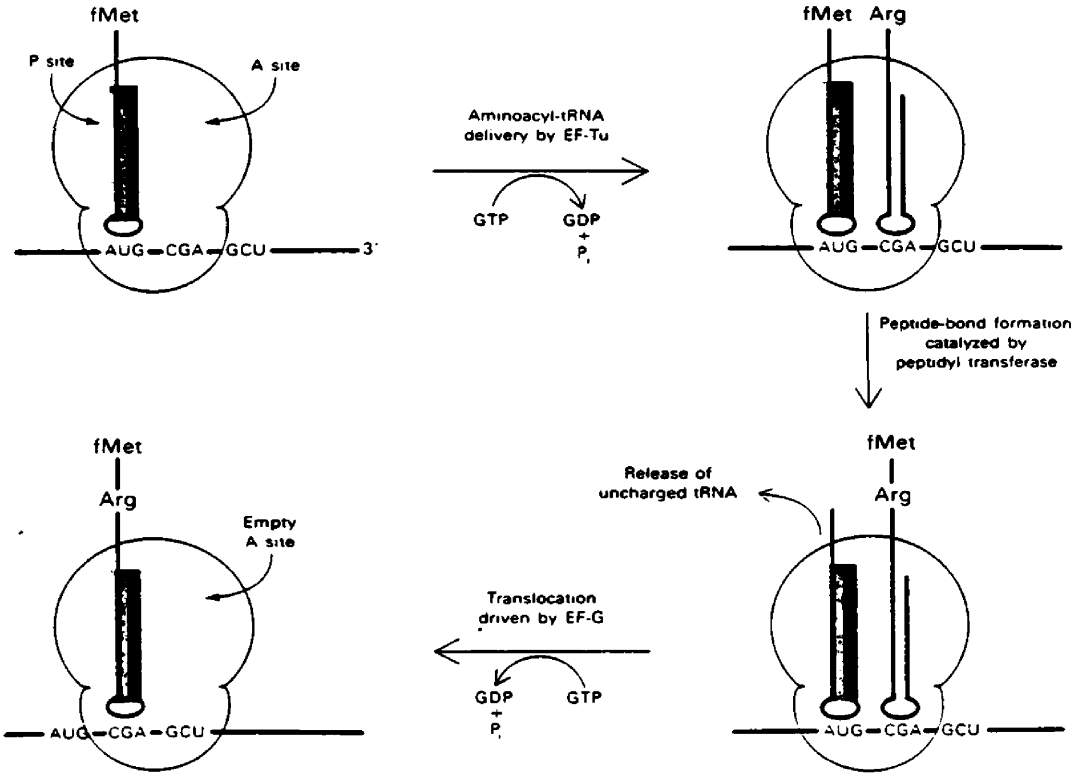

Figure 6: The classic model of the elongation cycle of protein synthesis, reproduced from Stryer (1988).

the P site (e.g. Roufa et al., 1970; Holschuh et al., 1980), but others thought of translocation as a synchronous displacement of tRNAs from the A to the P and a dislodging of the tRNA at the P site (e.g. Lucas-Lenard & Haenni (1969)).The result is that the ribosome is left ready for another cycle of elongation.

The classic Watson model for elongation has been extremely useful because it is simple, yet for about 20 years it adequately explained almost all experimental results involving tRNA binding to the ribosome. In the last 10 years or so, various researchers have produced experimental results which cannot be explained by the simple two site model, and several laboratories around the world are working on refining the model for the elongation cycle.

How many tRNA binding sites are there on a ribosome?

One of the earliest indications that the classic model of protein synthesis might not be complete came from Wettstein and Noll (1965). Using relatively simple techniques such as co-sedimentation analysis, they found that on rat liver polysomes, an average of two to three tRNAs were bound per ribosome. Since at some points during elongation there would be only one tRNA bound to the ribosome according to the classic model, statistically one should find an average of less than two tRNAs bound per ribosome. Wettstein and Noll suggested that there might be a third site on the ribosome

that serves an exit function for deacylated tRNA, but they could not conclusively prove it, and their idea was largely forgotten. Similar experiments in 1964 using rabbit reticulocyte polysomes suggested that an average of two tRNAs are bound to 80S ribosomes (which, using statistical analysis would suggest more than 2 binding sites), but the results were interpreted in terms of the Watson model, so an additional binding site was not suggested (Warner & Rich, 1964).

On the procaryotic ribosome, from only one (Gilbert, 1963) to up to four (Swan et al., 1969) tRNA binding sites have been reported, but most investigators generally accepted that there were two tRNA binding sites on the ribosome up until the 1980's, when several laboratories, using a variety of carefully controlled experiments, detected more than two tRNA binding sites (Rheinberger & Nierhaus, 1980; Rheinberger et al., 1981; Grajevskaja et al., 1982; Kirillov et al., 1983; Lill et al., 1984). The assertion that there were more than two tRNA binding sites on the ribosome did meet with considerable oppostition, however. Schmitt et al. (1984), using analytical ultracentrifugation, found exactly two sites on the ribosome for tRNA binding. They also found that the 30S subunit has no binding capacity in the absence of message and one site in the presence of mRNA (poly U). The 50S subunit bound a single tRNA in the presence or absence of message. Schmitt et al., contended that their experiments, represented a more accurate description of tRNA binding, since

they measured the equilibrium proportioning of tRNA and ribosomes, whereas the nitrocellulose filter binding assay was prone to "kinetic effects" of filtration and washing with buffer. Likewise, Spirin used a column-immobilized system to determine the stoichiometry of tRNA to ribosomes in various stages of the elongation cycle (Spirin, 1984). He found that on addition of EF-G-GTP, a deacylated tRNA was released, consistent with the classic model, and therefore he rejected any of the new models involving more than two non-overlapping tRNA binding sites. However, in the last few years, as data have accumulated, there have not been serious objections raised against the emerging models of the ribosome that include more than two sites for tRNA binding. Today, based on diverse research from several laboratories, it is generally accepted that there are at least three tRNA binding sites on the E. coli ribosome. In addition to the classical aminoacyl (A) and peptidyl (P) sites, ribosomes possess an additional site, the exit site whose exact characteristics are yet a matter of dispute among various labs.

A brief history of exit site research

Since the classic two-site model for elongation seemed to work so well for almost two decades, introducing a "new" site into the model required that it be accompanied by an explanation as to why the ribosome would need an additional tRNA binding site. The work of Wettstein and Noll (1965)

proposed three tRNA binding sites on the rat liver ribosome. They described the sites as the entrance or decoding site (now known as the A site), the condensing, or middle site (P site), and an exit (E) site. They made these conclusions based on binding characteristics of the three types of S-RNA (S for soluble, now called transfer RNA or tRNA). Aminoacyl-tRNA (called α S-RNA in the original work) and peptidyl-tRNA (β S-RNA) were stably bound to the rat liver ribosomes and were not removable by washing or exchange, and two of these could be bound to the ribosome at once. Deacylated tRNA (γ S-RNA), on the other hand, was removable by washing or exchange with more γ S-RNA only. Using these observations, they postulated a three-site model of elongation which described a third non-classical site serving an exit function. Other researchers presented multisite models subsequent to this proposal, (Arlinghaus et al., 1964; Kaji, 1967; Matthaei & Milberg, 1967) but none of these suggested the additional site was an exit site, as they all appeared to involve binding of an acylated tRNA.

In 1969, Hardesty et al., proposed the existence of an entry site that would bind the EF-Tu/tRNA/GTP ternary complex before hydrolysis of the GTP and dissociation of the EF-Tu. Lake (1977) used electron microscopy to support the notion of a ribosomal recognition site or pre-A site. He suggested that the recognition site shares the A site in the vicinity of the codon-anticodon interaction, but the rest of the tRNA remains

outside of the A site, on the outside surface of the ribosome. Because it is supposed that a pre-A, entry, or recognition site has some overlap with the A site, it did not truly represent an additional site.

In 1980, Rheinberger and Nierhaus detected 3 binding sites on E. coli ribosomes. Using a nitrocellulose filter binding assay, they found that they could reproducibly and stably bind 3 tRNAs per ribosome at the same time (Rheinberger & Nierhaus, 1980). They determined the tRNA binding site filling order to be P, then E, then A, by using the filter binding assay and different types of tRNA (i.e. deacylated, charged, and peptidyl). In addition to the "traditional" tRNA location test, the puromycin assay, which detects the presence of an aminoacyl or peptidyl tRNA in the P site (Traut & Monro, 1964), these researchers employed various radiolabeling schemes in an attempt to unambiguously determine the location of the tRNAs bound to the ribosomes. This paper was the first to attempt to rigorously quantify tRNA binding in the "new" site, and it stimulated much related research and a new thinking about the process of elongation.

Rheinberger et al. (1981) also reported that the E site is absolutely specific for deacylated tRNA, is sensitive to the codon present in the E site, and has an apparent affinity of ~9 X $10^6 M^{-1}$, similar to the P site and about 5 fold greater than the affinity of deacylated tRNA for the A site. They also brought forward the idea of an "exclusion principle" of

tRNA binding, which states that a peptidyl-tRNA analogue (N-acetyl-Phe-tRNA^{Phe}) can bind either the A site or the P site, but the ribosome cannot bind two N-Ac-Phe-tRNA^{Phe}s simultaneously. They argued that having a peptidyl tRNA in both sites would be an unnatural state of the ribosome, therefore it is logical that only one N-Ac-Phe-tRNA^{Phe} should bind to a ribosome. The exclusion principle and the relative importance of the codon-anticodon interaction in the E site have turned out to be points of controversy among various research groups over the years.

About a year after the Rheinberger et al. (1981) paper was published, Grajevskaja et al. confirmed that there are at least three tRNA binding sites on the Escherichia coli ribosome (Grajevskaja et al., 1982). They used ultracentrifugation and filter binding assays to find that ribosomes became saturated when an average of 2.5 tRNAs were bound per ribosome. Furthermore, they reported that poly U programmed ribosomes could hold exactly two N-Ac-Phe-tRNA^{Phe}s, in apparent violation to the exclusion principle. Unprogrammed ribosomes bound a total of 1.5 moles of deacylated tRNA per mole of ribosomes. They described two binding sites for deacylated tRNA: one site exhibited slow kinetics having an exchange rate of several hours at 0°C, and the other site showing rapid kinetics, having an exchange rate of several minutes at 0°C. The site with the slow exchange

rate they identified as the P site, since binding of deacylated tRNA in this site could be blocked with N-Ac-Phe-$tRNA^{Phe}$. The site with the rapid exchange rate they identified only as an "additional" site, and they reported that the presence or absence of message or cognate message did not affect binding in this site significantly. Another important observation they made was that a tRNA with its 3'-terminal adenosine removed no longer bound in the additional site, but it bound normally in the P site. In summary, Grajevskaja et al. were at odds with Rheinberger et al. in the number of N-Ac-Phe-$tRNA^{Phe}$s binding to a 70S ribosome, and regarding the importance of mRNA in E site binding, but the two groups independently confirmed the existence of more than 2 tRNA binding sites on the ribosome.

In 1983, a third lab reported three tRNA binding sites on the Escherichia coli ribosome. Kirillov et al. (1983) showed that the third site is associated with the 50S subunit and, unlike the A and P sites, the third site cannot be blocked using the antibiotics tetracycline (an A site blocker) or edeine (a P site blocker). Kirillov's group supported Grajevskaja et al. (1982) and refuted Rheinberger et al. (1981) in reporting that the additional site is practically codon-anticodon independent. They reported the K_{aff} of the additional site to be 5 X $10^7 M^{-1}$ with poly U present and 3 X $10^7 M^{-1}$ in the absence of poly U. These affinities were two to

three orders of magnitude less than the affinity of the P site (called the donor, or D, site in this paper) for deacylated tRNA.

Rheinberger et al. (1983) attempted to clear up some of the discrepancies between their data (Rheinberger et al., 1981) and those of Schmitt (1981), Grajevskaja (1982), and Kirillov (1983), concerning the exclusion principle and the number of binding sites for deacylated tRNA. First, they reported that the exclusion principle applies only if the Mg^{2+} concentration is 15mM or less. They also showed that using the binding conditions of Schmitt et al. (3.3μM ribosomes, 7-fold excess of tRNA), ribosomes saturated at 1.8 tRNA per 70S ribosome (as in Schmitt et al.), but at 0.33μM ribosomes and a 40-fold excess of tRNA, they were able to bind 2.7 tRNA/ribosome, as previously reported.

In 1983 came the first explicit proposal for the purpose or function of the new tRNA binding site. Rheinberger and Nierhaus (1983) suggested an alternative model to the classic model of elongation with the following features: i) In both the pre- and post-translocational states, there are two tRNAs bound to the ribosome and to the mRNA. ii) The new E site is adjacent to and upstream (relative to the message) of the P site. iii) Deacylated tRNA is released from the E site, not the P site. iv) The trigger for the release of E site bound tRNA is occupation of the A site. They suggested that the advantage for such a model is that, with two tRNAs involved in

codon-anticodon interactions at any given time, the message is better "fixed" in-frame on the ribosome. This model represented the first serious modification to the classic model of elongation, but it was still disputed by those who were loyal to the classic model (e.g. Spirin, 1984; Schmitt et al., 1984) and by those who believed that the codon-anticodon interaction in the new site was not important.

Lill et al. (laboratory of W. Wintermeyer) joined the foray in 1984, challenging the percent of active ribosomes, the exclusion principle, the message dependence in the E site, the stability of E site binding, and the A site occupation as a release mechanism for E site bound tRNA (Lill et al., 1984). Instead, they proposed that the purpose of the exit site is to serve as a thermodynamic escape route for deacylated tRNA from the very stable P site, that is, to divide the activation energy for dissociation of tRNA. Whereas Rheinberger and Nierhaus (1983) claimed that in the E site the tRNA is stably bound and dependent on codon-anticodon interaction, Lill et al. claimed that at physiological magnesium concentrations (i.e. less than 10mM Mg^{2+}) tRNA spontaneously dissociates from the P site and the E site is not appreciably occupied. They found that the effect of chasing E site bound tRNA with added deacylated tRNA was strong, but tRNA was not released when Phe-tRNA was added. In short, Lill et al. squared off firmly against almost every assertion Nierhaus' group made concerning the newly discovered tRNA binding site, and it was the

beginning of a rivalry which persists even today in the literature.

The importance of codon-anticodon interaction in the E site

One of the main differences between Nierhaus' and Wintermeyer's description of tRNA binding in the exit site was the relative importance of the codon-anticodon interaction. The implications of this interaction or lack of interaction were important, because each side of the dispute used their findings to help explain their own model. Using fluorescent derivatives of tRNA, Paulsen and Wintermeyer (Paulsen & Wintermeyer, 1986) sought to determine the exact distance between the anticodon loops in P- and E-site bound tRNAs. They derivatized $tRNA^{Phe}$ to contain wybutine and proflavine as the fluorescent donor and acceptor just 3' to the anticodon loop for A, P, and E site bound tRNAs and measured the distances. The distance from the A site to the E site was 42 +/- 10Å, and the distance from the P site to the E site was 34+/- 8Å. The distance between the A site and the P site was 24+/- 4Å (Paulsen et al., 1983), but the distance between the P site and the E site appears much larger, "rendering unlikely simultaneous codon/anticodon interaction in the P and E sites."

From these data, it would appear that the tRNAs in the A, P, and E sites are not aligned since the distances between the

three sites are not the same, and Paulsen and Wintermeyer interpreted this as an indication that there was no or very little codon-anticodon interaction in the E site. This discrepancy also harkened back to a proposal by Rich (1974) where it was suggested that the message would have to be kinked in order to accommodate two adjacent codon-anticodon interactions. (Lill et al., 1986) reported that on unprogrammed ribosomes, the non-coded P site is 100-fold more labile than the cognate codon-coded P site, and it displays binding characteristics similar to those of E site binding. Disruption of the codon-anticodon interaction in the E site was again proposed as a part of the Wintermeyer model of elongation where the E site serves as an easier thermodynamic and kinetic exit route for deacylated tRNA in the P site.

Robertson and Wintermeyer demonstrated the reversibility of tRNA binding in the E site by first binding ^{32}P-tRNA in the P site, then binding N-Ac-Phe-tRNA^{Phe} in the A site, then adding EF-G/GTP. After translocation, the labeled tRNA was followed and it was shown to be easily displaceable and chaseable with added cognate or non-cognate tRNA. The non-cognate tRNA was only marginally less effective at chasing the tRNA from the E site than the cognate tRNA. These results suggested that the mRNA/tRNA interaction in the exit site is relatively unimportant.

Gnirke et al. (1989), however, contended that the codon-anticodon interaction in the E site is very significant.

Using a heteropolymeric mRNA instead of poly U or poly A, the position of specific codons can be precisely determined. They found that when the cognate codon for a particular tRNA is located in the E site, the tRNA binds with a 20-fold higher affinity than if a non-cognate tRNA is located in the E site. The Nierhaus and Wintermeyer laboratories have tried preparing ribosomes according to each other's protocols, but they have not been able to reconcile experimental differences concerning the importance of the codon-anticodon interaction.

Major models of the elongation cycle

At this point in the history of E site research, one of the things that was generally agreed upon was that there exists an additional site on the ribosome that serves as an exit site in that it binds the deacylated tRNA before the tRNA dissociates from the ribosome. The issues of codon-anticodon interaction, stability of binding in the E site, effect of elongation factor G, the timing of deacylated tRNA release, and the general function of the exit site were (and in some respects still are) a matter of debate. This section will summarize experimental results from each of several labs working in the area of E site research, and give a description of that laboratory's model of the elongation cycle.

Laboratory of Knud Nierhaus (Berlin)

The first group to attempt to rigorously describe and

quantify tRNA binding in the additional site was Nierhaus' (Rheinberger et al., 1981; Rheinberger & Nierhaus, 1980). Their experimental approach has been to use the nitrocellulose filter binding assay, heteropolymeric synthetic mRNAs, and antibiotics to determine binding characteristics in each of the tRNA binding sites. In the nitrocellulose filter binding assay, incubation mixtures containing radiolabeled tRNA and ribosomes and mRNA are passed through the filter, where the ribosomes are retained with bound tRNA. Unbound tRNA passes through the filter. Originally, this group proposed that the function of an additional site was to assure the message was always fixed in frame on the ribosome by two codon-anticodon interactions. In the classic model of elongation, only one codon-anticodon interaction exists at times during the cycle, and Nierhaus argued this would be insufficient to keep the message firmly in frame (Rheinberger & Nierhaus, 1983).

Nierhaus has proposed and refined over the years an elaborate model for elongation called the allosteric three site model (see figure 7) (Gnirke et al., 1989). The model has the following features: 1) Three binding sites for tRNA, designated A, P, and E. The E site is specific for deacylated tRNA. 2) During translocation, the deacylated tRNA does not dissociate from the P site, but moves into the E site. 3) There are two tRNA molecules on the ribosome at all times, and both undergo codon-anticodon interaction. 4) The ribosome can assume two conformations, a high affinity A and P site with

low affinity E site (the pre-translocational state), or a high affinity P and E site with a low affinity A site (the post-translocational state).

One of the unique aspects of this model is the proposed allosterism between sites A and E. Nierhaus suggests that when the A site is occupied (i.e. in a pre-translocational state) the E site has an especially low affinity for tRNA. Before a cognate aminoacyl tRNA has been selected in the A

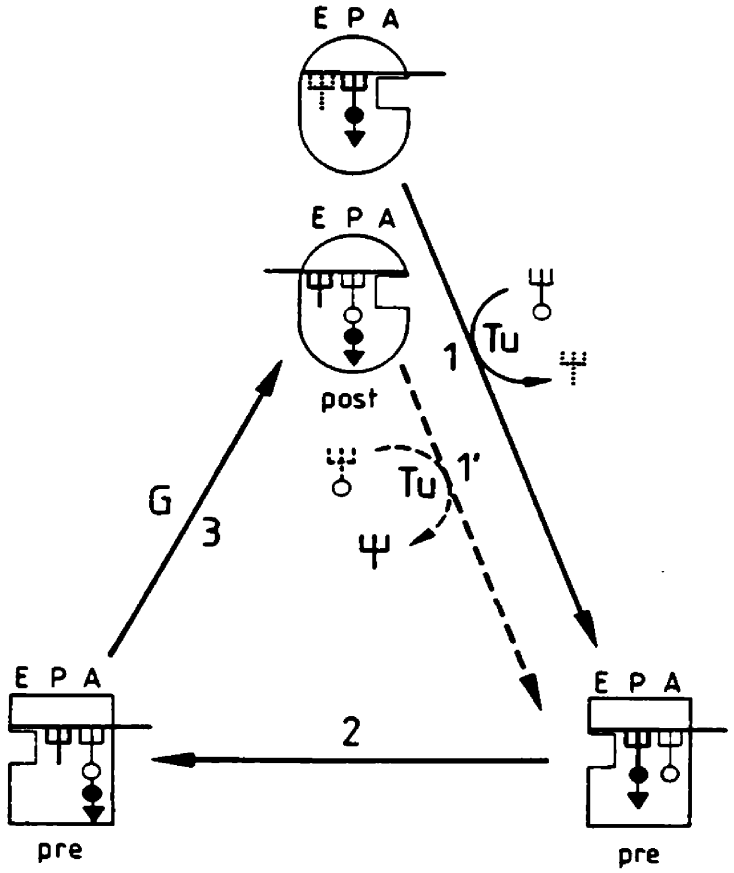

Figure 7: The allosteric three site model for the elongation cycle. Rectangular ribosomes are pre-translocational, oval ribosomes are post-translocational. Tu and G represent EF-Tu and EF-G. Step 1 is selection of an aa-tRNA, step 2 is peptidyl transfer, step 3 is translocation (Gnirke et al., 1989).

Conversely, when the ribosome has translocated, it assumes another conformation in which the A site is empty and has a low affinity for tRNA, and the E site is occupied and is in a high affinity state.

site, many non-cognate tRNAs must be screened. A low affinity A site would allow a more rapid screening of many tRNAs than would a high affinity A site (i.e. the low affinity site has a higher off-rate). This would be advantageous in proofreading/selection of the incoming aminoacyl-tRNA. Since translocation puts a tRNA in the E site, and after translocation is when the ribosome is "looking" for an aminoacyl-tRNA, using occupation of the E site as a switch for turning the A site to its low affinity state makes good sense. Once the cognate tRNA has been selected, the ribosome can bind it more tightly in preparation for peptidyl transfer (i.e. the A site now assumes its high affinity state). High affinity occupation of the A site in turn triggers the ejection of the tRNA from the E site. Peptidyl transfer ensues, and then translocation, with the concomitant switching of the E site to its high affinity state and occupation with a tRNA. A change in conformation of the E site to its high affinity state might also serve as thermodynamic "encouragement" for the deacylated tRNA in the P site to move to the exit site.

Some features that make Nierhaus' model different from other models are: Strong codon-anticodon interaction exists in all three sites (Wurmbach & Nierhaus, 1979; Gnirke et al., 1989);

E site tRNA binding of the post-translocational ribosome is stable and has an affinity comparable to that of P site tRNA binding (Rheinberger et al., 1981); Translocation and release of deacylated tRNA are separate, independent events (Rheinberger et al., 1983; Hausner et al., 1988); Allosterism in the form of negative cooperativity exists between the A and E sites, which may aid alternately in proofreading and promotion of translocation (Gnirke et al., 1989).

Laboratory of Wolfgang Wintermeyer (Munich)

Wintermeyer's first paper concerning exit site tRNA binding appeared in 1984, and his group has been very prolific in E site research ever since. They have used the nitrocellulose filter binding assay as well as fluorescence techniques to study tRNA binding to the ribosome. They argue that the fluorescence method is appropriate for studying the exit site, since, according to their results, tRNA binding in the E site is so labile that the nitrocellulose filter binding assay might not be as sensitive to E binding. Further, the fluorescence measurements are indicative of tRNA binding in equilibrium conditions, and are thus free of kinetic effects possible with the filter binding assay (Robertson & Wintermeyer, 1981). One problem with the fluorescence technique is that the tRNA must be modified to contain a fluorescent donor or acceptor, and this modified tRNA may have altered binding characteristics from native tRNA.

Wintermeyer's group has also used fluorescence to monitor tRNA binding to the ribosome in a qualitative way (Robertson & Wintermeyer, 1981; Robertson et al., 1986; Paulsen & Wintermeyer, 1986). Fluorescent tRNA derivatives give characteristic changes in the intensity and polarization of fluorescence upon binding in each of the A, P, and E sites. In addition to revealing the extent of binding, the fluorescence pattern of the tRNA derivatives bound in the various sites also yields information about the solvent environment at that site and about how constrained the site is. The P and A sites are shielded from the aqueous solvent (Robertson & Wintermeyer, 1981; Paulsen et al., 1982), but a tRNA in the exit site is exposed to an aqueous environment, which is one of the factors destabilizing binding there (Robertson & Wintermeyer, 1987).

Wintermeyer's group has not been in agreement with Nierhaus' group in several areas. Wintermeyer has proposed that the function of the exit site is to facilitate release of tRNA from the P site, to divide the activation energy for dissociation of tRNA (Lill et al., 1984). They reported that the E site is not message dependent, in agreement with Grajevskaja et al. (1982), and they did not detect any allosterism between sites A and E. They found that the exit site was only about 50% occupied at physiological magnesium concentrations in vitro, and therefore they proposed that the E site is not a stable binding site. A stopped flow

fluorescence study suggested that there are three distinct steps during translocation, and Wintermeyer's group proposed the following model (Robertson et al., 1986). Following peptidyl transfer, the peptidyl-tRNA in the A site and the deacylated tRNA in the P site are moved as a unit with the messenger RNA to the next site down (i.e. the peptidyl-tRNA to the P site and the deacylated tRNA to the E site), and this step is rapid. The next step is of intermediate speed, and it involves the rearrangement of the E site to a different conformation, perhaps disrupting the codon-anticodon interaction. The third, slow step is dissociation of the deacylated tRNA from the E site. The speed of the slowest step was slower than the overall in vitro elongation rate (measured in a separate experiment and in Gast et al. (1985)), suggesting that the ribosome does not have to wait for the slow step when it is functioning normally.

Paulsen and Wintermeyer reasserted their claim of no codon-anticodon interaction in the E site with distance measurements between anticodons in the A, P and E sites (Paulsen & Wintermeyer, 1986), then, in the following year, conceded that there was some interaction (Lill & Wintermeyer, 1987). They maintained that the E site was more labile than the other sites (Robertson & Wintermeyer, 1987), and that the ribosome could distinguish a tRNA in the E site by features other than the anticodon or presence of an acyl or peptidyl group (Lill et al., 1988). Lill et al. (1989) took this further and

found some evidence to suggest that the 3' end of the deacylated tRNA in the P site actually plays a part in promoting translocation by interacting with nucleotides of the rRNA in the E site (see figure 8).

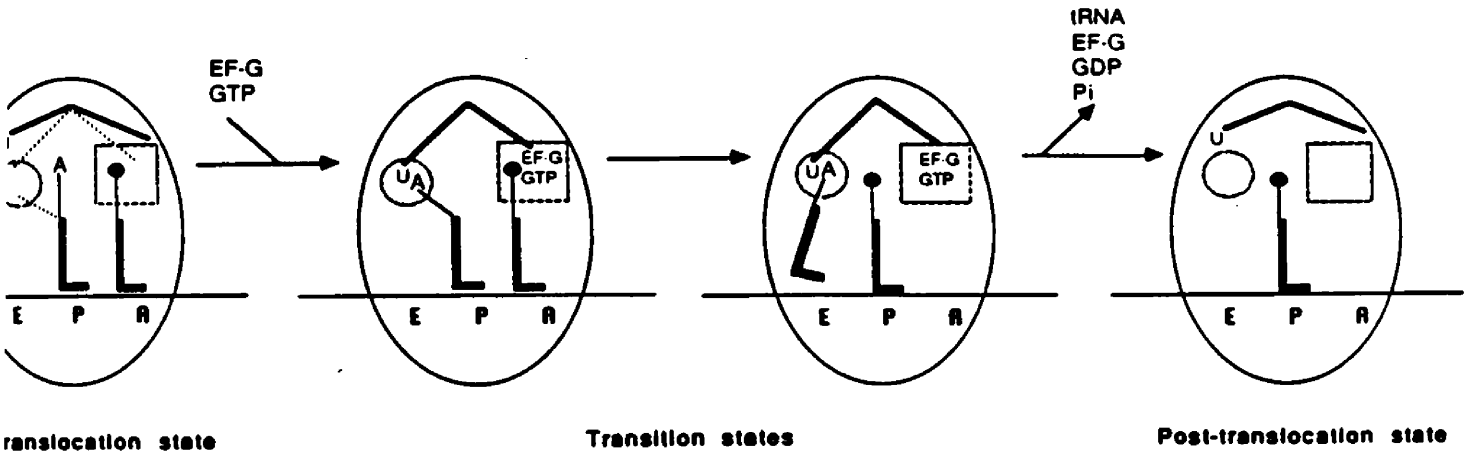

Figure 8: Model of translocation according to Lill et al. (1989). The L structures are tRNAs and the heavy kinked line is 23S rRNA. Nucleotide U2111 is shown interacting with the 3' terminal adenosine of the deacylated tRNA when EF-G is present and this interaction promotes translocation.

In summary, Wintermeyer's group believes that the E site is a labile tRNA binding site where the deacylated tRNA stays only transiently and the codon-anticodon interaction in this site is less significant than in the other sites. The function of the E site is to provide an thermodynamic escape route from the P site, and possibly also to promote translocation.

The laboratory of Harry Noller (Santa Cruz, CA)

Although Noller's lab was not involved in the early attempts to characterize the exit site, they have produced some of the most specific data concerning the interaction of tRNAs with ribosomal RNA using chemical probing techniques. In addition, they have proposed another model for elongation from which we get yet a different view of tRNAs moving through the ribosome.

Transfer RNAs actually shield some of the nucleotides of rRNA from attack by chemical modifiers such as kethoxal, dimethyl sulfate, and carbodiimide reagents. In fact, tRNAs bound in sites A, P, and E each protect a characteristic set of nucleotides in the 16S and 23S rRNA (Moazed & Noller, 1986; Moazed & Noller, 1989; Moazed & Noller, 1990). Interestingly, the 3' end fragment of an acylated tRNA, comprised of an amino acid and the five 3'-terminal nucleotides of the tRNA molecule, give the same protections in 23S rRNA as do intact tRNAs (Moazed & Noller, 1991). Using these protection data, Noller's group has proposed a model in which the ribosomal subunits slide back and forth relative to each other, creating hybrid tRNA binding sites in the process (see figure 9). Noller's work does not address the question of binding stability or codon-anticodon interactions, but it does unambiguously demonstrate the existence of three distinct tRNA binding sites on the Escherichia coli ribosome, and it provides a list of nucleotides in the rRNA that may be involved in tRNA/rRNA interaction in the three sites. The

The function of the E site remains obscure

The process of elongation is not as simple as was once thought. With many laboratories around the world studying various aspects of elongation, it seems that with each new result comes several new questions. The gross picture is fairly complete, but the intricacies and the fine control mechanisms that allow the ribosome to do its job with such speed and precision are being uncovered but slowly. In the research presented below, some new aspects of ribosome function are proposed which will hopefully help push our overall understanding of translation toward the realm of molecular detail. In particular, evidence will be presented on the locations of interaction of tRNA with rRNA in the ribosomal exit site, and for conformational changes that occur on the ribosome upon tRNA binding.

Experimental outline

The research presented here addresses several questions pertaining to tRNA interactions with the ribosome. Moazed and Noller (1989) identified several nucleotides in 23S rRNA that may be involved in tRNA binding in the exit site using chemical footprinting techniques. In this study, DNA probes complementary to those regions on the 23S rRNA were synthesized and mixed with ribosomes or subunits to ascertain if those regions of 23S rRNA were accessible for probe hybridization. If the region was available for probe binding,

the effect of adding different species of tRNA on probe binding was examined. In addition, the effect of antibiotics (chloramphenicol and tetracycline) on probe binding was studied.

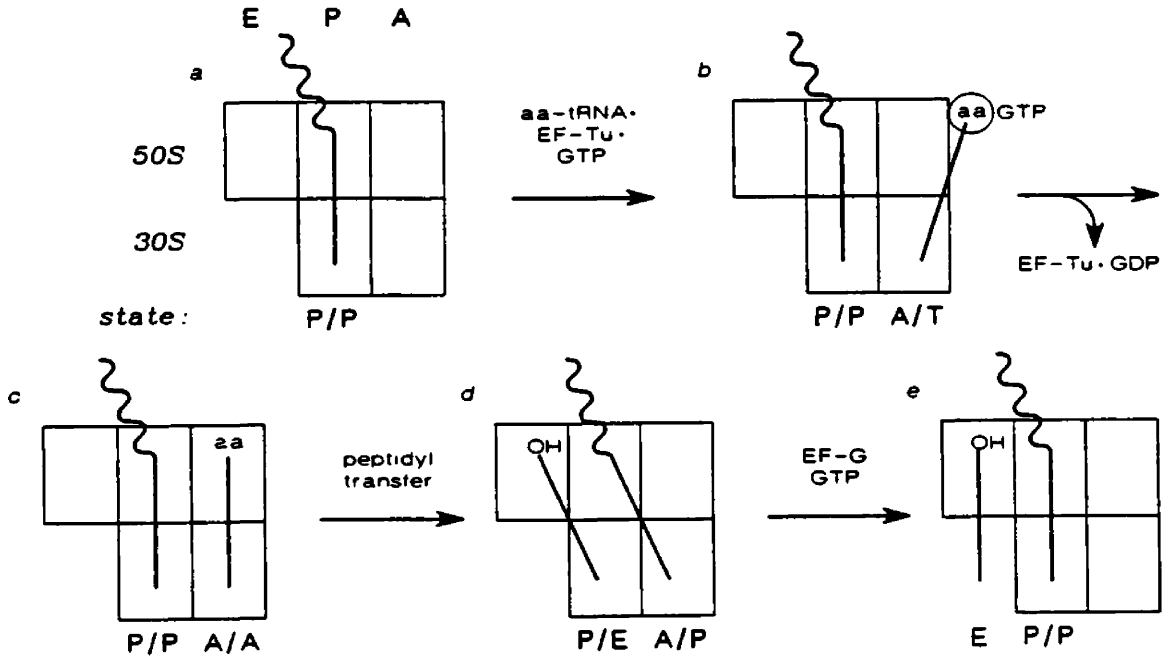

Figure 9: The Moazed and Noller (1989) model of elongation. In the process of the subunits moving relative to each other, hybrid tRNA binding sites are formed.

Chapter II

Materials and Methods

Preparation of ribosomes and ribosomal subunits

Ribosomes or ribosomal subunits were isolated from E. coli strain MRE600 by the methods of Hill et al. (1969). Cells were grown in trypticase soy broth at 37°C with vigorous aeration. When the A_{600} of the broth reached 0.5-0.8, the cells were concentrated using a Millipore Pellicon Cassette System, then pelleted in a Sorvall GSA rotor for 5 minutes at 5,000 rpm. The cells were ground using a mortar and pestle with twice the cells' weight of alumina in a buffer containing 10mM Tris-HCl, pH 7.4, 15mM $MgCl_2$, and 100mM KCl in the cold for 30 minutes. The suspension was centrifuged briefly to remove the alumina, the alumina was washed once again with the same buffer, and the supernatants were pooled. The cell extracts were centrifuged at 50,000 x g for one hour to remove large cellular debris and unlysed cells. The pellets were discarded and the supernatants were centrifuged again at 250,000 x g for three hours to pellet ribosomes. The pellets were resuspended in 10mM Tris-HCl, pH 7.4, 15mM $MgCl_2$, and 0.5M NH_4Cl and subjected to another 50,000 x g (one hour) and 250,000 x g (three hour) centrifugation. The pellets were then resuspended in either tight-couple 70S (TC70S) buffer

(10mM Tris-HCl, pH 7.4, 6mM $MgCl_2$, 60mM KCl) to isolate intact 70S ribosomes, or in 30-50 buffer (10mM Tris-HCl, pH 7.4, 1.5mM $MgCl_2$, 60mM KCl) to isolate ribosomal subunits. These crude preparations were purified further by zonal centrifugation through a gradient of diethylpyrocarbonate (DEPC) treated sucrose (10-38%) (Voorma et al., 1971). Appropriate fractions were pooled and the ribosomes or subunits were pelleted by centrifugation. The pellets were resuspended in either TC70S buffer or 30-50 buffer and dialyzed against 4 liters of the same buffer over 8 hours with one change of buffer. The ribosomes or subunits were stored in small aliquots at -70° C.

To check the purity of the subunits, i.e. for contamination with the opposite subunit, a sample (~50 pmol) was phenol extracted two times, ethanol precipitated, dried, resuspended in 15$\mu\ell$ tracking dye (7M urea, 0.025% xylene cyanol, 0.025% bromphenol blue) and loaded on a 5% polyacrylamide gel. The sample was electrophoresed for 4 hours at 15 mA, using 1X Tris-Borate (TBE) (0.09M Tris-Borate, 0.002M EDTA) as the running buffer. The gel was checked for degradation of the rRNA or for the presence of rRNA from the "wrong" subunit.

As another check for homogeneity of the sample, about 1000 pmol ribosomes or subunits were suspended in 700$\mu\ell$ of either TC70 buffer or 30-50 buffer and centrifuged at 52,000rpm in a Spinco Model E analytical ultracentrifuge (AND rotor) equipped

with schlieren optics. A homogeneous solution of subunits yielded a single peak that migrated from left to right across the screen. Contamination with the opposite subunit or evidence of subunit degradation was detected by the occurrence of multiple peaks. Tight couple 70S ribosomes usually started migrating as a single peak, but often partially dissociated into 30S and 50S subunits toward the end of the 20-30 minute run.

Synthesis and purification of oligodeoxynucleotides

DNA oligomers were synthesized on a Biosearch 8600 DNA Synthesizer using phosphoramidite chemistry. The oligomers were cleaved from the solid support by emptying the contents of the column into a screw-cap microcentrifuge tube and incubating in NH_4OH for 5 hours to overnight. The oligomers (probes) were then purified using NENPrep nucleic acid purification cartridges (DuPont). The crude probe mixture was loaded onto the column in 0.1M triethylamine acetate (TEAA) pH 7, then washed with acetonitrile:TEAA 1:9 to remove failure sequences while the tritylated probe remained bound to the column. The column was then washed with 0.5% trifluoroacetic acid (TFA) to cleave the trityl group from the probe and washed again with TEAA to remove excess acid. The probe was then eluted using 35% methanol into several fractions, which were measured spectrophotometrically (260nm) to determine the fractions containing DNA. The fractions were pooled, dried,

and resuspended in 500$\mu\ell$ water. The concentration of probe at this point was typically 1000-2000pmol/$\mu\ell$.

5'-end labeling of the DNA probes

Radiolabeling of the probes was accomplished using 3 units T4 polynucleotide kinase (United States Biochemicals), 10$\mu\ell$ γ^{32}P-ATP (3000 Ci/mMol, 3.3μM, New England Nuclear) and 35pmol probe in a total volume of 30$\mu\ell$ 1X reaction buffer (10X buffer supplied with enzyme by U.S.B.), according to the method of Chaconas and Van de Sande (1980). The reaction mixture was incubated at 37°C for 30 minutes and purified on NENSorb 20 nucleic acid purification cartridges as follows. The column was pre-washed with 100% methanol, then pre-equilibrated with 0.1M triethylamine (TEA) pH 7.7. The samples were loaded onto the column in 0.1M TEA pH 7.7 and washed with the same buffer. During this wash, the unincorporated nucleotides (ATP and ADP) and the polynucleotide kinase were removed, while the probe (labeled and unlabeled) remained bound to the column. The probe was eluted with 50% methanol into two fractions (the first contained the probe), dried and resuspended in 150$\mu\ell$ water.

To ensure that the radioactivity detected after the labeling reaction and purification was associated with the probe and not just residual unincorporated γ-^{32}P-ATP, approximately 100,000cpm of the labeled probe was loaded onto a 20% polyacrylamide/7M urea gel and electrophoresed for 45 minutes

at 40mA constant current. After 40 minutes, another 100,000cpm aliquot was added to an adjacent well and electrophoresed for the remaining 5 minutes to see if any fast-migrating molecules (e.g. degraded probe or unincorporated nucleotides) were present in the sample that may have run completely off the gel during the full length 45 minute electrophoresis.

This labeling procedure yielded probe with a very high specific activity (~3 x 10^6 cpm/pmol), much higher than necessary for the binding experiments. In order to reduce the specific activity, the appropriate quantity of unlabeled probe was added to the labeled probe to bring the specific activity down to about 500 cpm/pmol. Water and 10X binding buffer were then added to bring the concentration of the probe to 100 pmol/$\mu\ell$.

5'-end labeling of $tRNA^{Phe}$

Native tRNA is phosphorylated at its 5' end, therefore, the 5' terminal phosphate must be removed prior to treatment with polynucleotide kinase. Two hundred pmol deacylated $tRNA^{phe}$ (Subriden) were dephosphorylated using calf intestinal alkaline phosphatase (CIAP)(Boehringer-Mannheim) in 50$\mu\ell$ 20mM Tris-HCl, pH 8.0 at 50°C for 30 minutes. The mixture was then extracted twice with phenol to remove the CIAP, then extracted once with diethyl ether to remove traces of phenol. The

dephosphorylated tRNA was then ethanol precipitated with 3 volumes of 95% ethanol at -70°C for at least 30 minutes. After a 20 minute spin in a microcentrifuge, the pellet was washed with 70% ethanol and spun again for 10 minutes. The ethanol was then decanted and the pellet was dried in a spin-vac until just dry (about 10 minutes). The dried product was then phosphorylated using T4 polynucleotide kinase (USB) first using cold ATP and then γ^{32}P-ATP, so that the smaller fragments would be "cold-labeled", and only the intact tRNA would carry the ^{32}P label, since smaller fragments undergo phosphorylation more rapidly than intact tRNA. Labeled tRNA was purified using Nensorb 20 nucleic acid purification cartridges (Dupont), as described above. Homogeneity of the labeled product was checked by gel electrophoresis and autoradiography, as above (method of Lill and Wintermeyer, 1986).

Nitrocellulose filter binding assay

Binding of probes to ribosomes was quantified by filter binding assay (Backendorf et al., 1981; Tapprich and Hill, 1986). Increasing amounts (up to 40:1 probe:ribosomes) of radiolabeled probe (specific activity 500cpm/pmol, 100pmol/$\mu\ell$) were mixed with 25pmol ribosomes or subunits in a binding buffer containing 15mM $MgCl_2$, 10mM Tris-HCl, pH 7.4, 150mM KCl, 50$\mu\ell$ total volume. The mixtures were incubated at 37°C

for 20 minutes, then at room temperature for 20 minutes, and then on ice for 1 hour. The mixtures were then diluted to 1mℓ and immediately filtered through nitrocellulose filters (Millipore HAWP 45μm pore size) and washed with 2mℓ of the same buffer. Under these conditions, the nitrocellulose filters bind ribosomes and subunits, but very little free tRNA or probe. The radioactivity retained on the filters (less the radioactivity retained on control filters) therefore represents the probe or tRNA bound to the ribosomes or subunits, and is measured by liquid scintillation counting (Packard Tri-Carb 1500).

tRNA binding to ribosomes

Increasing amounts of deacylated $tRNA^{Phe}$, $Phe\text{-}tRNA^{Phe}$, or $N\text{-}Ac\text{-}Phe\text{-}tRNA^{Phe}$ were added to a constant amount of ribosomes or ribosomal subunits (with or without mRNA) in the binding buffer described above. The reaction mixtures were incubated at 37°C for 20 minutes, followed by a 20 minute room temperature incubation, then on ice for one hour. The samples were filtered and washed as described above, and the filters were assayed for radioactivity using liquid scintillation counting.

Antibiotic binding to ribosomes

Increasing amounts of ^{14}C-chloramphenicol (ICN Biomedicals,

100μCi/mℓ, 1850pmol/$\mu\ell$) were added to a constant amount of ribosomes or ribosomal subunits in binding buffer. The reaction mixtures were incubated at 37°C for 20 minutes, then at room temperature for 20 minutes, then allowed to incubate on ice for one hour. The reactions were diluted to 1mℓ and filtered immediately, followed by two additional 1mℓ washes with the same buffer. Chloramphenicol binding to the ribosomes or subunits was quantified by liquid scintillation counting of the filters.

The effect of tetracycline binding on probe binding was also tested, but the tetracycline was unlabeled, so its exact binding characteristics could not be determined. Ribosomes or subunits were incubated with increasing amounts of tetracycline (Sigma) up to 2,000:1 (tetracycline:ribosomes), and then saturating amounts of probe were added. Incubation was at 37°C for 20 minutes, room temperature for 20 minutes, ice for one hour, followed by filtration and liquid scintillation counting.

Multiple ligand binding experiments

It was determined in the course of this study that the order of addition of ingredients in multicomponent experiments did not affect their outcome. That is, a "chase" type experiment (i.e. pre-bind ligand A and try to dislodge with ligand B) yielded the same results as a "competition" type experiment (add both ligands simultaneously to vie for binding site).

Therefore, the order of addition was standardized as follows. Ribosomes or subunits were pre-incubated with increasing amounts of tRNA (up to 5:1 tRNA:ribosomes or subunits) or mRNA (up to 20μg/reaction) for 20 minutes at 37°C followed by addition of a saturating amount of probe (amount varies according to probe, but usually 20:1 probe to ribosomes or subunits) and incubated at 37°C for a further 10 minutes. Mixtures were then allowed to sit at room temperature for 20 minutes and then on ice for 1 hour before filtration, as above.

Ribonuclease H cleavage of probe-rRNA hybrid

RNase H cleavage reactions were carried out by incubating 50 pmol ribosomes, subunits, or rRNA with an excess of probe and 2 units of RNase H (Wako) in 50$\mu\ell$ of RNase H reaction buffer (40mM Tris-HCl, pH 7.4, 10mM $MgCl_2$, 60mM KCl) and incubated at 37°C for 30 minutes (modified procedure from Donnis-Keller, 1979). The reaction mixture was then extracted twice with phenol, ethanol precipitated, dried, resuspended in tracking dye and subjected to electrophoresis on a 5% polyacrylamide/7M urea gel. The size of the RNA fragments on the gel were estimated by comparison to RNA size markers (Bethesda Research Laboratories). The size of the RNase H cleavage fragments were an indicator of the site of hybridization of the probe on the rRNA.

Aminoacylation of deacylated tRNA

Aminoacylation of the tRNA was accomplished by incubation of $tRNA^{phe}$ (1 A_{260} unit) with 3500 pmol ^{14}C-Phe (NEN) and 2 units of aminoacyl-tRNA synthetase (Sigma) in a buffer containing 2mM ATP, 30mM HEPES pH 7.4, 15mM $MgCl_2$, 25mM KCl, 4mM DTT (Kristi Harrington, personal communication). The mixture was incubated for 15 minutes at 37°C, then phenol extracted and the phenol was back-extracted with water to enhance recovery of acylated tRNA. The aqueous portion was then ethanol precipitated and the free tRNA was separated from the aminoacyl-tRNA on a 1 x 15cm benzoylated DEAE cellulose (Serva) column (Rheinberger et al., 1983). The benzoylated DEAE cellulose was equilibrated for two hours at 4°C on a stir plate in a buffer containing 50mM NaAc, pH 5.0, 10mM $MgCl_2$, and 500mM NaCl. After slowly packing the column, the sample was loaded onto the column and washed with the same buffer (50 mℓ), followed by another wash with 80 mℓ of a buffer containing 50mM NaAc pH 5.0, 10mM $MgCl_2$, 580mM NaCl. The aminoacylated tRNA was then eluted with 50mM NaAc ph 5.0, 10mM $MgCl_2$, 2M NaCl, and 10% ethanol. Fractions (1.5 mℓ) containing the charged tRNA were collected and pooled, ethanol precipitated, dried, resuspended in 10mM Tris-HCl, pH 7.4 and stored at -70°C.

Acetylation of aminoacylated tRNA

N-Acetyl-Phe-tRNA^{Phe} was prepared by resuspending 1000 pmol of aminoacylated tRNA in 200$\mu\ell$ of 200mM NaOAc pH 5.0 followed by the addition of 2.5$\mu\ell$ acetic anhydride and incubation on ice for one hour. After one hour, an additional 2.5$\mu\ell$ acetic anhydride were added and incubated on ice for an additional hour. The N-Ac-Phe-tRNA^{Phe} was precipitated with ethanol, washed with ethanol, dried, and resuspended in 50$\mu\ell$ water (Moazed and Noller, 1989).

Chapter III

Results

Probe binding to 50S subunits

The binding of oligodeoxynucleotide probes to 50S subunits was assayed by nitrocellulose filtration, which under the buffer conditions used, retains ribosomes or ribosomal subunits and any attached probe, but not free, unbound probe. The percent binding of probe to subunits or ribosomes, therefore, is representative of the molar ratio of probe retained on the filter to ribosomes or subunits. Probe binding in this study varied from 3% to about 40%, depending on the probe and the hybridization conditions used. Probe binding typically attained its maximal value at a probe to ribosome or subunit ratio of about 20:1. Figure 10 shows the probe binding saturation curves for several of the more important probes used in this study, while table 1 shows the maximum percent binding of all probes assayed.

Probe binding to 70S ribosomes

Probe binding to 70S ribosomes was assayed in the same way as probe binding to 50S subunits, except that 25 pmol 70S ribosomes were used instead of 50S subunits. Several of the more important probes' saturation curves are shown in figure 11, and a more complete list of maximum binding values is

shown in table 1.

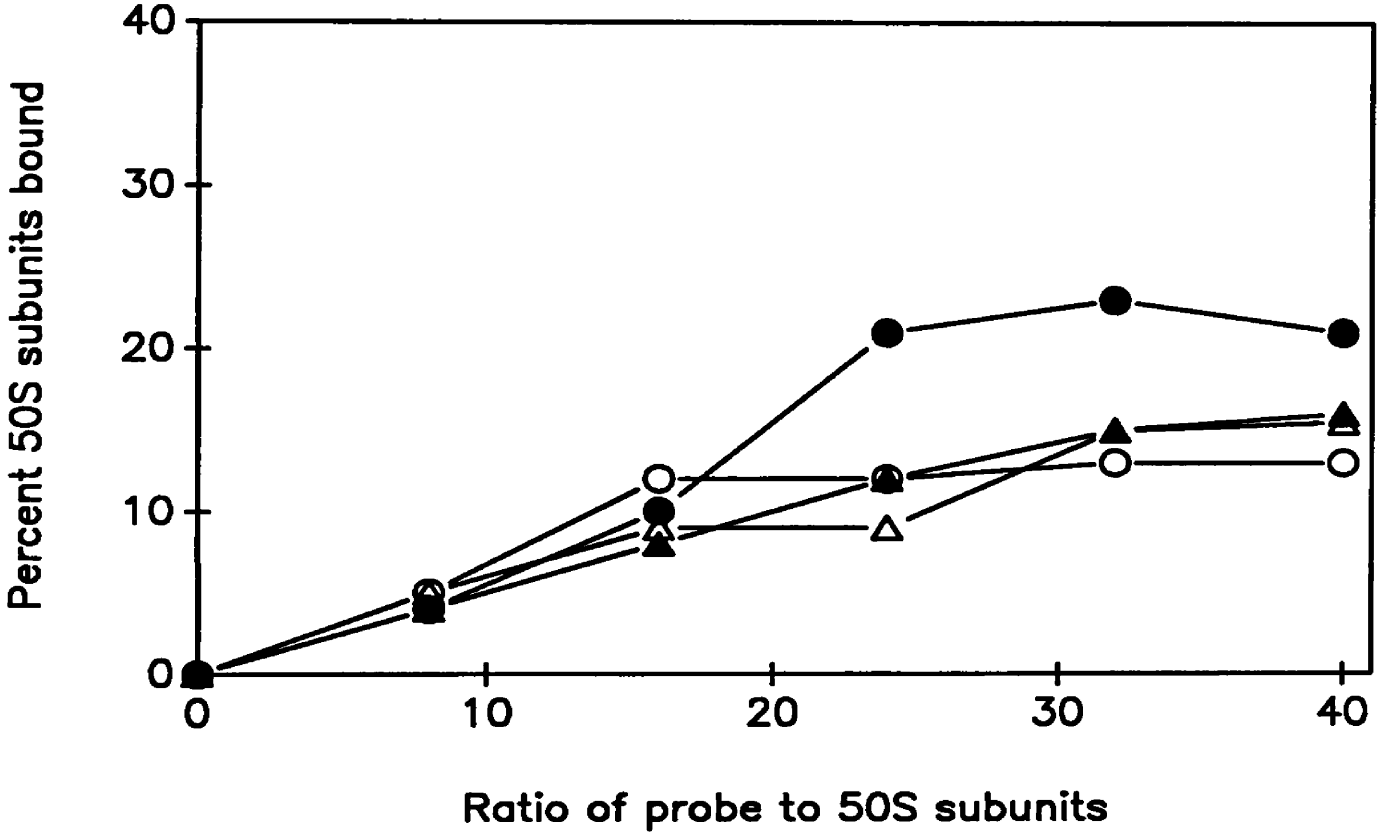

Figure 10: Saturation curves for probes 2109-2117 (open circles), 2109-2119 (closed circles), 2111-2117 (open triangles), and 2165-2171 (closed triangles). Increasing amounts of labeled probe were added to 25 pmol 50S subunits in binding buffer (15mM $MgCl_2$, 10mM Tris-HCl, pH 7.4, 150mM KCl), then incubated and filtered as described in Chapter II.

Table 1: Maximum binding values of probes to ribosomes or subunits. The double dash (--) means not determined.

Probe	Max. % binding 50S	Max. % binding 70S	Saturation ratio (probe:50S or 70S) 50S	Saturation ratio (probe:50S or 70S) 70S
1) 1834-1840	13	--	24:1	--
2) 1866-1876	18	--	32:1	--
3) 1882-1893	27	--	32:1	--
4) 1900-1905	19	--	24:1	--
5) 1906-1912	3	--	16:1	--
6) 1912-1918	8	--	16:1	--
7) 1931-1940	25	--	24:1	--
8) 2109-2115	9	12	16:1	16:1
9) 2109-2117	13	19	16:1	32:1
10) 2109-2119	22	26	24:1	32:1
11) 2111-2117	15	33	40:1	20:1
12) 2112-2118	--	40	--	26:1
13) 2113-2119	--	24	--	20:1
14) 2162-2173	--	8	--	32:1
15) 2165-2171	18	23	32:1	26:1
16) 2382-2394	9	12	40:1	32:1
17) 2386-2394	--	7	--	40:1

Binding of tRNA to ribosomes or ribosomal subunits

Binding of deacylated $tRNA^{Phe}$, Phe-$tRNA^{Phe}$, or N-Ac-Phe-$tRNA^{Phe}$ to ribosomes or subunits was assayed by adding increasing amounts of tRNA to 25 pmol ribosomes or subunits in the presence or absence of 12.5 μg poly U. Buffer conditions were as with probe binding (15mM $MgCl_2$, 10mM Tris-HCl, pH 7.4, 150mM KCl) and incubations were as described in Chapter II.

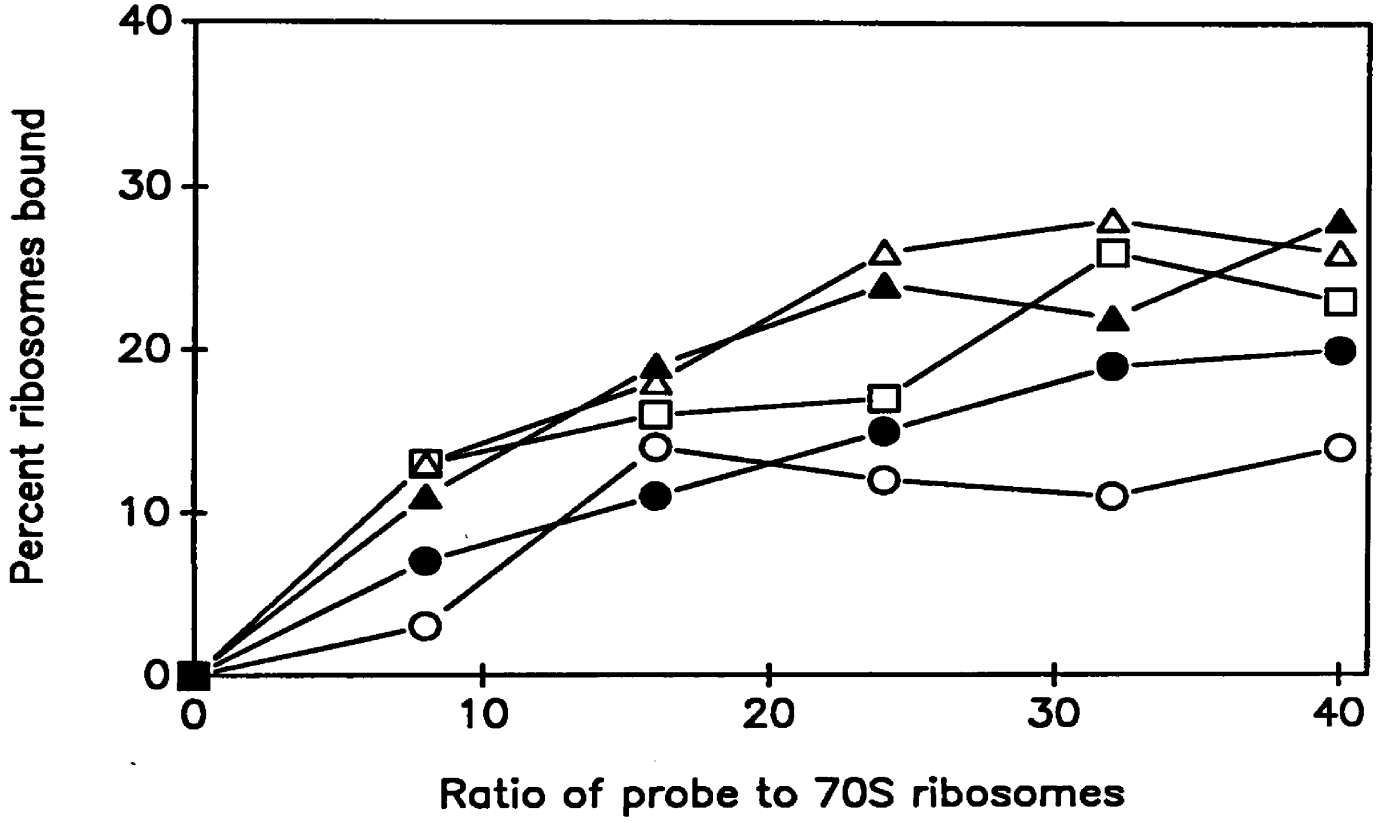

Figure 11: Binding curves of selected ^{32}P-labeled probes to 70S ribosomes. Probes 2109-2115 (open circles), 2109-2117 (closed circles), 2109-2119 (open triangles), 2113-2119 (closed triangles), 2165-2171 (open squares) were added to 70S ribosomes in increasing amounts, as described in the text.

Figure 12 shows binding of deacylated tRNA to 50S subunits in the absence of message, so that the tRNA binds only the P and E sites. Increasing amounts of 5'-labeled deacylated tRNA were incubated with 25 pmol 50S subunits up to a ratio of 5:1 tRNA:subunits in binding buffer. Incubation periods were as with probe binding assays, as was the filtration. Maximum tRNA binding was 40-45%, saturating at 3:1 tRNA to subunits.

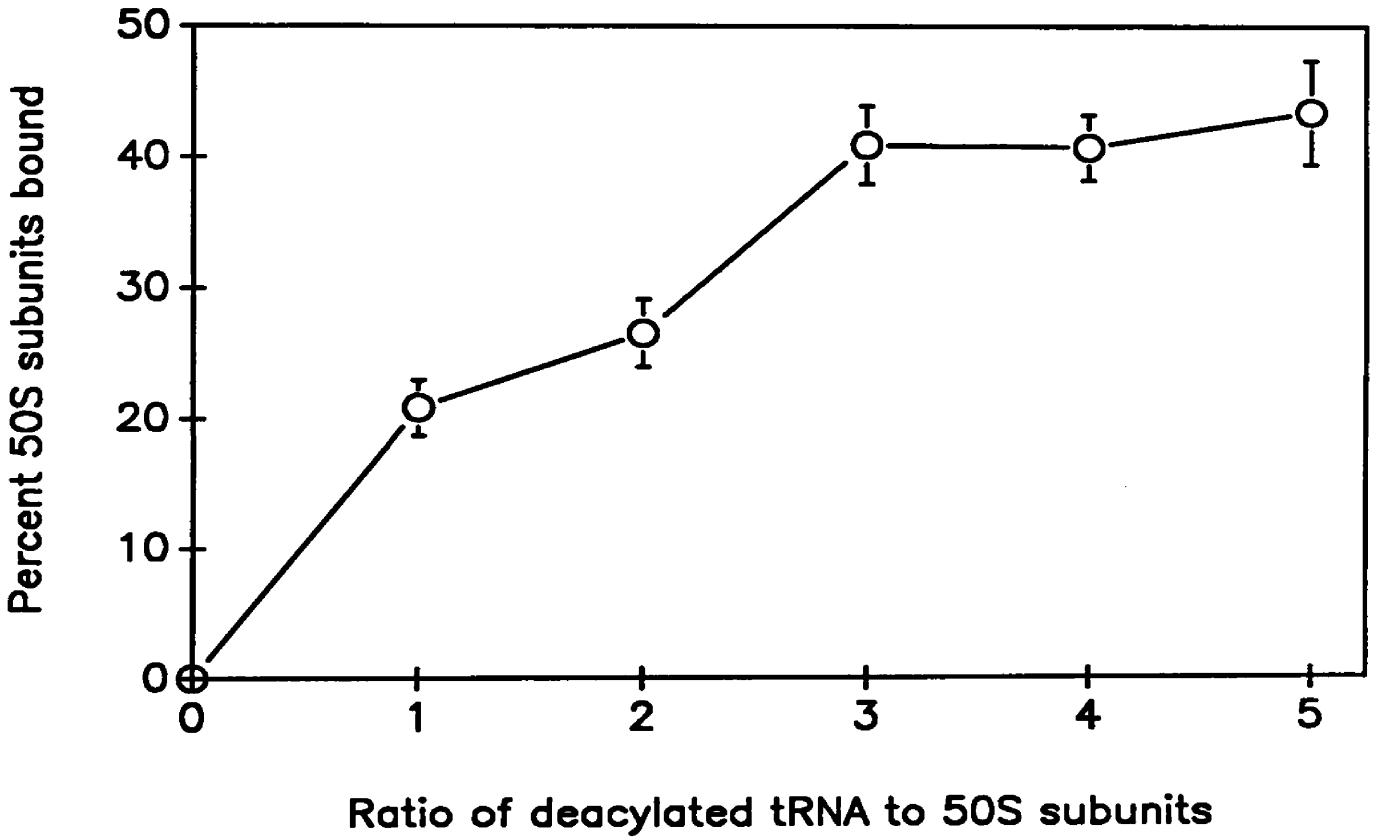

Figure 12: Deacylated $tRNA^{Phe}$ from E. coli binding to 50S ribosomal subunits. Increasing amounts of labeled deacylated tRNA were added to 25 pmol subunits, as described in text.

Binding of various species of tRNA from E. coli or from yeast to 70S ribosomes from E. coli is shown in figures 13a and 13b. The assorted tRNAs have different binding levels because each has a different affinity for the tRNA binding sites on the ribosome. Deacylated tRNA from E. coli binds at 87%, while deacylated tRNA from Saccharomyces cerevisiae has a maximum binding level of about 60% at a stoichiometric excess of 5:1 tRNA to ribosomes (see figure 13a). The difference in binding levels can be attributed to the inability of the yeast tRNA to bind the exit site of the E. coli ribosome (Lill et al., 1988). In the absence of message, E. coli deacylated tRNA binds the P and E sites only, as the A site is absolutely message dependent (Grajevskaja et al., 1982; Kirillov et al., 1983; Lill et al., 1984).

Figure 13b shows that Phe-$tRNA^{Phe}$ binds to the ribosome at 38% in the absence of poly U or at 103% in the presence of poly U (both cases in binding buffer consisting of 20mM $MgCl_2$, 10mM Tris-HCl, pH 7.4, and 150mM KCl with the same incubation and filtration scheme as described previously). N-Ac-Phe-$tRNA^{Phe}$ binds to 70S ribosomes at about 12 or 69%, in the absence or presence of poly U, respectively, in 15mM $MgCl_2$, 10mM Tris-HCl, pH 7.4, 150mM KCl. In a buffer containing 25mM $MgCl_2$, 10mM Tris-HCl, pH 7.4, 150mM KCl, the N-Ac-Phe-$tRNA^{Phe}$ binds at about 37% in the absence of poly U (data not shown). The binding of acylated and acetyl-acylated tRNAs is

restricted to the P site in the absence of message and to the P and A sites in the presence of message. Neither will bind the E site since the E site is specific for deacylated tRNA only. It should be noted that the binding of Phe-tRNA^{Phe} in the presence of poly U does not saturate in the tRNA concentration range used as shown in figure 13b. A possible explanation for this is the spontaneous formation of peptide

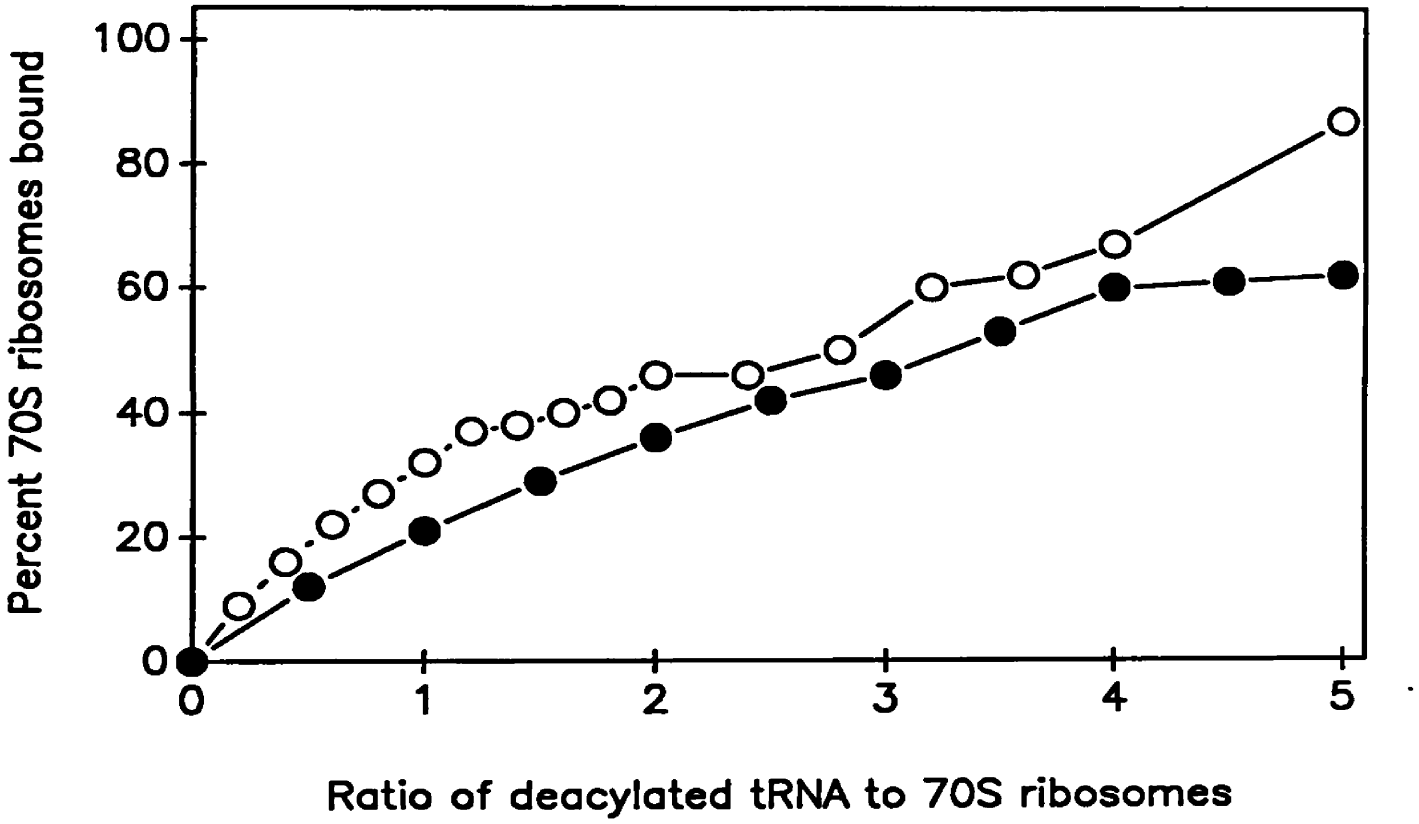

Figure 13a: Binding of deacylated tRNA^{Phe} from E. coli (open circles) and from S. cerevisiae (closed circles). Increasing amounts of ^{32}P-labeled tRNA were added to 25 pmol 70S ribosomes, followed by incubation and filtration as described in the text.

bonds that can occur even without elongation factors in artificial systems. Therefore, some of the ribosomes may be carrying short chains of ^{14}C-labeled phenylalanine at the higher tRNA concentrations, making the apparent binding level higher than expected.

Effect of deacylated tRNA binding on probe binding to subunits

The effect of tRNA binding on probe hybridization was assayed on 50S subunits by pre-incubating a saturating amount

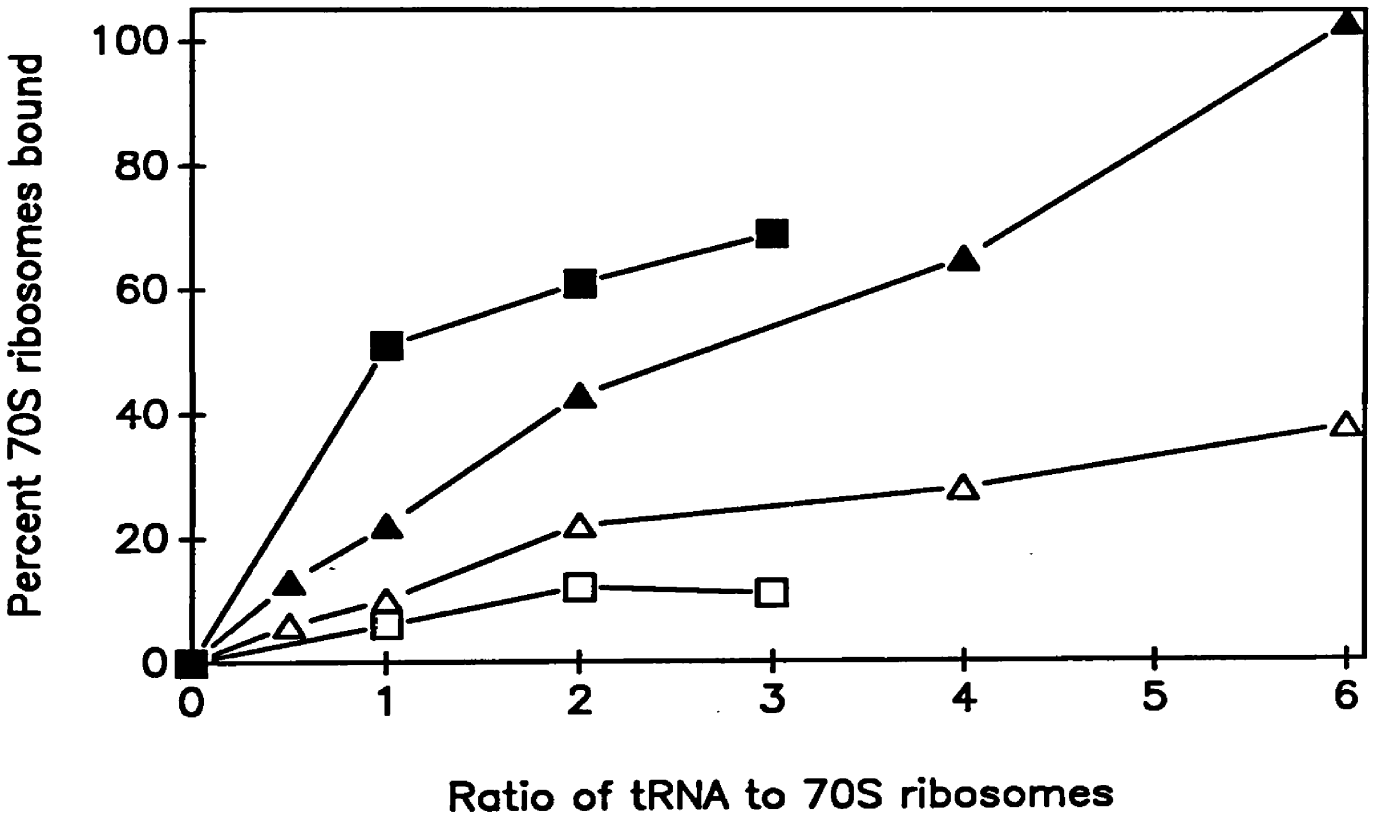

Figure 13b: Binding of acylated and N-acetyl-Phe-tRNA^{Phe} to 70S ribosomes in the presence and absence of poly U. Increasing amounts of Phe-tRNA^{Phe} without poly U (open triangles), and Phe-tRNA^{Phe} with poly U (closed triangles) or N-Ac-Phe-tRNA^{Phe} without poly U (open squares) or with poly U (closed squares) were added to 70S ribosomes. Binding conditions were as described in the text.

of labeled probe 2109-2119 or 2111-2117 with 25 pmol 50S subunits, then adding increasing amounts of deacylated tRNA. After a further incubation on ice, the reactions were filtered through a nitrocellulose filter and washed twice with binding buffer (25mM $MgCl_2$, 10mM Tris-HCl, pH 7.4, 150mM KCl) and assayed for radioactivity using liquid scintillation counting.

As figure 14 shows, the extent of probe binding is

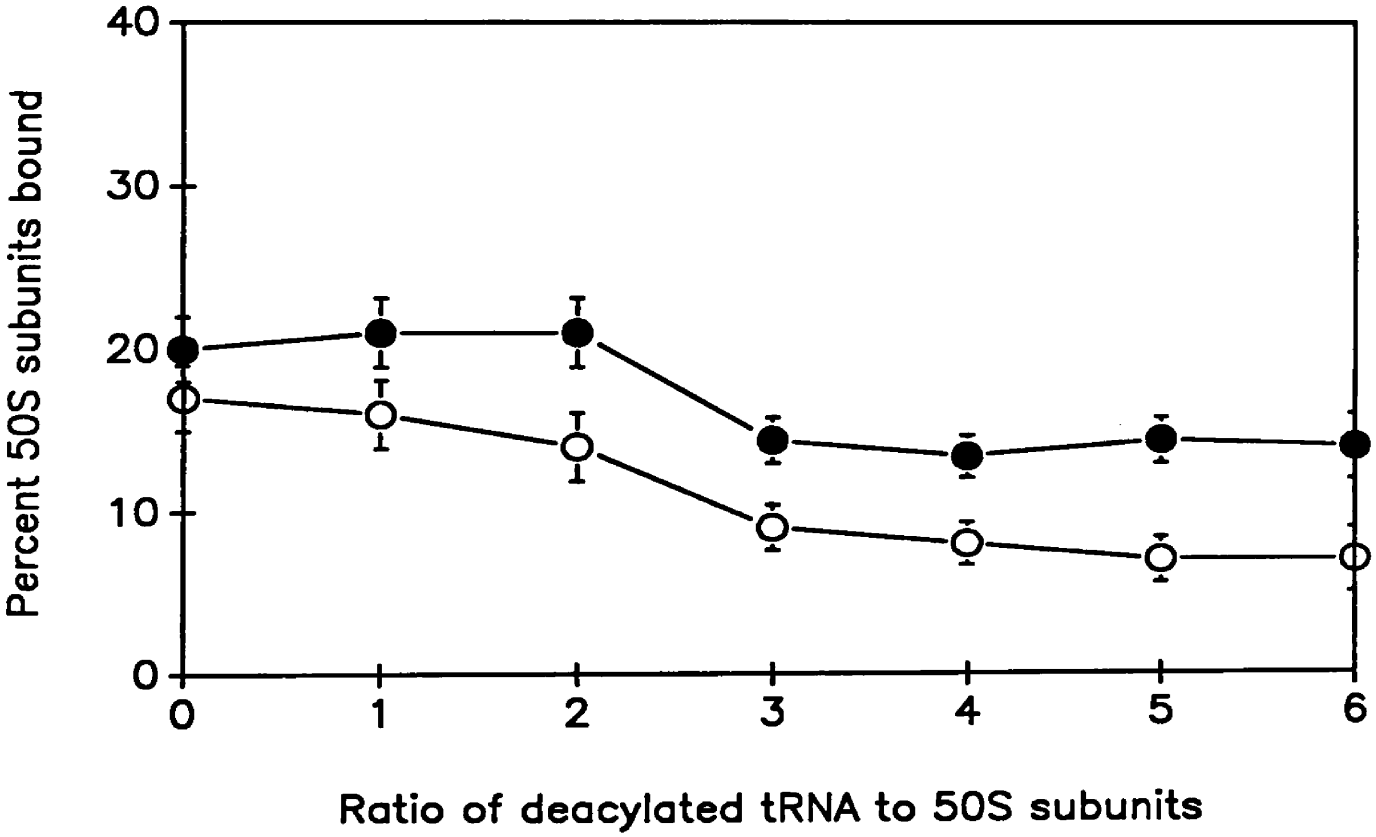

Figure 14: Effect of increasing deacylated tRNA on probe 2109-2119 (open circles) and probe 2111-2119 (closed circles) binding to 50S subunits.

attenuated, beginning when the tRNA:50S ratio is 2:1. Probe binding is cut roughly in half for probe 2109-2119, and is reduced by about 30% (compared to binding in the absence of tRNA) for probe 2111-2117. This strongly suggests that probe and deacylated tRNA compete for the same binding site in this location, or that tRNA causes a conformational change in the ribosome that inhibits probe binding. Furthermore, since these are conditions favoring E site tRNA binding, it appears that probes 2109-2119 and 2111-2117 hybridize in the E site. The effect of tRNA binding on probe 2165-2171 binding was investigated as well, but no effect was seen. This may indicate that the 2165-2171 probe does not bind in the same region as tRNA in the E site, or that the probe binds with an affinity greater than the afffinity of tRNA for this region and therefore cannot be displaced. Similarly, the effect of increasing deacylated tRNA on 2382-2394 binding was examined, and no effect was seen, although probe binding levels were quite low for precise measurement (data not shown).

Effect of tRNA binding on probe binding to 70S ribosomes

Several methods, probes, and tRNAs were used to examine the effect of adding tRNAs on probe binding. Figure 15 shows the results of a dual label experiment wherein probe 2109-2119 was 5'-labeled with ^{35}S and the deacylated tRNA was 5'-labeled with ^{32}P, so that simultaneous binding data could be obtained. Increasing amounts of labeled deacylated tRNA were pre-bound

to 25 pmol 70S ribosomes by incubating 20 minutes at 37°C, 20 minutes at room temperature, then 10 minutes on ice. An excess of ^{35}S-labeled probe 2109-2119 was then added and allowed to incubate for two additional hours on ice. Unlike the effect of deacylated tRNA on probe binding to 50S subunits, the addition of the first increments of tRNA (up to a ratio of 2:1 tRNA:ribosomes) actually enhanced binding of probe 2109-2119 to 70S ribosomes. At higher stoichiometries

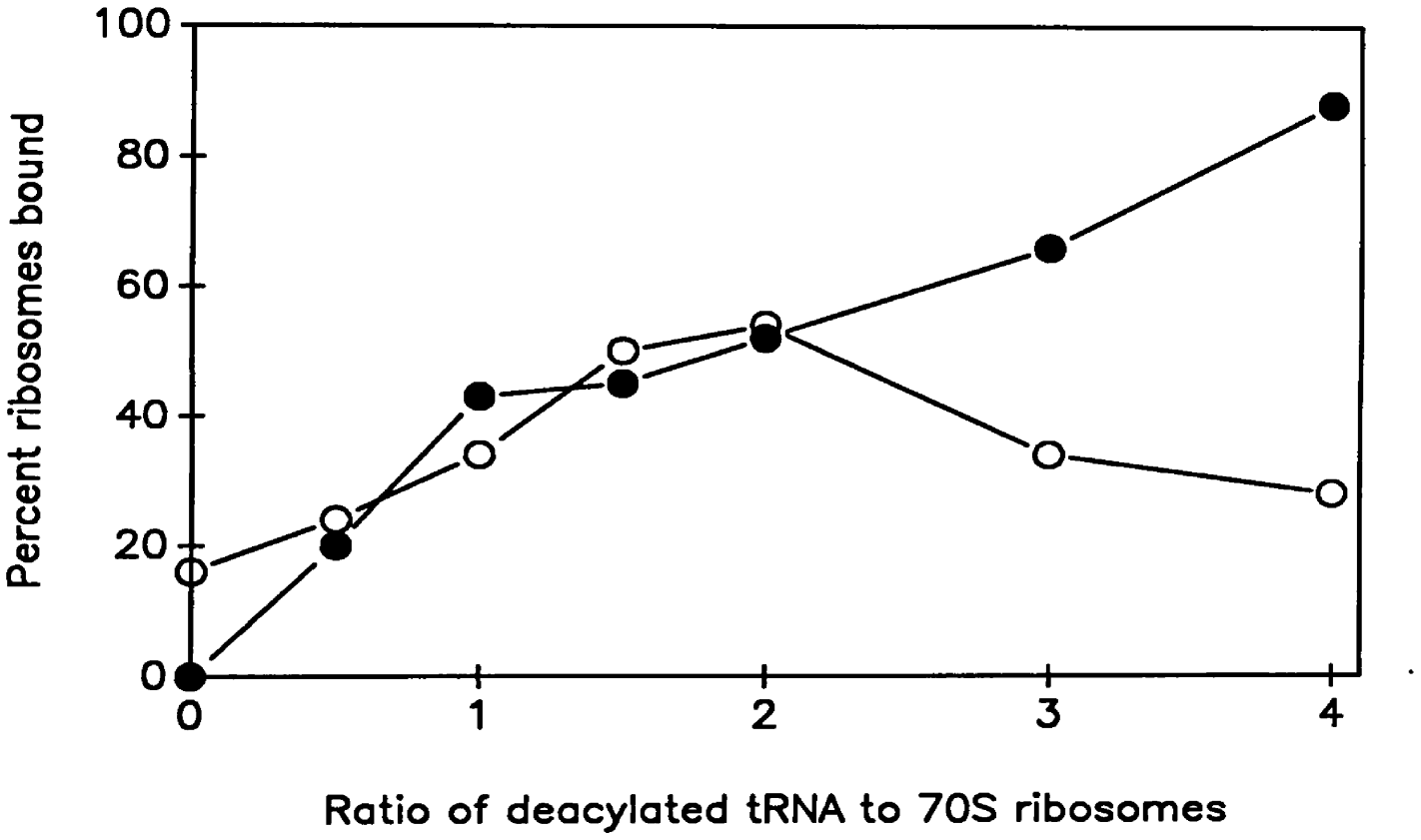

Figure 15: The effect of deacylated tRNA binding on probe 2109-2119 binding to 70S ribosomes. Ribosomes were pre-incubated with increasing amounts of ^{32}P-labeled tRNA (closed circles) and then a saturating quantity of ^{35}S-labeled probe (open circles) was added and allowed to incubate for an additional hour.

of tRNA, however, the probe binding was attenuated. The results of the dual label experiment were exactly what was seen in single label experiments in which either the tRNA or the probe (not both) was labeled. As explained before, binding of deacylated tRNA in the absence of mRNA in this binding buffer can be ascribed to the P and E sites, with the P site filling first. Rheinberger et al. (1981) and Lill et al. (1984) have shown that the E site is not significantly populated until the tRNA:ribosome ratio exceeds 1.5-2:1. This result is intriguing, as it shows that deacylated tRNA binding in the P site makes the 2109-2119 region, showed in the last experiment to be located in the E site, more available for probe hybridization.

Figure 16 shows the effect of increasing amounts of deacylated tRNA on probes 2111-2117, 2113-2119 and 2165-2171 binding. This experiment is the same as the last one, except that the tRNA is not labeled in this assay. tRNA binding was measured in separate reaction tubes, in contrast to the dual label experiment depicted in figure 15. The effect of tRNA on probes 2113-2119 and 2165-2171 binding is similar to the effect on probe 2109-2119 binding in that binding seems to be enhanced at low levels of tRNA, but probes 2113-2119 and 2165-2171 are not markedly displaced at higher stoichiometries of tRNA as is probe 2109-2119. In these experiments, probe binding and tRNA binding were monitored in separate tubes, as opposed to the dual label experiments where the binding of

both ligands was monitored simultaneously. Binding and incubation conditions were as described in Chapter II.

Effect of yeast deacylated tRNA on probe binding to ribosomes

The effect of binding deacylated $tRNA^{Phe}$ isolated from S. cerevisiae on probe 2109-2119 binding was also investigated, and the results are presented in figure 17. The previous experiments with deacylated tRNA from E. coli showed that its binding to the P site has a pronounced effect on the probe

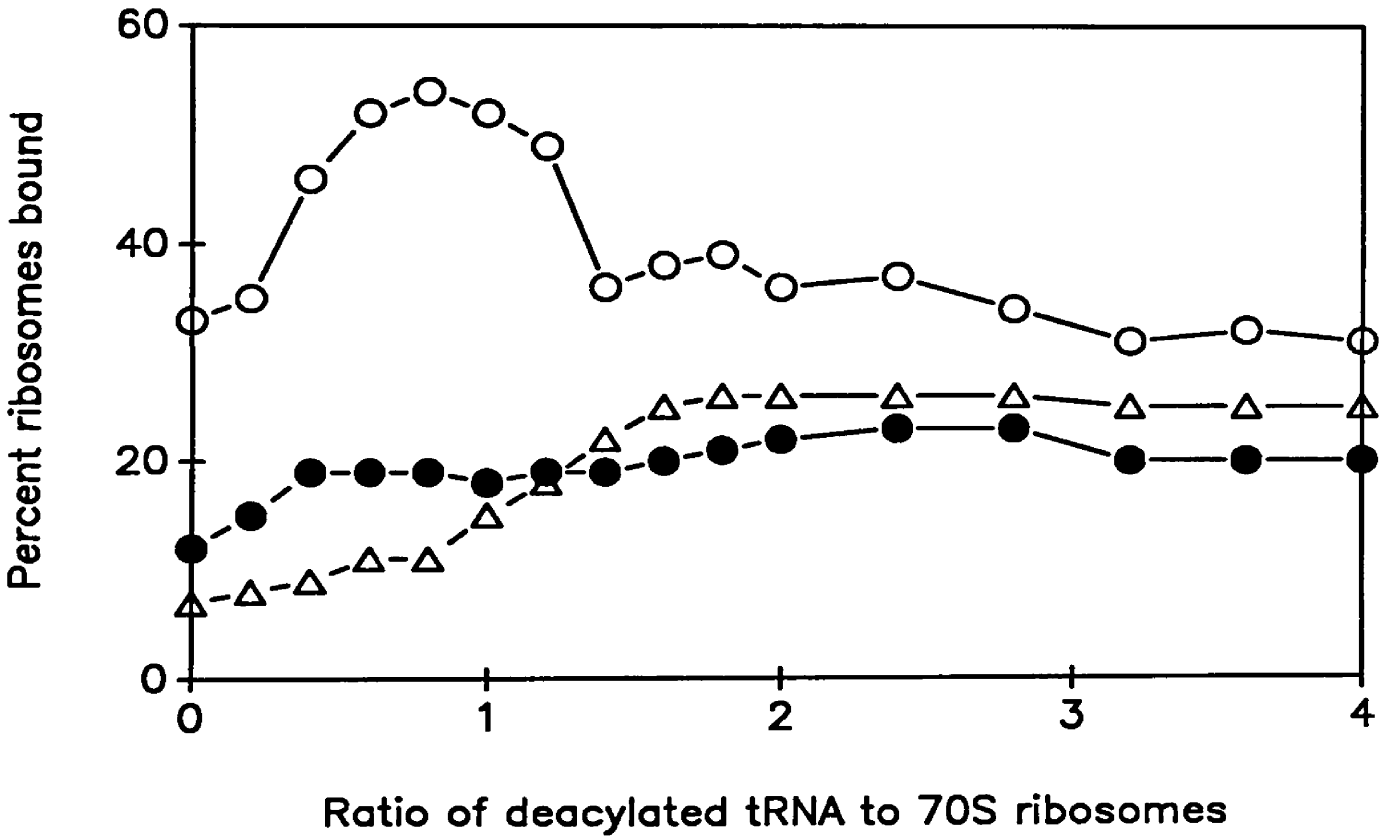

Figure 16: The effect of increasing tRNA concentration on probes 2111-2117 (open circles), 2113-2119 (closed circles), and 2165-2171 (open triangles) binding. Increasing amounts of unlabeled tRNA were added to 70S ribosomes followed by addition of an excess of labeled probe. Incubation and filtration were as described in chapter II.

binding environment in the E site. tRNA isolated from yeast does not bind the E site well (Lill et al., 1986), so it was desirable to see what effect, if any, yeast tRNA has on E site probe binding. As with the binding of E. coli tRNA to 70S ribosomes, the first increments of yeast tRNA to bind actually increases probe 2109-2119 binding, almost two-fold. However, the probe is not displaced at higher yeast tRNA concentrations, unlike the previous case. Binding and incubation conditions were as described in Chapter II.

Effect of Phe-tRNA and N-Ac-Phe-tRNA binding on probe binding

To determine the effect of binding acylated and acetyl-acylated tRNAs on probe binding to 70S ribosomes, E. coli $tRNA^{Phe}$ was acylated with ^{14}C-labeled phenylalanine. In the case of N-Ac-Phe-$tRNA^{Phe}$ binding studies, the acylated product was then acetylated, as described in chapter II. To make either Phe-$tRNA^{Phe}$ or N-Ac-Phe-$tRNA^{Phe}$ bind efficiently to ribosomes, it was necessary to either program the ribosomes with mRNA or an mRNA analogue (e.g. add poly U to the system), or to raise the magnesium concentration. Adding mRNA interfered with probe binding in some cases, so in these instances, the binding buffer used was 25mM $MgCl_2$, 10mM Tris-HCl, pH 7.4, 150mM KCl. In the absence of message, binding of either Phe-$tRNA^{Phe}$ or N-Ac-Phe-$tRNA^{Phe}$ to ribosomes could be only in the P site, since the A site is message dependent, and

the E site binds only deacylated tRNA.

Figure 18 shows that binding of Phe-tRNA^{Phe} does not enhance or inhibit probe 2109-2119 binding. Probe binding remains essentially constant at about 20% as Phe-tRNA^{Phe} binding increases to a maximum of about 80%. This result is striking,

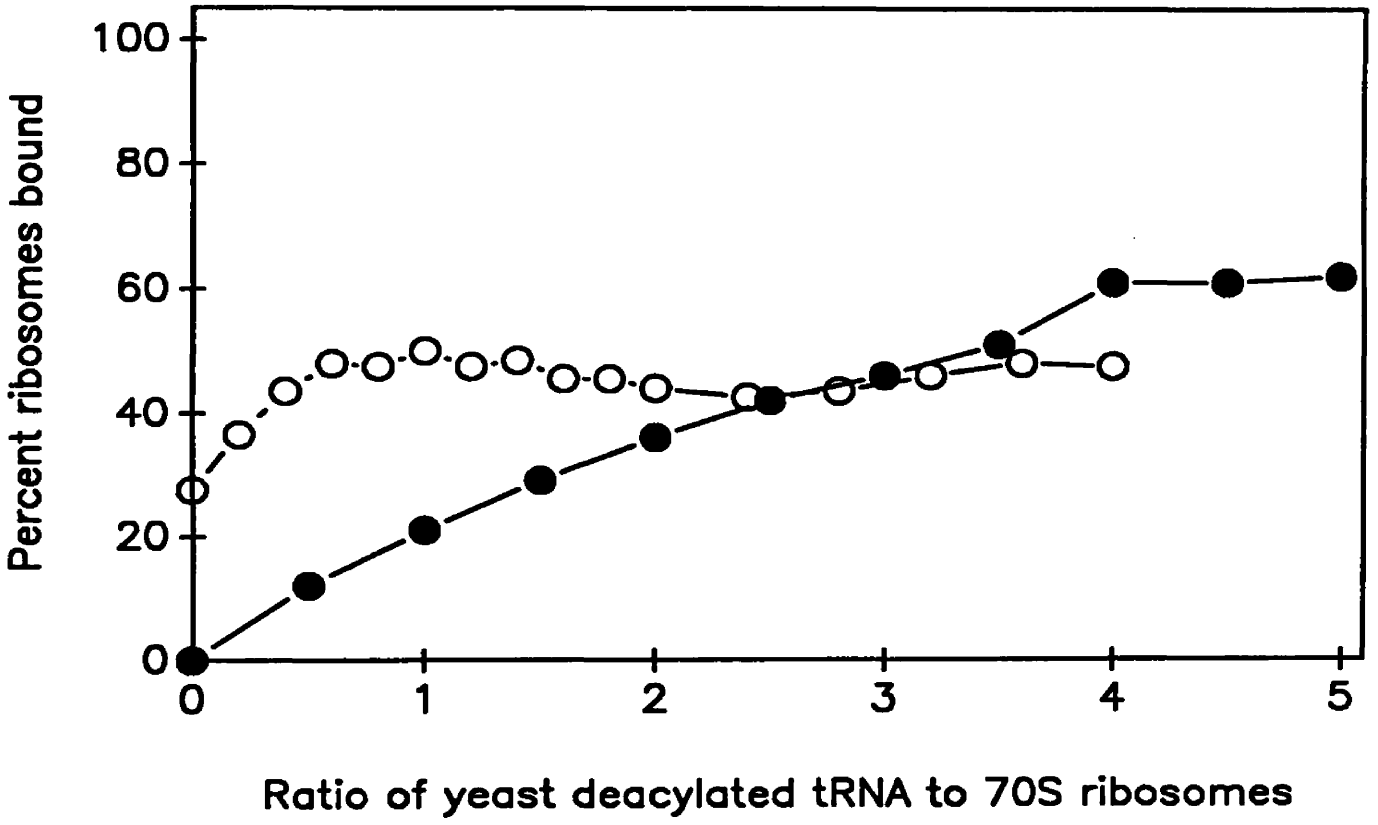

Figure 17: Effect of yeast tRNA binding on probe 2109-2119 binding to 70S ribosomes. Increasing amounts of yeast deacylated tRNA (solid circles) were pre-bound to ribosomes, then an excess of probe 2109-2119 (open circles) was added and incubated an additional one hour on ice.

since the acylated tRNA binds the P site, as the deacylated tRNA does, but this binding does not have the same effect on probe 2109-2119 binding. From this experiment it appears that only the presence of deacylated tRNA in the P site causes a change in the E site facilitating probe binding. Because poly U was used in this experiment, some binding to A site is likely, however, the P site fills first before A site binding occurs.

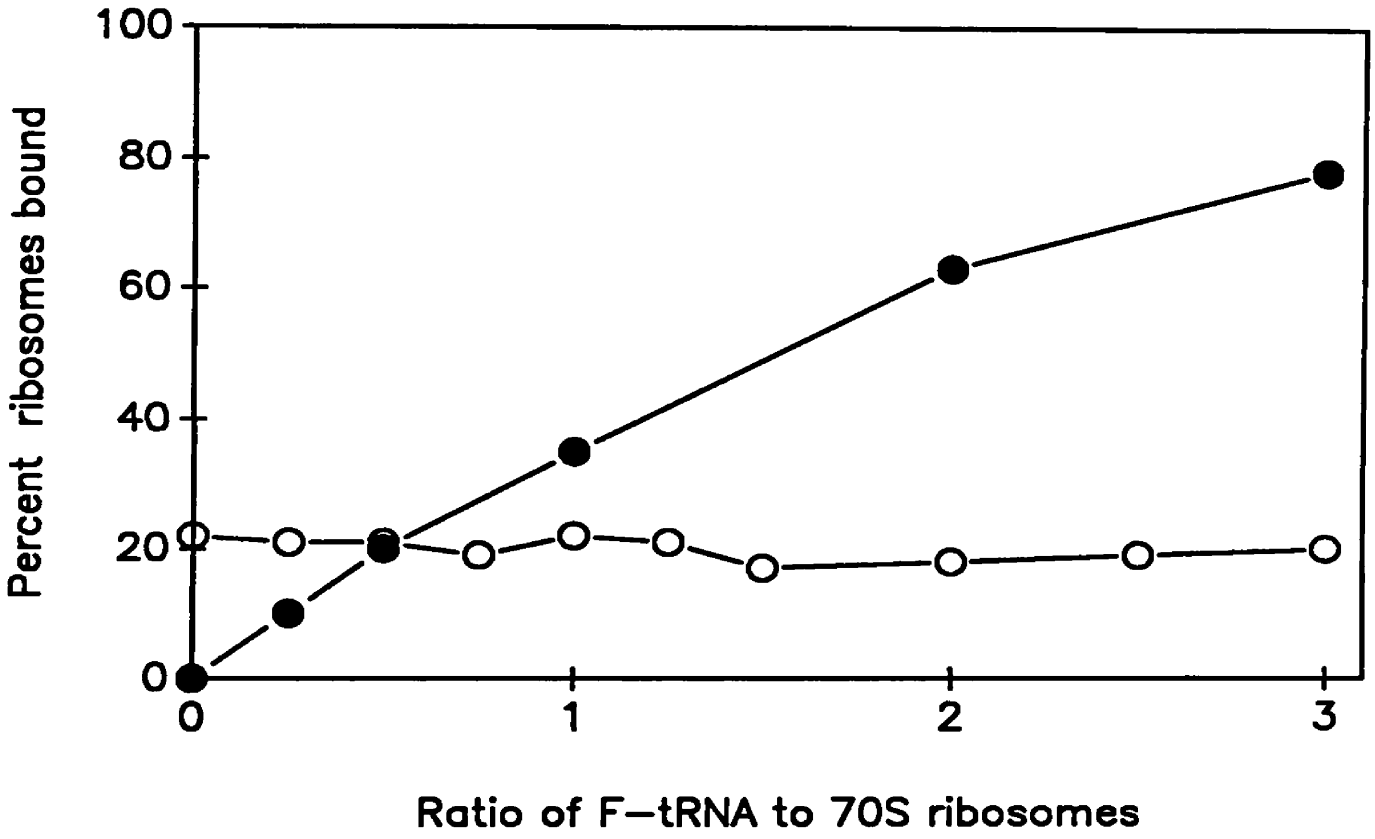

Figure 18: Effect of acylated $tRNA^{Phe}$ binding to ribosomes on probe 2109-2119 binding. Phe-$tRNA^{Phe}$ binding (closed circles) does not significantly affect the binding of probe 2109-2119 (open circles). Binding buffer included 12μg poly U per 25 pmol ribosomes, otherwise, binding and incubations were as described in Chapter II.

Similarly, figure 19 demonstrates that as increasing amounts of N-Ac-Phe-tRNA^{Phe} are added to ribosomes, the binding of probe 2109-2119 is not affected. In this experiment, no poly U was used, so only P site binding would be possible.

Effect of chloramphenicol on probe binding

Chloramphenicol is an inhibitor of the peptidyltransferase

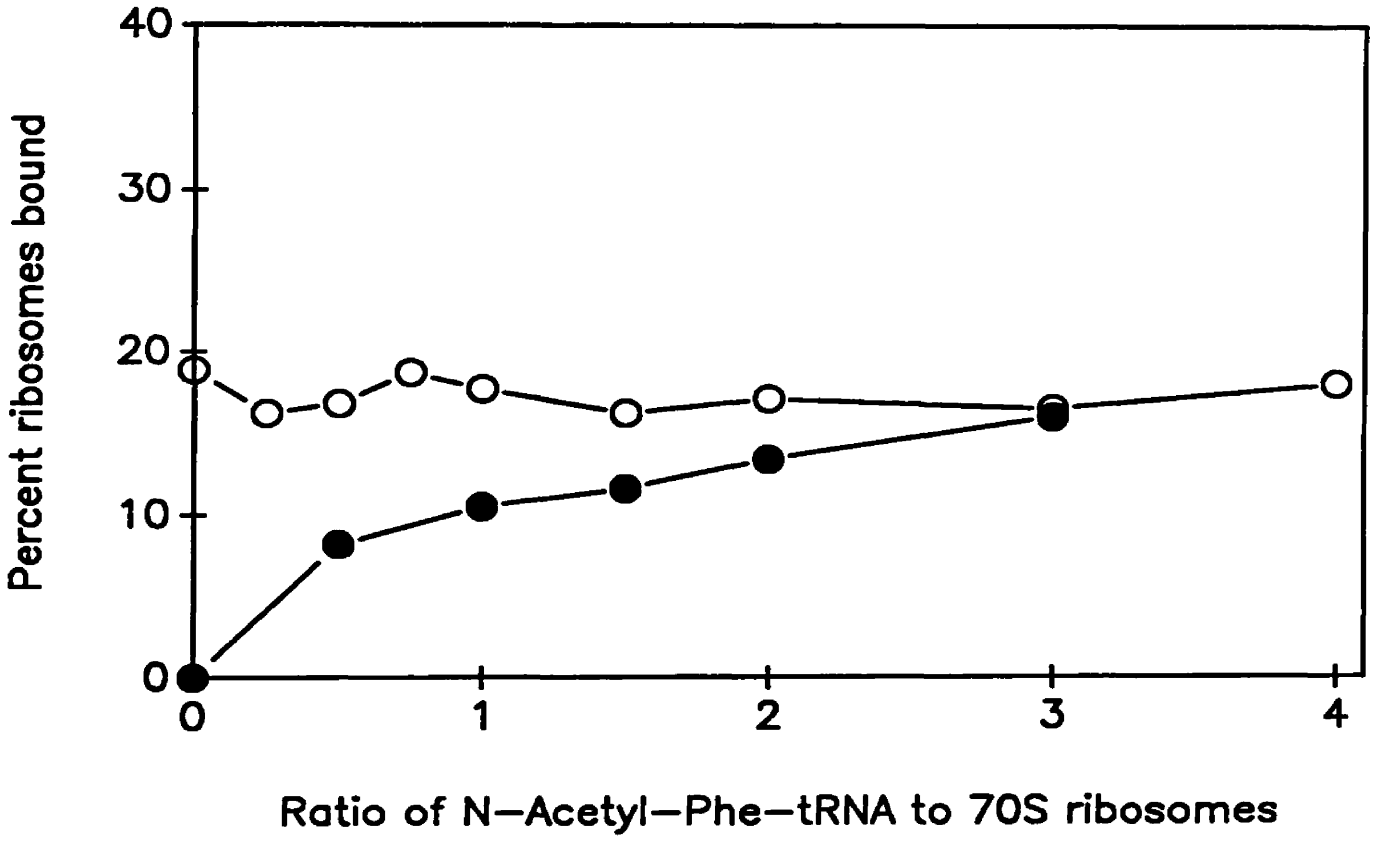

Figure 19: Effect of increasing N-Ac-Phe-tRNA^{Phe} on probe binding. Increasing amounts of N-Ac-Phe-tRNA^{Phe} (closed circles) were added to 25 pmol 70S ribosomes. An excess (20:1 probe:ribosomes) of probe 2109-2119 was then added and its binding was monitored (open circles). Incubation and filtration was as described in text.

reaction, and it may act as a tRNA analogue in the P site. The effect of chloramphenicol binding to 70S ribosomes on probe 2109-2119 binding was measured to see if its effects mimic those of P site bound tRNA. In figure 20, simultaneous binding data for chloramphenicol and probe 2109-2119 are presented. Increasing amounts of chloramphenicol were incubated with 15 pmol 70S ribosomes and a 20:1 excess of

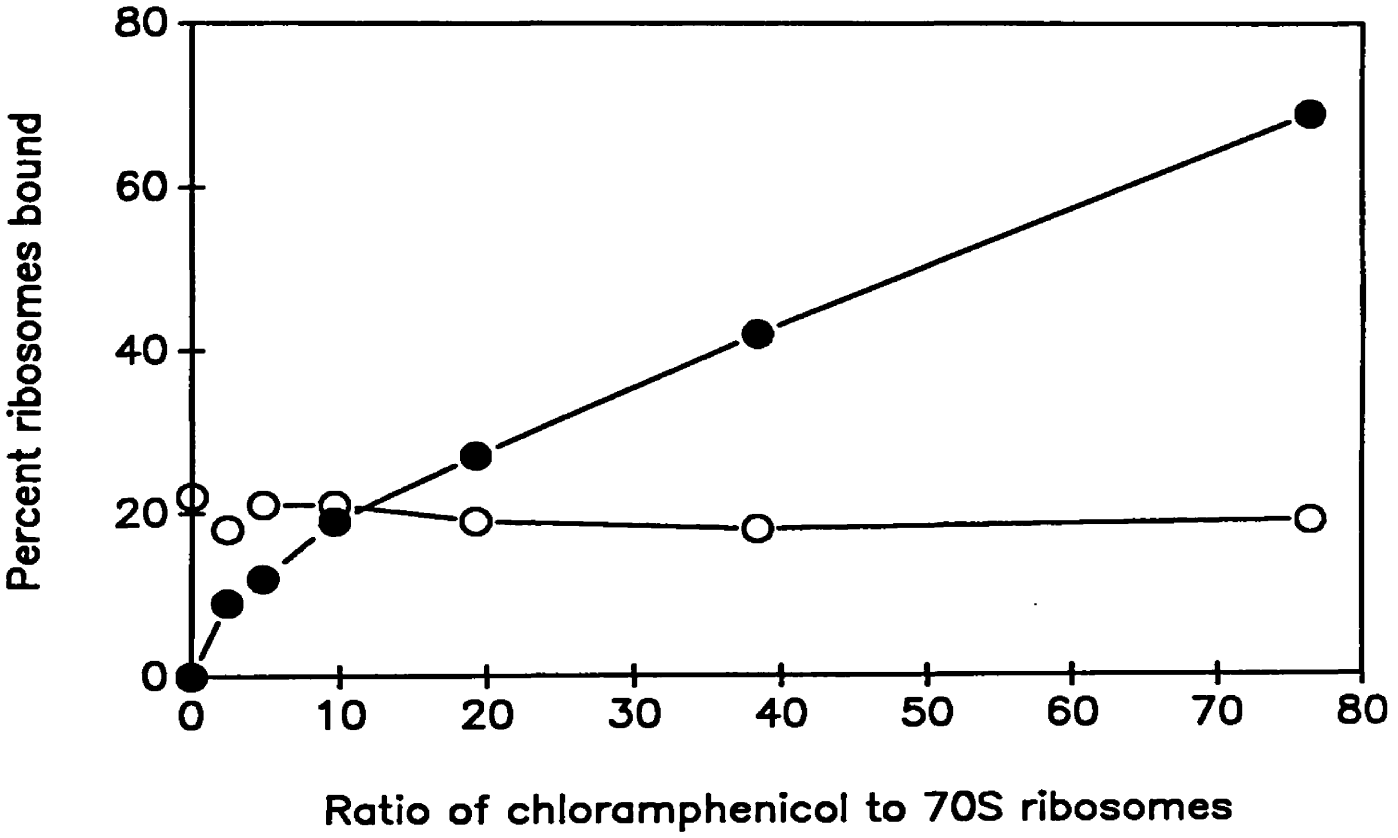

Figure 20: Effect of chloramphenicol on probe 2109-2119 binding to 70S ribosomes. Increasing amounts of ^{14}C-labeled chloramphenicol were incubated with 70S ribosomes, then a 20:1 excess of ^{32}P-labeled probe was added. Incubation and nitrocellulose filtration were as described in text.

probe was subsequently added. Following a further incubation, the reaction mixtures were subjected to nitrocellulose filtration and liquid scintillation counting as described in Chapter II. The results show that even at high concentrations of chloramphenicol, probe 2109-2119 binding is not affected by chloramphenicol binding.

Effect of tetracycline on probe 2109-2119 binding to ribosomes

Tetracycline is an inhibitor of A site tRNA binding, and the effects of its binding to 70S ribosomes on probe 2109-2119 binding was determined. The tetracycline was unlabeled, so its precise binding characteristics could not be determined. Increasing amounts of tetracycline (up to 2000:1 tetracycline:70S ribosomes) were pre-incubated with 25 pmol ribosomes then an excess of ^{32}P-labeled probe 2109-2119 was added, followed by a 20 minute 37°C incubation, a 20 minute room temperature incubation, and a one hour incubation on ice. The samples were then filtered and counted as usual, and the results are shown in figure 21. Even at very high concentrations of tetracycline (about 2000:1 tetracycline to ribosomes), probe 2109-2119 binding was unaffected.

Effect of probe binding on tRNA binding

The effects of various tRNAs binding on probe binding has been demonstrated in this chapter, but the question of whether probe binding affects tRNA binding has not yet been addressed.

The following experiments were performed to determine various probes' effects on tRNA binding.

Effect of increasing probe on tRNA binding to 50 subunits

The ability of probes 2109-2119 and 2165-2171 to displace tRNA was tested by incubating 50S subunits with a three-fold excess of ^{32}P-labeled deacylated tRNA and adding increasing quantities of cold probe. tRNA binding was not affected by the addition of up to 40 pmol probe per pmol subunits (data

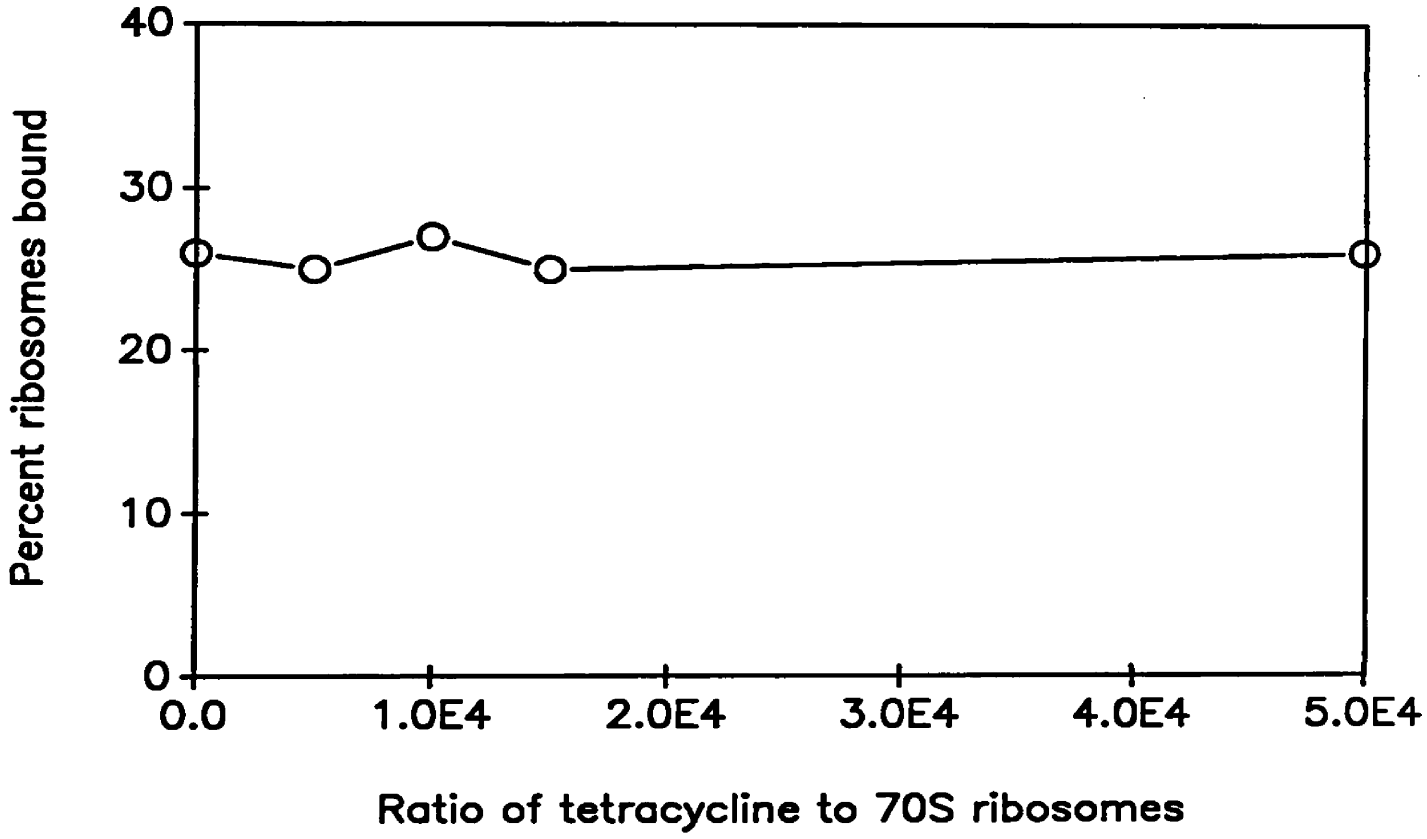

Figure 21: Effect of tetracycline on probe 2109-2119 binding to 70S ribosomes. Increasing amounts of unlabeled tetracycline were pre-incubated with ribosomes, then an excess of ^{32}P-labeled probe 2109-2119 (open circles) was added. Incubation and filtration were as described in the text.

not shown).

Effect of E site probe binding on N-Ac-Phe-tRNA^{Phe} binding

To investigate the possibility that probes bound in the E site may have an allosteric effect on N-Ac-Phe-tRNA^{Phe} binding in the A site, 25 pmol of 70S ribosomes were pre-incubated with 25 pmol deacylated tRNA (to fill the P sites) and an excess (6:1) of either probe 2109-2119 or 2165-2171 or both. After pre-incubation, a five-fold excess of N-Ac-Phe-tRNA^{Phe} was added to the reaction mixtures, incubated on ice for a further 30 minutes and filtered as usual. N-Ac-Phe-tRNA^{Phe} binding in the A site was not affected by the presence or absence of either or both probes (data not shown).

Cleavage of 23S rRNA using probes and RNase H

When the probes used in this study were incubated with either 50S subunits or 70S ribosomes and treated with RNase H, none of the probes produced a reproducible cleavage of the rRNA as determined by polyacrylamide gel electrophoresis (data not shown). Since deacylated tRNA binding to the ribosome enhances probe binding, the prospect that the vicinity of probe binding may become more available for RNase H cleavage in the presence of deacylated tRNA was tested. 50 pmol of 70S ribosomes were pre-incubated with 50 pmol deacylated tRNA in the presence of an excess of probes 2109-2119 or 2165-2171 in

RNase H buffer (10mM $MgCl_2$, 40mM Tris-HCl, pH 7.4, 60mM KCl) or in binding buffer. After 30 minutes 1 unit of RNase H was added, and the reaction mixtures were incubated at 37°C for 30 minutes, then at 4°C for one hour. The RNA was then isolated and electrophoresed on a 5% polyacrylamide gel. No RNase H cleavage of the rRNA was detected.

Probes hybridized to naked 23S rRNA and subjected to RNase H cleavage all yielded fragments of the expected sizes, as determined by comparison to migration of known size markers in the gel. Figure 22 shows representative clips from various probes used in this assay.

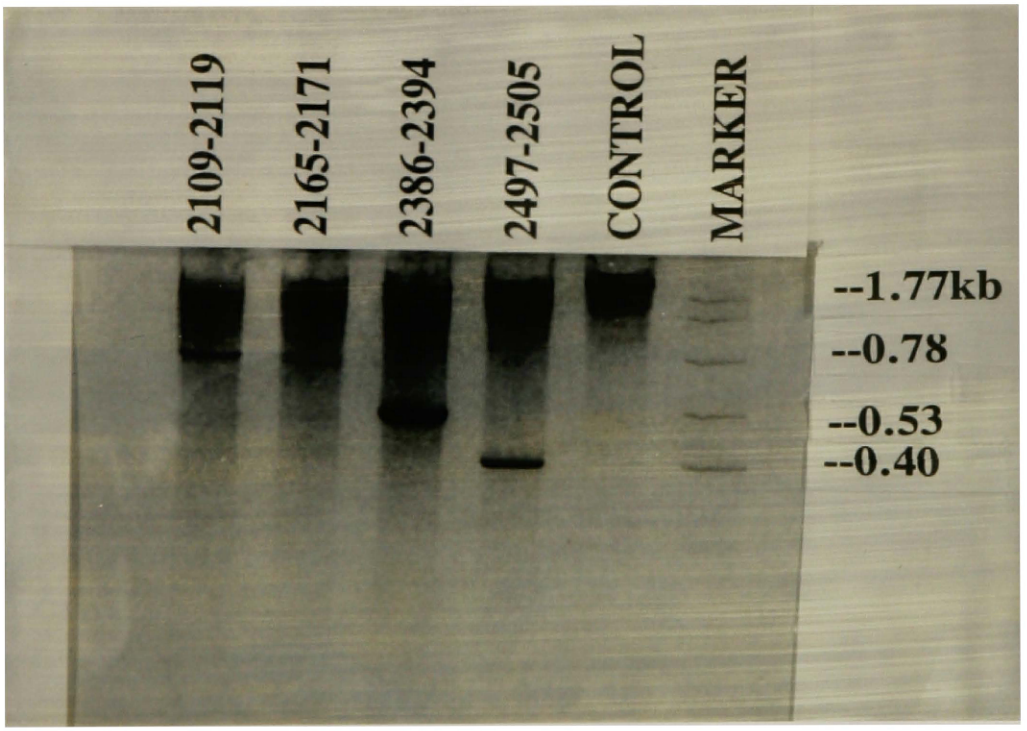

Figure 22: RNase H cleavage of 23S rRNA with various probes. From left to right: probes 2109-2119, 2165-2171, 2386-2394, 2497-2505. Reaction conditions are described in text.

Chapter IV

Discussion

New evidence for an allosteric linkage between tRNA binding sites P and E in the *Escherichia coli* ribosome

Whereas the overall functioning of the ribosome is fairly well understood, its workings at the molecular level are still elusive. In particular, the rapidity and accuracy with which this macromolecule does its job requires that it have finely tuned control mechanisms to regulate individual steps in protein synthesis with a light enough touch to ensure speed, yet a firm enough grasp to maintain accuracy. In this study, evidence is presented that strongly suggests a new control mechanism in the process of translocation during the elongation cycle. The research outlined here demonstrates that the P site can discriminate between different types of tRNA, and depending on the tRNA bound, can effect a conformational change in the local environment of the E site.

Specifically, evidence is presented that upon binding a deacylated tRNA in the P site, the E site spontaneously becomes more available for cDNA probe binding, and by analogy, tRNA binding. This is appealing teleologically, since in the process of elongation, a deacylated tRNA in the P site immediately precedes translocation, therefore the exit site should be made ready to accept a deacylated tRNA. Taken one step further, the presence of a deacylated tRNA in the P site

triggers the exit site to attain a high affinity conformation to "entice" discharged tRNA from the P site. Such an affinity-switch linkage between the P and E sites has not been described before.

Probing ribosome structure and function with cDNA probes

The use of complementary oligodeoxynucleotides to investigate various properties of nucleic acids in general and ribosomes in particular has a fairly extensive history. These cDNA probes have been useful in elucidating the structure of 5S rRNA ((Lewis & Doty, 1970)), determining the general morphology of the 30S ribosomal subunit using cDNA hybridization electron microscopy (reviewed in Oakes et al., (1990), in crosslinking studies of the peptidyltransferase center (Muralikrishna & Cooperman, 1991), and they have been used extensively in our laboratory to investigate the fine structure and function of local environments on the Escherichia coli ribosome (see reviews in Hill et al. (1988) and Gryaznov & Sokolova (1990)).

In this study, cDNA probes complementary to single stranded regions in Domains IV and V were constructed and tested for their ability to hybridize to either 50S subunits or 70S ribosomes. Of the probes listed in Table 1, probes numbered 1 through 7 are directed toward single stranded segments of a tRNA-like region in Domain IV. Because of the region's apparent similarity to a tRNA molecule, it appeared to be a

logical target for probing to see if it had any discernable functions. In this study, binding data were obtained for probes in this region, but it still has not been extensively investigated. Probes spanning nucleotides 1882-1893 and 1931-1940 bind at 27 and 25%, respectively, which means these regions are available for further investigation using cDNA probes.

Probes numbered 8-17 in Table 1 span different segments of three single stranded regions in Domain V thought to be important in binding tRNA in the ribosomal exit (E) site. The bulk of the research presented here pertains to interactions between the cDNA probes, rRNA in Domain V, and tRNAs.

Availability of rRNA for hybridization with cDNA probes

Table 1 shows the maximum binding values for each of the probes tested in this study. Binding values range from 3% to 33%, none of the probes gives quantitative hybridization with ribosomes or subunits. The reason (or reasons) for this sub-stoichiometric hybridization has not been unambiguously determined, but several plausible explanations are discussed below.

One possible explanation for less than quantitative probe binding is that our ribosome preparations contain a mixed population of ribosomes. In this view, one can imagine that a certain percentage of ribosomes in solution are available for (quantitative) probe binding, and the others are not. In

growing cells, because there is a constant recycling of cellular machinery, including ribosomes, it seems obvious that there would be ribosomes present in any preparation that are in various stages of assembly or degradation. Therefore, the mixed population theory probably contributes to the overall effect. However, some probes have been shown to bind quantitatively to ribosomes from several different preparations (reviewed in Hill et al., 1990) so this is not an adequate explanation in all cases.

Another related theory is that the mixed populations of ribosomes in a preparation are representatives of different conformational states of the ribosome. Some of the conformers would be available for hybridization with probes, others would not. This view is supported by evidence presented in this study (discussed further below) and by experiments done by Merryman (discussed in Hill et al., 1990), showing that some regions of 23S rRNA are available for RNase H cleavage only at certain times during the elongation cycle.

A third factor contributing to sub-stoichiometric binding of probes to ribosomes could be the so-called kinetic effect of the filter binding assay. Briefly, the kinetic effect (discussed in Schmitt et al. (1984) and Robertson & Wintermeyer (1981)) is a general term used to describe what may happen on the molecular level when the equilibrium of a binding experiment is perturbed by dilution and washing on the filter. For example, in the equilibrium conditions of the

test tube, nearly all the ribosomes may be bound with a probe at any given time, but the probe may have a rapid on/off rate, so when the reaction mixture is diluted, filtered, and washed, the apparent binding value is diminished.

Strong evidence for the kinetic effect phenomenon using cDNA probes has recently been put forth by Charles Rettberg of Hill's group. Several of his experiments have shown that probes showing negligible binding by filter binding assay give strong RNase H clips under the same binding conditions as the filter binding assay (unpublished results). One interpretation of this result is that the probe hybridizes to its target site sufficiently well to permit cleavage of the rRNA by RNase H under equilibrium binding conditions, but when the solution is diluted and the filter is washed, most of the probe diffuses away. In other words, if the binding half-life of a probe is sufficiently short, significant quantities of bound probe can be removed from the ribosome during the filtration procedure. If this is the case, then binding data must be interpreted in relative terms, that is, the final binding values must be seen as some definite fraction of the binding in equilibrium conditions.

Binding levels of probes to ribosomes or ribosomal subunits is quite reproducible (i.e. within 10% error on replicate samples) between experiments using a given preparation of ribosomes or subunits. Occasionally there is variation in the absolute binding values for a given probe on different

preparations of ribosomes, due perhaps to some subtle difference in the way the ribosomes were prepared. Binding experiments for all the probes were conducted using several preparations of ribosomes or subunits, and results reported are representative of only the reproducible experiments.

According to chemical protection data produced by Moazed and Noller (1989), several nucleotides have been implicated in tRNA binding in the exit site. Probes were constructed that were complementary to the single stranded regions of 23S rRNA containing these nucleotides. In particular, nucleotides U_{2111}, G_{2112}, and G_{2116} are located in the 2109-2119 single stranded region, nucleotide A_{2169} is located in the 2162-2173 single stranded region, and nucleotide $^{5}{}_{m}C_{2394}$ lies at the 3' end of the 2382-2394 single stranded region. Probes used in this study spanned all three of these single stranded regions, covering different combinations of the implicated nucleotides. Probe 2109-2119, for example, spans three tRNA-protected nucleotides, whereas 2113-2119 spans only one.

Probe 2109-2119 gave the most consistent binding characteristics of the probes described in this study, and therefore was used in all of the experiments. Shorter versions of this probe (i.e. 2109-2115, 2109-2117, and 2111-2117) generally gave similar results, but with more experimental noise. The increased stability of hybridization one would expect using a longer probe probably accounts for

the more consistent results. This probe bound at 22 and 26% on 50S subunits and 70S ribosomes, respectively, demonstrating that this region is available for probe binding studies.

Probe 2165-2171 bound 50S subunits at 18% and 70S ribosomes at 23%. In this region, no greater binding was afforded by using a longer probe. A probe spanning 2162-2173, for example, bound 70S ribosomes at only 8%. This suggests that there may be some steric constraint in this region (e.g. a ribosomal protein may bind here), or that some secondary or tertiary structure exists here that is not shown on the 23S rRNA secondary structure maps. Probe 2165-2171 bound ribosomes and subunits in a reproducible manner, suggesting that in most preparations of ribosomes or subunits, this region was available for hybridization with a cDNA probe.

In contrast to the two single stranded regions described above, the 2382-2394 region of 23S rRNA was not available in most ribosome or subunit preparations for hybridization with cDNA probes. In several experiments, some low-level binding was detected, but often probes in this region simply would not bind. Maximum binding for probe 2382-2394 was 9 and 12% on 50S and 70S, respectively, and with probe 2386-2394, binding to 70S ribosomes was only 7%. These numbers are marginally too low to interpret with confidence.

Some relatively new secondary structure features have been proposed for this region which may help explain the low binding. According to the secondary structure model of

(Egebjerg et al., 1990), a portion of this historically-ascribed single stranded region is actually involved in base pairing with nucleotides 2328-2330, and has two more base pairs with nucleotides 2284 and 2285, which are located just "across the way" on the secondary structure map (see figure 4b). Since binding with this probe was so sporadic, it did not yield meaningful experimental results, besides noting that this region is not available for cDNA probe binding.

tRNA binding to 50S subunits and 70S ribosomes

Using specific binding conditions, one can titrate specific tRNA binding sites on the ribosome with different species of tRNAs. On the 50S ribosomal subunit, there are either one or two sites available for binding with deacylated tRNA, the P and E sites. Nierhaus' group argues that since 50S subunits do not bind acylated tRNAs to any great extent, binding on this subunit is representative of the "prospective" E site (it becomes the "real" E site upon association with the 30S subunit) (Rheinberger et al., 1990). A definitive test of where tRNA binds on the 50S subunit has not been established. However, in the absence of message, it is generally agreed that no binding occurs in the A site.

Saturation of deacylated tRNA binding to 50S subunits occurs at about 40% in the absence of mRNA, at an excess of tRNA over 50S subunits of 3:1. This binding curve has been very reproducible among researchers in our laboratory. One

interesting feature of the curve is the inflection point at around 2:1 tRNA:50S (see figure 12). A possible interpretation of this inflection point is that it represents filling of the P, then the E site (or the corresponding prospective P and E sites).

Deacylated tRNA fills the tRNA binding sites on 70S ribosomes sequentially, with the order P, E, A (Rheinberger et al., 1990; Robertson et al., 1986). In the absence of message, the A site is not significantly populated with deacylated tRNA (Grajevskaja et al., 1982; Kirillov et al., 1983; Moazed & Noller, 1989), so only the P and E sites are filled when unprogrammed ribosomes are titrated with deacylated tRNA. Deacylated tRNA isolated from yeast (Saccharomyces cerevisiae) binds the E. coli ribosomal P site with comparable affinity as the E. coli deacylated tRNA, but it does not bind the E site of an E. coli ribosome to any significant degree (Lill et al., 1986; Lill et al., 1988). This specificity for the P site is an important feature in interpreting the effects of yeast tRNA on probe binding.

In our hands, yeast tRNA binds to 70S ribosomes slightly less than the E. coli tRNA through the range of tRNA concentrations (see figure 13a). However, since the binding curves essentially parallel one another, and the yeast tRNA binding curve represents P-only binding, it is difficult to assign any single portion of the E. coli tRNA binding curve as being representative of P-only or E-only binding. However,

experiments described below, and evidence presented by Lill et al. (1984) and Rheinberger et al. (1981) demonstrate that occupation of the E site with deacylated tRNA does not start until there is a 1.5-2:1 stoichiometric excess of tRNA over ribosomes.

The binding of acylated and N-acetyl-acylated tRNAs to ribosomes can be assigned to the A and P sites exclusively, since the E site is absolutely specific for deacylated tRNA. In general, the P site is occupied by either Phe-tRNA^{Phe} or N-Ac-Phe-tRNA^{Phe} first, followed by occupation of the A site if messenger RNA is present. Binding of these two species is greatly enhanced by the addition of mRNA (poly U). There is some controversy about the number of N-Ac-Phe-tRNA^{Phe}s that can be bound simultaneously to the ribosome. Rheinberger et al. (1981) proposed that not more than one N-Ac-Phe-tRNA^{Phe} can be present on the ribosome at any given time (the "exclusion principle"), but Grajevskaja, et al. (1982) and Lill et al. (1984) claim that 1.5 and 2 N-Ac-Phe-tRNA^{Phe}s can simultaneously bind the ribosome, respectively. On the other hand, there is no controversy over how many Phe-tRNA^{Phe}s can be present on a programmed ribosome at once. All labs report two binding sites for Phe-tRNA^{Phe}, with the P site filling first, followed by occupation of the A site.

Figure 13b shows binding data for N-Ac-Phe-tRNA^{Phe} and Phe-

$tRNA^{Phe}$ to 70S ribosomes in the presence or absence of poly U. The unprogrammed binding of Phe-$tRNA^{Phe}$ to ribosomes saturates at about 30-40% at a molar excess of 2:1 Phe-$tRNA^{Phe}$:70S (20mM $MgCl_2$). This is representative of P-only binding, since in the absence of message, the A site is not bound. In the presence of poly U, binding just exceeds 100%, and represents both A and P site binding. N-Ac-Phe-$tRNA^{Phe}$ binds 70S ribosomes at about 12% in the absence of poly U (in 15mM Mg^{2+}), which, again, represents P-only binding. In the presence of message, the binding is about 70% at 3:1 N-Ac-Phe-$tRNA^{Phe}$:70S. This may represent A and P site binding, or P-only binding, depending on whether or not the exclusion principle applies to these experimental conditions. In this study, differentiating between these possibilities is not necessary for interpreting experiments.

Probe/deacylated tRNA competition on the 50S subunit

One of the early goals of this research was to determine if the nucleotides implicated by Moazed and Noller (1989a) as being important in E site tRNA binding were, indeed, in the E site as determined by a probe/tRNA competition assay. The rationale for these experiments was that if nucleotides involved in tRNA binding were "covered" by a site-specific probe, then either tRNA binding should be disrupted or the

probe should be displaced. Figure 14 shows that at a stoichiometry of about 2:1, deacylated tRNA to 50S subunits, probe binding was significantly attenuated. This strongly suggests that the single stranded region spanned by these two probes are involved directly or indirectly in tRNA binding. These results are in agreement with those of Moazed and Noller (1989), who implicated nucleotides 2111, 2112, and 2116 in E site binding by their work using chemical probes.

The stoichiometry of displacement of these two probes is worth noting, too. No effect of adding tRNA was noted until the ratio of tRNA to subunits was 1:2 or greater. This is in line with assertions by Lill et al. (1984) and Kirillov et al. (1983), that the E site is not occupied until a 1.5 to 2-fold excess of tRNA is present. Since the probes are displaced at higher ratios of tRNA to subunits, it appears that probes 2109-2119 and 2111-2117 either reside in the exit site or at least in an area that is affected by binding of tRNA in the E site. On the other hand, if 50S subunits bind only tRNA in the prospective E site (as per Rheinberger et al., 1990), then the fact that the probes are displaced at any concentration of deacylated tRNA suggests that they lie in the E site or are at least affected by tRNA bound in the exit site.

A probe spanning nucleotides 2165-2171, which includes nucleotide 2169, also shown to be protected against chemical modification with E site bound tRNA, was not significantly displaced by the addition of tRNA (data not shown). There are

several possible explanations for this. The simplest explanation is that none of the nucleotides spanned by 2165-2171 are involved, directly or indirectly, in tRNA binding, but as is shown in experiments below, this explanation is unlikely. Another possibility is that the nucleotides spanned by 2165-2171 are not absolutely necessary for tRNA binding, and hybridizing a probe across this region does not compromise tRNA binding. Finally, it is possible that hybridizing a probe across this single stranded region inhibits or precludes tRNA binding. This last option can be ruled out, since none of the probes used in this study could attenuate tRNA binding, even at a high stoichiometric excess of probe (data not shown). A probe complementary to the 2382-2394 single stranded region did not bind well enough to perform this experiment.

Effect of deacylated tRNA on probe binding to 70S ribosomes

The results of a dual label experiment using ^{32}P-labeled deacylated tRNA and ^{35}S-labeled probe 2109-2119 binding to 70S ribosomes were very surprising. In contrast to the case with the 50S subunits, addition of the first two equivalents of tRNA to the ribosomes actually enhanced probe binding (figure 15). At ratios higher than 2:1 tRNA:70S, the probe binding was attenuated. The attenuation of probe binding at tRNA ratios higher than 2:1 tRNA:ribosome was an expected result after the experiments with the 50S subunits. If the probe

2109-2119 competes with E site bound tRNA for the same binding site, one would expect to see probe binding decrease in conditions that favor E site tRNA binding. However, the enhancement of probe binding in conditions favoring P site binding was certainly an unexpected result.

Figure 16 shows results of experiments in which ribosomes were titrated with increasing amounts of unlabeled deacylated tRNA and incubated with saturating amounts of labeled probes 2111-2117, 2113-2119, and 2165-2171. Probe 2111-2117 displayed the same general behavior as probe 2109-2119 in the tRNA titration experiment. Binding of the probe was enhanced at low tRNA concentrations, but was attenuated at higher concentrations.

Probe 2113-2119 binding, on the other hand, showed a weaker dependence on tRNA binding than probe 2111-2119 or 2109-2119. Although some enhancement of probe binding was observed at lower concentrations of tRNA, no significant attenuation of probe binding was observed even at a 4:1 molar excess of tRNA. A likely reason for this is that probe 2113-2119 does not span nucleotides 2111 and 2112, which are two of the three nucleotides in this single stranded region thought to be important in E site tRNA binding, the other being nucleotide 2116. Further, whereas nucleotide 2112 is strongly protected from chemical modification by E site bound tRNA, nucleotides 2111 and 2116 are more weakly protected (Moazed and Noller, 1989). If the strength of protection for a particular

nucleotide is proportional to its importance in tRNA binding, then one would expect that a probe spanning a more weakly protected nucleotide should show a weaker dependence on tRNA binding. The enhancement of probe binding at lower tRNA levels is interesting, since it seems to reflect an overall change of environment as tRNA binds the P site.

The probe spanning nucleotides 2165-2171 exhibits three-fold enhancement of binding at tRNA:ribosome ratio of about 0.5 to 1.5:1, and no attenuation of probe binding is evident even at high tRNA concentrations. Recalling that this probe was not displaced by tRNA on the 50S subunits, this is an interesting result. The local environment is apparently changed to favor probe hybridization as tRNA binds the P site, but even in conditions favoring E site binding, the probe is not displaced, indicating again that this probe does not compete directly or indirectly with a binding site for tRNA in the E site.

Effect of yeast tRNA binding on probe binding

To ensure that the enhancement in probe binding seen in the last several experiments was indeed caused by P site tRNA binding, a similar experiment was conducted using $tRNA^{Phe}$ isolated from yeast. As described above, this tRNA species binds the P site of E. coli ribosomes with about the same affinity as tRNA isolated from E. coli, but it has a low affinity for the E site. When the effect of adding yeast

deacylated tRNA on probe 2109-2119 binding was measured, it was found that as yeast tRNA binds the P site, probe 2109-2119 binding is enhanced, but even at 5:1 $tRNA_{yeast}$:70S ribosome, there is no attenuation of probe binding (figure 17). This experiment provides additional evidence that the presence of tRNA in the P site causes a change in a local environment shown previously to be involved in E site tRNA interaction.

Effect of Phe-$tRNA^{Phe}$ binding on probe binding to 70S ribosomes

With the enhancement of probe binding upon P site deacylated tRNA binding clearly established, the next logical step was to examine the effect of acylated tRNA binding on probe binding. Figure 18 shows simultaneous binding data for Phe-$tRNA^{Phe}$ and probe 2109-2119 on poly U programmed 70S ribosomes. Since the order of binding under these conditions is P site then A site (there would be no E site binding, since the E site is specific for deacylated tRNA), enhancement of probe binding should appear in the lower ranges of Phe-$tRNA^{Phe}$ concentrations if the effect is similar to that of deacylated tRNA. As the graph shows, however, there is neither enhancement nor attenuation of probe binding at any level of Phe-$tRNA^{Phe}$ binding.

The lack of effect of Phe-$tRNA^{Phe}$ on probe binding has several implications. First, the absence of attenuation of

probe 2109-2119 binding, even at high tRNA binding levels, supports the assertion that 2109-2119 does lie in the exit site, since it can be displaced by deacylated, but not acylated tRNA. Second, the lack of enhancement of probe binding upon P site binding of Phe-tRNA^{Phe} suggests that deacylated tRNA induces a change in the exit site that acylated tRNA does not. A ribosome with an acylated tRNA in the P site resembles an elongating ribosome prior to peptidyl transfer (or a ribosome just after initiation is complete), whereas a ribosome with a deacylated tRNA in the P site resembles a ribosome in the post-peptidyltransfer state. This raises a question as to what is the trigger or mechanism that causes a change in the E site? Why should a deacylated tRNA in the P site cause a change in the E site, while an acylated tRNA in the P site causes no change in the E site, at least as detected by probe binding?

A third ramification of this experiment concerns the effect of A site bound tRNA on probe binding in the E site. Under these binding conditions, Phe-tRNA^{Phe} binds both the P and the A sites, with the P site filling first (Rheinberger et al., 1981; Gnirke and Nierhaus, 1989). Therefore, at the higher molar ratios of Phe-tRNA^{Phe} to 70S ribosomes shown in figure 18, some A site binding is inevitable. The (putative) A site binding does not affect probe binding in the E site.

Effect of N-Ac-Phe-tRNA^{Phe} binding on probe binding

The effects of deacylated and acylated tRNA binding on probe binding were vastly different, as described above. Next, the effects of acetyl-acylated tRNA (a peptidyl tRNA analogue) binding on probe binding were tested. In the absence of message and at moderate magnesium concentrations, N-Ac-Phe-tRNA^{Phe} binds only the ribosomal P site (Rheinberger et al., 1990). This binding condition resembles the state of the ribosome prior to peptidyl transfer, since the peptidyl group is still attached to the tRNA in the P site. We have shown that probe 2109-2119 binding is not significantly affected by binding of N-Ac-Phe-tRNA^{Phe}, although the overall binding of the N-Ac-Phe-tRNA^{Phe} was fairly low (figure 19). This result was like the effect of Phe-tRNA^{Phe} binding to the ribosome on probe 2109-19 binding, and different than the effect of deacylated tRNA binding. This confirms that only deacylated tRNA in the P site affects probe 2109-2119 binding in the E site.

Effect of chloramphenicol on probe binding

The antibiotic chloramphenicol is an inhibitor of the peptidyltransferase reaction, and its binding site has been localized on the 50S subunit in the peptidyltranferase center (Vasquez, 1964; Marconi et al., 1990). Because chloramphenicol's mode of action pertains to the

peptidyltransferase reaction, and because its binding site on the 23S rRNA coincides with some of the nucleotides implicated in P site tRNA binding (Moazed and Noller, 1989), it seemed a likely candidate to serve as a P site tRNA analogue. However, as figure 20 shows, despite good binding by chloramphenicol, there was no effect on probe 2109-2119 binding to 70S ribosomes. This result can be interpreted in at least two ways. The most solid conclusion that can be drawn from the lack of effect on 2109-2119 binding is that chloramphenicol does not mimic a deacylated tRNA in the P site. If it did, probe binding would have increased as chloramphenicol bound. On the other hand, with these data it is impossible to determine if the chloramphenicol mimics an acylated or a peptidyl tRNA in the P site, or even if it binds the P site like a tRNA at all. In this experiment, a positive result may have led to some insight into the mode of action of chloramphenicol, or led to its use as a P site tRNA analogue, but the negative result is difficult to interpret.

Effect of tetracycline on probe binding

Tetracycline is an antibiotic that specifically blocks A site tRNA binding (Kirillov *et al.*, 1983), and in that capacity, may act as an A site tRNA analogue. This would be very convenient, since binding the A site usually requires pre-binding the P site, so the effects of the two sites are hard to separate (see experiment using Phe-tRNA^{Phe}, for

example).

Figure 21 shows the effects of adding increasing amounts of tetracycline to 70S ribosomes in the presence of an excess of probe 2109-2119. The tetracycline was not radiolabeled, so its precise binding characteristics are unknown in this experiment, but conditions were similar to those used in Kirillov et al. (1983). Figure 21 shows that tetracycline does not affect probe 2109-2119 binding.

As was the case with the chloramphenicol experiments, the lack of effect of tetracycline on probe binding is difficult to interpret. The only definite conclusion we can draw is that tetracycline does not successfully compete with the probe for the same binding site which is an expected result. At this point it is not possible to say whether tetracycline binds the A site like a tRNA and an A site bound tRNA has no effect on probe binding, or that the tetracycline does not act as a tRNA analogue in the A site.

A model for P site-E site interaction during elongation

The experimental findings presented above, when examined as a whole, suggest a model for active interaction between tRNA binding sites P and E on the Escherichia coli 70S ribosome during elongation. This model complements existing models by the Nierhaus, Wintermeyer, and Noller groups, and may help to bring them closer together.

In the cycle of elongation, the ribosome must move three

different types of tRNAs and mRNA rapidly and precisely through at least three tRNA binding sites. The various steps along the way can take different lengths of time, depending on the availability of incoming tRNAs, the number of proofreading steps required to screen out mismatched codon-anticodon pairs, etc., so the ribosome must be sensitive to molecular cues as to when to proceed with the next step. In the present study, the finding that P site deacylated tRNA binding enhances E site probe binding but acylated tRNA binding in the P site has no effect on the E site is suggestive of just such a cueing mechanism.

During elongation the position of the various types of tRNAs on the ribosome is indicative of the position of the ribosome in the cycle. For example, a post-peptidyltransfer/pre-translocational ribosome would hold a peptidyl tRNA in the A site (or hybrid A/P site by Noller's model), and a deacylated tRNA in the P site. Therefore, a deacylated tRNA in the P site could serve as an indicator that it is time for translocation. Conversely, the presence of an aminoacyl or a peptidyl tRNA in the P site is characteristic of a pre-peptidyltransferase ribosome and translocation would not be appropriate in such a state.

With these conditions in mind, and in light of the experimental results presented in this study, several striking conclusions can be drawn. First, this study provides strong evidence that the ribosomal P site can discriminate between

different types of tRNA, and react to the different types accordingly. Second, it clearly demonstrates a linkage between the P and the E sites on the E. coli ribosome. More specifically, the presence of a deacylated tRNA in the P site makes the E site more available for probe binding, and by extension, tRNA binding. This may be a mechanism to promote translocation, to open or change the conformation of the E site to favor tRNA binding.

Lill et al. (1989) have proposed that the 3' end of deacylated tRNA in the P site is involved in actively promoting translocation. Their assay for this involved measuring translocation rates and measuring EF-G dependent GTP hydrolysis rates. They found that only the deacylated and intact 3' end of the tRNA was capable of promoting translocation. By contrast, this study directly measures the availability for probe hybridization in the local environment of the ribosomal E site, and finds that a significant change occurs there only when a deacylated tRNA is positioned in the P site. Interestingly, Lill et al. found that tRNA isolated from yeast was 7-fold less effective in promoting translocation than the E. coli tRNA, but in this study, enhancement of probe binding was comparable using either tRNA. Thus, this study proposes that the presence of a deacylated tRNA in the P site actively promotes translocation by altering the binding environment of the exit site.

The allosteric three site model proposed by Gnirke et al.

(1989) predicts a high and a low affinity state for the A and E sites. This study demonstrates the existence of two states of the ribosomal exit site, with the high affinity state occurring when a deacylated tRNA is located in the P site. This could provide thermodynamic "encouragement" for translocation of the tRNA in the P site to move to the E site. The present study does not completely corroborate the allosteric three site model, however. For example, this study suggests an allosteric link or a conformational switching mechanism between the P and E sites, a feature lacking in the allosteric three site model. An additional side note is that Ulbrich et al. (1978) found that binding of deacylated tRNA to the ribosome caused a 7-fold stimulation of A site affinity for acylated tRNA. Although results of the present study cannot speak to the tRNA affinities in the A site, this would mean that both the A and E sites' affinities would be stimulated by the addition of deacylated tRNA, in apparent violation of the allosteric three site model.

The data presented here generally are in agreement with the hybrid states model of Moazed and Noller (1989b). The change in availability of the 2109-2119 region for cDNA hybridization may be caused by a shift between the subunits to accommodate a deacylated tRNA either in a P/E state or an E state (see figure 9). In either case, it appears that the 2109-2119 region is indeed involved in E site binding, since probes spanning that region are displaced in conditions favoring E

site tRNA binding in both subunits and ribosomes. There is the possibility that the displacement of probes is a secondary effect, caused by a change in conformation of the rRNA upon E site binding. This possibility cannot be unambiguously resolved at this time. The 2162-2173 probe was not displaced by deacylated tRNA, so its role in E site tRNA binding is nebulous. It is clear, however, that both the 2109-2119 and 2162-2173 regions are involved in some way with E site binding, since their availability for probing is altered in E site binding conditions or when translocation is imminent. Since the 2382-2394 region was not generally available for probing, its role in E site binding remains unclear in the context of this study.

In summary, this study helps to bring closer together several of the major models of elongation, and adds several new insights into the mechanism of translocation and its control. Just as this study relied heavily on previous work by researchers in the field, this research should provide another foothold or starting point for future investigations in this area.

Future prospects

One of the most powerful aspects of cDNA probing is its reversible nature. The fact that the probes can hybridize and dissociate makes possible dynamic studies that are not achievable with covalently bound probes. By the same token,

the effects of dilution and filtration on probe binding are not completely defined, and makes absolute interpretation of the binding values difficult. Fluorescence studies using cDNA probes should help solve these problems, and allow a glimpse into the equilibrium conditions of the probe-ribosome complex.

Another rich vein for future research is the complete tracking of tRNAs through the ribosome using probes complementary to rRNA thought to be involved in A and P site binding. When data such as these are combined from the 30S and the 50S subunits and the 70S ribosome, one should be able to almost unify the competing models for the elongation cycle, since this method can (shown in this study) detect switching mechanisms as well as actual locations of the tRNAs on the ribosome.

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