1991

Dynamic ribosome

Chuck Merryman

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The Dynamic Ribosome

by

Chuck Merryman

B.A., University of Montana, 1986

Presented in partial fulfillment of the requirements
for the degree of

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UNIVERSITY OF MONTANA

1991

Approved by:

[Signature]
Chairman, Board of Examiners

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Dean, Graduate School

[Signature]
Date

June 30, 1992
ABSTRACT

The accessibility of the single stranded α-sarcin site in 23S RNA (2653-2667) was investigated during translation and under certain conditions reflecting stages of the elongation cycle.

Hydrolysis of 23S rRNA complexed to DNA probes by RNase H produces a fragment similar to the one produced by the action of α-sarcin. This hydrolysis occurs on ribosomes which are actively synthesizing protein but not on isolated ribosomes. The fragment can also be generated when N-acetyl-Phe-tRNA_phe occupies the A/A and P/P sites of Poly (U) programmed ribosomes. Interestingly, the binding of deacylated tRNAs does not expose the site to probe directed RNase H cleavage. Thus, a condition present in functioning ribosomes causes a structural conformation that is not present on isolated ribosomes or ribosomal subunits. This indicates that the ribosome is a dynamic molecule and that it produces during protein synthesis at least one conformation which allows cDNA probes and RNase H access to the α-sarcin site.
ACKNOWLEDGEMENTS

I would like to thank Dr. Walter E. Hill for his friendship and guidance throughout my stay at the university. I am deeply indebted. I would also like to thank his lab and Dr. Tom North's lab for the excitement and camaraderie which made my work so enjoyable. Bill, Kathy, Rich, Jeff, Steve, and the Seat of the Pants Gang all deserve special thanks for their help.
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<tr>
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<td>amino acyl transfer ribonucleic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<td>ethylenediamine tetra- acetic acid</td>
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<td>elongation factor G</td>
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<td>EM</td>
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</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
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Chapter 1. Introduction

The ribosome, identified in 1952 and named apparently because the word has "-a pleasant sound" (Nomura, 1990), is the piece of cellular machinery universally responsible for translation, the process of synthesizing protein from chromosomally encoded messenger ribonucleic acid (mRNA). Translation, although essentially a ribosomal function, requires extra-ribosomal components including mRNA, initiation factors, elongation factors, release factors, ATP, GTP, the appropriate transfer RNAs (tRNA) and other molecules for which we have inadequate or non-existent terminology (Atkins et al. 1990; Forchhammer et al. 1990; Hayes et al. 1990; Wada et al. 1990; Sachs, and Davis, 1990; Moradbakhti et al. 1990; Struck et al. 1990). Although many differences do occur, the fundamental properties of the ribosome are conserved throughout phylogeny. All ribosomes belong to the ribonucleoprotein class of macromolecules, which are constructed from protein and RNA. Ribosomes are constructed from small and large subunits and represent a large proportion of cellular material, a tremendous energy investment. Even though ribosomes from many organisms have been studied, the most extensive information has accumulated on ribosomes from \textit{E. coli}. Unless stated otherwise, nomenclature and data will be based on this organism. In \textit{E. coli}, as elsewhere, the ribosome has generally been investigated by identifying
contributions from its individual components and so it is appropriate to construct a model of the ribosome by assembling its elements until a coherent picture emerges.

1.1 Ribosomal RNA structure

Ribosomal RNA (rRNA) is a ribonucleic acid polymer containing the nucleotides adenosine, cytidine, guanosine, and uridine. Additionally it often contains several modified bases, including ribothymine. Ribosomal RNA is formed by transcription from highly expressed, chromosomally encoded sequences, with unusual nucleotides being manufactured by post-transcriptional modification. The sequence and continuity of rRNAs can vary immensely between organisms (Hsu et al. 1990) but portions of rRNA have been conserved throughout evolution. In *E. coli*, ribosomes contain three RNAs: 5S, 120 nucleotides in length (4 X 10^4 g/mol); 16S, 1542 nucleotides in length (5 X 10^5 g/mol), and 23S, 2904 nucleotides in length (1.1 X 10^6 g/mol). The nomenclature is based on the sedimentation coefficient of each RNA. In organisms where more than three rRNAs exist, they can be shown to be non-continuous fragments which associate to form structures reminiscent of the three elemental RNAs (Boer, and Gray, 1988; Gray et al. 1984). For instance 5.8S rRNA in all eukaryotes, is a fragment of the large subunit rRNA (Vaughn et al. 1984). Even though the structure of rRNAs appear to be
quite different, upon further study they reveal exciting similarities.

The sequences, or primary structure, for hundreds of rRNAs have been determined directly or by inference from genomic sequence (Gutell et al. 1990; Specht et al. 1990; Neefs et al. 1990). Ribosomal RNAs show great diversity in primary structure. However there exist a few regions and many bases which are phylogenetically conserved to varying degrees (Figures 1,2,3). These universal sequences and nucleotides of rRNA were correctly identified as sites which would most likely be involved in translation and have been subjected to intense scrutiny. The primary structure of rRNA is well known, but how this sequence relates to overall conformation is unknown.

The secondary structure of rRNA is formed by folding the RNA chain such that it associates with itself to form base paired double strands and various single strand regions (Figures 1,2,3). The rRNA appears to be grouped into domains which are connected by loosely defined "hinges". Secondary structure maps are based on chemical, oligonucleotide and enzymatic probing, phylogenetic analysis, thermodynamic minimilization, and nuclear magnetic resonance (Baudin et al. 1987; Gutell et al. 1990; Specht et al. 1990; Neefs et al. 1990). Interestingly all secondary structures can be subdivided into two basic classes, core and variable regions (Gray et al. 1984). The core regions are common to all
Figure 1. Secondary structure map of *E. coli* 16S rRNA. The phylogenetic conservation of each base is shown decreasing in the order orange > red > green > blue. Tertiary interactions identified by covariance are connected with lines. Adapted from Noller and Nomura (1987).
Figure 2. Secondary structure map of the 5' half of *E. coli* 23S rRNA. The phylogenetic conservation of each base is shown decreasing in the order orange > red > green > blue. Tertiary interactions identified by covariation are connected with lines. Adapted from Noller and Nomura (1987).
Figure 3. Secondary structure map of the 3' half of E. coli 23S rRNA. The phylogenetic conservation of each base is shown decreasing in the order orange > red > green > blue. Tertiary interactions identified by covariance are connected with lines. Adapted from Noller and Nomura (1987).
organisms and have the same or similar structures, although their sequences may be significantly different (Gray et al. 1984). The variable regions exhibit only limited secondary structure preservation and are responsible for length heterogeneity within a particular rRNA class. Presumably they are responsible for making an organism's ribosome unique. The concentration of highly conserved sequences within single-stranded regions of secondary structures reinforced the supposition that the conserved sequences were important for ribosomal function. In general, secondary structure is reasonably well understood, especially in the case of 5S and 16S.

The tertiary structure of rRNA is made by folding the secondary structure into three dimensions. Formation of the tertiary structures must be stabilized by protein interactions, coordination of metal ions, hydrogen bonding, base pairing, and coaxial stacking. The affect of proteins on tertiary structure is demonstrated by the compacting of rRNA upon formation of ribosomal subunits (Serdyuk et al. 1983; Gongadze et al. 1986) and by a few protein rRNA interactions which have been examined in great detail (Mougel et al. 1988; Baudin et al. 1987; Egebjerg et al. 1990). The role of metal ions and hydrogen bonding involving phosphate oxygens, the ribose 2'-OH, and bases is at this time mostly an extrapolation from tRNA and oligonucleotide structure (Rich, and Rajbhandary, 1976). Recently the participation of
phosphate oxygens in tertiary structure has been elegantly demonstrated (Baudin et al. 1989).

Base pairing of a helical nature between single-stranded regions of rRNA, though important, must by necessity be extremely short, in a manner reminiscent of pseudoknots (Pleij, 1990). This is because, except at the 5' and 3' ends, the single stranded regions of rRNA are constrained by their associated double stranded segments and lack of free termini. The single strands therefore cannot wrap around one another in forming tertiary interactions. This includes possible triple helices. This arrangement is supported by studies on a limited set of high-resolution hairpin loops which exhibit no similarity to helical moieties (Puglisi et al. 1988; Puglisi et al. 1990). Base pairing, within the above limitations, has been directly identified by sequence comparison and compensatory base mutations (Gutell, and Woese, 1990; Woese, and Gutell, 1989; Melançon et al. 1990) (Figures 1,2,3) and indirectly by crosslinking (Baudin et al. 1987).

The importance of stacking interactions in nucleic acid structure cannot be overstated (Turner et al. 1987; Jaeger et al. 1990). Coaxial stacking effects tertiary structure and is formed when two independent helices align so that their axes essentially become continuous. Evidence for the involvement of coaxial stacking in rRNA comes from model building studies and chemical modification (Stern et al. 1988; Schüler, and Brimacombe, 1988). Tertiary structure models of rRNA have
recently emerged (Stern et al. 1988; Schüler, and Brimacombe, 1988) and although encouraging, the resolution is still limited (Figure 4). The positions of a few nucleotides relative to the ribosomal outline have been discovered by electron microscopy (Kossel et al. 1990; Walleczek et al. 1990) (Figures 5, 6) and have been incorporated into the models. In general the tertiary structure of rRNA is poorly understood, but significant advances have recently been made.

Interactions of rRNA with extra-rRNA factors have been identified in several instances. They are formed by rRNA, ribosomal protein and extra-ribosomal component binding and have been shown to affect rRNA tertiary structure (see above). Inter-rRNA interactions have been identified by mutagenesis, crosslinking, chemical and cDNA probing (Chapman, and Noller, 1977; Brow, and Noller, 1983; Herr et al. 1979; Herr, and Noller, 1979; Tapprich, and Hill, 1986; Tapprich et al. 1989). The sites of ribosomal protein binding have been demonstrated by fragmentation, crosslinking and chemical probing which provides footprints (Morgan, and Brimacombe, 1972; Szekely et al. 1973; Barta, and Kuechler, 1983; Roth, and Nierhaus, 1973; Roth, and Nierhaus, 1975; Greuer et al. 1987; Osswald et al. 1987; Gulle et al. 1988; Wiener et al. 1988; Powers et al. 1988; Stern et al. 1988; Mackie, and Zimmermann, 1975; Moine et al. 1988; Spitnik-Elson, and Elson, 1979). The binding sites of extra-ribosomal factors including tRNA, mRNA, EF-Tu, EF-G, IF-1, IF-2, and IF-3 have been partially identified by
Figure 4. Stereoview of 30S subunit model. The 5' domain is blue, central domain red, and 3' domain yellow. Proteins are included in the lower figure. Adapted from Noller et al. (1990).
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crosslinking, phylogenetic analysis, cDNA probing, and chemical modification (Ehresmann et al. 1984; Hsu et al. 1984; Thanaraj, and Pandit, 1989; Zucker, and Hershey, 1986; Tate et al. 1988; Tate et al. 1990; Acharya et al. 1973; Sander, 1977; Skold, 1983; Girshovich et al. 1986; Bochkareva, and Girshovich, 1984; Langer, and Lake, 1986; Ehresmann et al. 1986; Nag et al. 1987; Moazed, and Noller, 1986; Moazed, and Noller, 1989; Moazed, and Noller, 1990). These interactions of rRNA have been used to indirectly locate regions of RNA within the ribosome by electron microscopy and model building. Even though much useful information has been accumulated as to which bases are involved, the structures formed are not well understood.

1.2 Ribosomal protein structure

Ribosomal proteins are formed by peptide linkages between the canonical twenty amino acids. They also contain unusual residues which are formed by postranslational modification. Ribosomal proteins vary greatly in number and sequence and although phylogenetic conservation undoubtedly occurs, in contrast to rRNA, an inclusive picture has not surfaced. In general ribosomal proteins are basic and range in molecular weight from $5 \times 10^3$ to $6.1 \times 10^4$ g/mol. The nomenclature of ribosomal proteins is based on their presence in the small or large subunit and their movement in two-dimensional electrophoretic gels. For *E. coli* this results in a
nomenclature in the (S)mall subunit of S1 through S21, and in
the (L)arge subunit of L1 through L32. Comparatively few
specific data exist on the structure of ribosomal proteins.

The complete or partial primary structures of ribosomal
proteins from many organisms are known (Wittmann-Liebold et
al. 1990). A degree of ribosomal protein homology exists
within a kingdom and there is limited correlation between the
kingdoms. For many reasons it has been difficult to
phylogenetically align ribosomal proteins and so information
on conserved residues is limited. In contrast to rRNA,
ribosomal protein sequences have not been as useful in
identifying important aspects of ribosomal protein structure
and function.

Presumably the forces which guide ribosomal protein
secondary structure are similar to those which influence other
proteins but at this point in time, that is more speculative
than factual. Ribosomal proteins are generally described as
having thirty percent alpha-helix and less beta-sheet
(Boublik, 1987). The proteins of the large subunit apparently
do have a more variable structure than the 30S proteins for a
review see (Giri et al. 1984). Secondary structures of
ribosomal proteins have not been very informative.

In contrast to other proteins the hydrophobic effect may
not be a large contributor to ribosomal protein tertiary
structure (Wilson et al. 1986). The forces responsible for
folding ribosomal proteins in three dimensions are not well
understood. The tertiary structure of some ribosomal proteins has been studied intensively by low resolution techniques and these investigations have provided some data on overall protein shape, which is generally globular but can be highly elongated (Giri et al. 1984). High resolution crystal structures of only three ribosomal proteins have been obtained a portion of L7/L12, L6, and L30 (Leijonmarck, and Liljas, 1987; Liljas, and Gudkov, 1987; Horjales et al. 1987; Sedelnikova et al. 1987; Wilson et al. 1986). From these data it is hard to form a model of ribosomal protein structure although there are indications that it is fundamentally different from other proteins (Wilson et al. 1986).

Ribosomal proteins also interact with other ribosomal components and factors. Although extensive data on spatial relationships between proteins have been obtained (Capel et al. 1987), very little detail is known about the method of interaction. Recent evidence indicates, as had been supposed earlier, that basic and hydrophobic "patches" are present which may bind RNA and protein respectively (Wilson et al. 1986). The structure of ribosomal proteins, when viewed from a teleologic standpoint, has been very disappointing, but this may reflect their inherent complexity rather than a lack of significance. The present data are incomplete and much work remains to be done in the study of ribosomal proteins.
1.3 Small subunit structure

The small ribosomal subunit is made by interactions between 16S RNA and twenty-one ribosomal proteins. As 16S rRNA is being transcribed, ribosomal proteins begin to associate and stabilize its three dimensional structure, a process referred to as "assembly". At our present level of understanding, assembly is a semi-ordered process in which the binding of certain ribosomal proteins induces structural alterations which allow subsequent proteins to bind (Held, and Nomura, 1973; Held et al. 1974; Stern et al. 1988; Powers et al. 1988) (Figures 7,8). The procedure may be more sequential in vivo. Assembly experiments are performed with intact rRNA. In contrast transcription presents rRNA in the 5' to 3' direction, which would allow assembly involving ribosomal proteins and 5' regions of RNA before the 3' sequences are even transcribed. A portion of 30S subunits reconstituted with full-length 16S RNA are capable of synthesizing protein.

Interactions formed by assembly between constituents of the 30S subunit induce structural changes in rRNA (Stern et al. 1988; Powers et al. 1988) and undoubtedly in ribosomal proteins as well. These structural alterations are presumably important but, except in a few cases, they have not been investigated extensively. Once assembled the small subunit (1 X 10^6 g/mol; Hill, et al. 1969), consists of RNA intermingled with 21 proteins and has approximate dimensions of 22x22x10 nM in solution (Moore, and Engelman, 1974; Van Holde, and Hill,
Figure 7. Assembly map of the E. coli 30S subunit. Arrows and their thickness indicate dependence of one molecule on the presence of another, for assembly. Adapted from Noller and Nomura (1987).
Figure 6. Assembly map of the E. coli 50S subunit. Proteins encircled by the dotted line are involved in 5S rRNA assembly. Adapted from Rohl and Nierhaus (1982).
The subunit has been viewed successfully by electron microscopy (Boublik, 1987; Lasater et al. 1989) and its morphological features are summarized in (Figure 5). The relative positions of proteins within the subunit have been determined by neutron scattering (Capel, and Moore, 1988; Moore, 1987; Capel et al. 1987), immune electron microscopy (Stoffler-Melicke, and Stoffler, 1990; Oakes et al. 1990), crosslinking (Traut et al. 1980; Stoffler-Melicke, and Stoffler, 1990), and singlet-singlet energy transfer (Huang et al. 1975). In general these near neighbor assignments are internally consistent and support the ribosomal protein assembly map. It is probably the case that three dimensional protein positions are substantially accurate for the 30S subunit. However, information about ribosomal protein shape, orientation, and atomic interactions is virtually unknown.

Several models of the 16S RNA path through the 30S subunit have recently been presented (Stern et al. 1989; Schüler, and Brimacombe, 1988; Malhotra et al. 1990). The models use ribosomal protein tertiary positions, protein binding sites on rRNA and RNA A-form helix parameters to constrain the locations available to a portion of rRNA. Ribosomal RNA is first associated with specific ribosomal proteins by protein-RNA crosslinking or by ribosomal protein mediated rRNA protection from chemical or enzymatic modification. Once a region of rRNA has been placed in the
vicinity of a ribosomal protein, its position is further restricted by RNA A-form structure. With the application of these constraints to many data points a reasonably accurate model begins to emerge (Figure 4). A great deal is known about the nucleotides and even the atoms of 16S RNA which are part of 30S subunit structure. These data are invaluable, but until more information is available on spatial relationships between these sites, or atoms, residues and nucleotides with which these sites interact more detailed models of the subunit will be hard to construct.

1.4 Large subunit structure

The large subunit is constructed from 23S and 5S RNA plus 32 ribosomal proteins. The ribosomal proteins are not present in molar ratios as they are in the small subunit and thus their stoichiometry deserves some attention. To begin with protein L8, which was previously defined, does not exist, but is actually the complex formed between L10 and L7/L12. Protein L7 is the same as L12 except that its amino terminal serine residue is acylated. These two proteins are also present on the ribosome in a tetrameric complex not a dimeric one as previously believed (Georgalis et al. 1989). Finally protein L20 is identical to S8 which is a protein of the small subunit, and may be shared between the subunits.

The parameters involved in the assembly of 50S subunits are essentially the same as for the 30S subunit. The main
difference seems to be that the 50S subunit is simply more complicated as is demonstrated by a comparison of the assembly maps (Figures 7,8). The large subunit (50S, \(1.8 \times 10^6\) g/mol) has a maximum dimension in solution of 25 nm and occupies \(1.8 \times 10^3\) cubic nm (Van Holde, and Hill, 1974; Trakhanov et al. 1987). The conformation of the large subunit has been determined by image reconstruction electron microscopy and crystallography (Wagenknecht et al. 1988; Lotti et al. 1989) and general accord has been reached on several features (Figure 6). Information on the positions of 50S ribosomal proteins within the subunit has been obtained by essentially the same methods as for the positions of proteins in the 30S subunit (Nowotny et al. 1989; Walleczek et al. 1989; Scheibe, and Wagner, 1986; Walleczek et al. 1989), although conflicting results obtained by the various methods is more apparent. As with the 30S subunit information about individual ribosomal protein shapes, orientation, and atomic interactions is virtually unknown.

Due to its small size, very detailed studies on 5S RNA have been possible and many of these have addressed its conformation, which is still not completely resolved (Kime, and Moore, 1982; Kime, and Moore, 1983; Kime, and Moore, 1984; Zhang, and Moore, 1989; Zhang et al. 1989). Here it is sufficient to note its location in the central protuberance (Figure 6) and association with proteins L5, L18, and L25. The path of 23S RNA through the 50S subunit has not been
mapped to any great degree, but model building studies have begun. The 50S subunit is not as well understood as the 30S subunit, but this is due to the relative complexity which is more a reflection of size than an inherently different structure.

1.5 Ribosome structure

The ribosome is formed by the association of a 30S subunit and a 50S subunit to make a 70S particle (Figure 9). A large range of chemical interactions, similar to those used in subunits, are undoubtedly used in making the ribosome. Except in a few circumstances, these interactions have not been adequately defined. The ribosome has dimensions of approximately 17X23X25 nm and has been studied by techniques similar to those used for the subunits (Yonath et al. 1986; Shevack et al. 1985; Yonath, and Wittmann, 1988; Chistyakov et al. 1988; Wagenknecht et al. 1989; Hardesty et al. 1985; Yusupov, and Spirin, 1986; Scheibe, and Wagner, 1986; Uchiumi et al. 1986). The ribosome cannot be thought of as combined 30S and 50S subunits because the subunits apparently do not maintain their initial conformations upon assembly (Baudin et al. 1989; Ghosh and Moore, 1979). With high resolution images it has become apparent that the 30S and 50S subunits contact only in a single region (Figure 9) (Verschoor, and Frank, 1990). This forms a striking feature of the ribosome a large cavity termed the "interface canyon" (Wagenknecht et al.
Figure 9. Electron microscopy based model of the E. coli 70S ribosome. Adapted from Noller and Nomura (1987).
1989). The canyon is a vast open space accessible to solvent and its presence appears to promote efficient binding of translational factors (Verschoor, and Frank, 1990; Girshovich et al. 1986; Kastner et al. 1990; Tate et al. 1990; Tate et al. 1986; Tate et al. 1984; McCaughan et al. 1984).

The interaction of mRNA with the ribosome provides some loosely defined constraints on ribosome structure. Evidence indicates that the mRNA passes between the subunits (Santer, 1963; Olson et al. 1989) into the cleft of the 30S subunit. The passageway of mRNA cannot be through a permanent tunnel since covalently closed circular mRNA is translated. Additionally phage T4 topoisomerase subunit gene 60 mRNA (gene 60) contains an untranslated internal sequence of 50 nucleotides, and less dramatic segments of 12 and 20 nucleotides have also been found (Atkins et al. 1990; de Smit, and Van Duin, 1990). Some simple calculations indicate that the distance occupied by the gene 60 untranslated segment, perpendicular to the mRNA axis, could range from approximately 25 to 65 nm. The actual value is probably intermediate (Burke-Agüero, and Hearst, 1990). Gene 60-like structures eliminate any possibility that mRNA moves through the ribosome in a rigid corkscrew like manner since the resulting propeller motion would prevent association of mRNA with the ribosome. It is probable that mRNA is in an extended conformation and has a reasonably large volume available perpendicular to its axis. Presently, a gross morphological understanding of the
The ribosome is all that is possible. A more detailed picture can only be obtained by solving the spatial relationship between the subunits and identifying the conformational changes which occur upon subunit association.

1.6 Ribosomal function

The function of the ribosome is to make protein and to act as an environment signalling device (VanBogelen, and Neidhardt, 1990; Roesser, and Yanofsky, 1988; Roland et al. 1988). The individual roles of RNA and protein in ribosome function have not been completely separated, although some general statements are possible. RNA is the binding site of antibiotics (Table 1) and some of the ligands involved in protein biosynthesis. Proteins are responsible for altering the structure of rRNA but have no identifiable enzymatic activities. It is most likely the case that rRNA is capable of performing most of the important functions in protein synthesis. The efficiency of translation is greatly enhanced by ribosomal proteins and it seems likely, though unproven, that they are directly involved in some aspects of ribosome function.

Protein synthesis is essentially the process of properly aligning and decoding mRNA (Atkins et al. 1990) while forming peptide bonds. Several intermediate steps can be at least partially associated with a specific subunit. The 30S subunit, mRNA, tRNA, EF-Tu, and EF-G are involved in the
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<tr>
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<td>752, 2058, 2062, 2439, 2451, 2505</td>
<td>CM</td>
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Table 1. Antibiotic binding sites of ribosomes. Bases are translated to equivalent position in *E. coli*. Methods are, M = mutant; CH3 = methylation confers resistance; -CH3 removal of postranscriptional methyl group confers resistance; CM = protection from chemical modification; XL = crosslinking.
alignment and decoding of mRNA. Peptide bond formation is an intimate aspect of the 50S subunit and aminoacyl-tRNA (aatRNA). Translation begins with functions specific to the 30S subunit during the process of initiation. After choosing a mRNA, protein synthesis continues by the concerted efforts of both subunits during elongation and termination.

In prokaryotes, initiation (Figure 10) begins with the separation of 70S ribosomes into subunits. Though definitive data do not exist, this action is apparently performed by initiation factor 3 (IF-3) and initiation factor 1 (IF-1). The initiation factors, including initiation factor 2 (IF-2), have been located on the 50S side of the 30S subunit near the 3' end of 16S rRNA (Figure 5). Either mRNA, with the help of S1, or the complex fMet-tRNA_f^met•GTP•IF-2 (IF-2 complex) is bound next. The order seems to be unimportant. The efficiency of mRNA binding to 30S subunits, and thus translational efficiency, is determined by a variety of factors including mRNA structure (Guan, and Weiner, 1989; Shpaer, 1985), mRNA-rRNA base pairing (Thanaraj, and Pandit, 1990; Thanaraj, and Pandit, 1989; Morel-Deville et al. 1990) and mRNA-protein interactions (Philippe et al. 1990; Cerretti et al. 1988; Climie, and Friesen, 1987). Approximately 40-70 (Gualerzi et al. 1990) nucleotides of mRNA are associated with the 30S subunit although the association may not be continuous. The mRNA may actually have two binding sites during initiation, the final binding site is formed when the
Figure 10. Initiation of translation in prokaryotes.
IF-2 complex is present (Figure 10) (Gualerzi et al. 1990). The mRNA contains the initiation codon which is almost always an internal 5'-AUG-3'. The initiation codon is properly placed with respect to the 30S subunit, partially, by base pairing formed between the mRNA and the rRNA. This pairing is between the Shine and Dalgarno sequence (SD) at the 3' end of 16S rRNA and the anti-SD sequence located 5' to the initiation codon of most prokaryotic mRNAs. When the IF-2 complex and mRNA are both present on a 30S subunit containing IF-1 and IF-3, the positioning of the initiation codon with respect to the 30S subunit becomes exact (Hartz et al. 1990). The result is that a codon-anticodon interaction is formed between mRNA and fMet-tRNA^met in the P/P site (Figure 11). There is a general consensus that IF-3 is responsible for evaluating the codon-anticodon interaction and that IF-2 is responsible for the selective binding of fMet-tRNA^met. However discrete functions cannot be entirely assigned to a single initiation factor and it is quite possible that a degree of cooperativity exists for all steps in the formation of 30S initiation complexes. Initiation is complete when the 50S subunit is added, GTP is hydrolyzed and the initiation factors released. The resulting 70S initiation complex is now ready to begin elongation. It is important to note that the initiation of protein synthesis in eukaryotes is quite different.

The first step in elongation is the random association of EF-Tu•GTP•aATRNA (ternary complex) with the ribosome. This
occurs at the A/T tRNA binding site (Odom and Hardesty, 1987) (Figure 11). The anticodon interaction of ternary complex tRNA with the second codon is checked on the 30S subunit by a two-step mechanism which uses internal kinetic standards (Thompson, 1988). Non-cognate and even near-cognate tRNAs are successfully rejected based upon their codon-anticodon interactions. Acceptance of the proper tRNA is accompanied by the release of EF-Tu•GDP from the ribosome and movement relative to the 50S subunit of the A/T tRNA into the A/A binding site. The ribosome now consist of initiator tRNA bound to the P/P site and elongator tRNA bound to the A/A site. The nitrogen of the A/A tRNA amino acid is presented in the proper orientation and environment to perform nucleophilic attack at the ester bond carbon of the P/P tRNA amino acid. The 50S subunit peptidyl transferase center (Figure 6) promotes this reaction by an unknown mechanism, although the pH profile indicates histidine may be involved. The peptidyl transferase center is a non-specific binding pocket which can form ester, polyester, thioester, thioamide, and phosphino-amide bonds (Lamond, and Gibson, 1990; Lim, and Spirin, 1986). During peptide bond formation both tRNAs move relative to the 50S subunit which results in the P/P tRNA moving into the P/E site and the A/A tRNA moving into the A/P site. The growing peptide is now covalently attached by a carboxy terminal ester linkage to the tRNA in the A/P site. Translocation of the tRNAs and message relative to the 30S subunit is now performed
Figure 11. Diagram of translational elongation cycle. Adapted from Moazed and Noller (1989).
with the help of EF-G. This results in tRNAs shifting from the P/E to /E site and A/P to P/P site. Once the A/A site is empty and EF-G is removed the ribosome is ready to investigate more ternary complexes until a cognate tRNA for the third codon of the mRNA is found. This cyclic process continues for each codon until a termination signal is reached.

Termination occurs with the hydrolysis of the peptidyl-tRNA-ester bond and subsequent release of the peptide from the ribosome. Termination is performed in response to UAA or UAG codons by release factor 1 (RF-1) or by release factor 2 (RF-2) in the case of UAA or UGA codons. Release factor 3 (RF-3) appears to stimulate the actions of RF-1 and -2 but have no direct effect. The release factors work by allowing nucleophilic attack of water at the P/P site tRNA ester bond carbon. This effectively terminates protein synthesis by generating a peptide carboxy terminus and an unacylated tRNA. The peptide is released from the ribosome by the action of EF-G and ribosome release factor (RRF). The subunits are dissociated and then recycled by formation of another initiation complex or they can be degraded.

1.7 Ribosomal dynamics

A plethora of suggestive information exist on the possibility that the ribosome is a dynamic particle (Marion, and Marion, 1989; Spirin, 1987; Verschoor, and Frank, 1990; Gross, and Jaenicke, 1990; Thanaraj et al. 1988; Dahlberg,
1974). The conformational changes can be divided into two groups, those occurring during subunit association and those occurring within the elongation cycle. There are probably conformational changes within the termination cycle but these have not been identified. The sequence of events which cause a conformational change are entirely unproven but some data do exist on the constituents involved.

When the 50S subunit associates with the 30S initiation complex to form the 70S ribosomes, both subunits undergo conformational changes (Lee, and Horowitz, 1987). The most thoroughly studied aspect of this change involves the "active-inactive" switch of the 16S RNA 3' end. During this process, 16S becomes sensitive to colicin E3 (Kossel et al. 1990), nucleotide 1542 moves away from S21 (Kossel et al. 1990), and S1 shows increased reactivity with antibodies (Walleczek et al. 1990). Sequence analysis (Kossel et al. 1990) and crosslinking (Ericson, and Wollenzien, 1989) indicate alternative base pairing and chemical probing shows differential reactivities of bases (Prescott, and Dahlberg, 1990) as do cDNA probing and antibody reactivity (Ericson, and Wollenzien, 1989). Despite the intense investigation of the "active-inactive" switch, a model which is consistent with all or even most of the data has not emerged. Structural changes probably occur at other positions within the 30S and 50S subunits during 70S formation, but they have not been investigated very thoroughly.
Structural alterations within the elongation cycle have been investigated most thoroughly in the case of ribosomal proteins L7/L12. Although a plausible model is not available, NMR studies (Cowgill et al. 1984) and trypsin hydrolysis (Spirin, 1987) both indicate that the proteins have multiple conformations. Several other sites have been identified as possibly having multiple conformations: resistance to streptomycin may be due to a mutation which interferes with a conformational change in the 530 loop of 16S (Leclerc, and Brakier-Gingras, 1990); availability of bases to chemical and cDNA probes may change upon antibiotic and tRNA binding (Marconi et al. 1990); and crosslinking of mRNA analogues is different in the post-translocation state (Vladimirov et al. 1990). This circumstantial proof makes it very unlikely that the ribosome is a static particle. A ribosomal site with two or more structures remains to be identified, at reasonably high resolution, before dynamics can be unequivocally established however.

1.8 The α-Sarcin site

Nucleotides 2653-2667 of 23S RNA form the α-sarcin site of E. coli ribosomes. The site is named for the cytotoxin α-sarcin which causes hydrolysis of the phosphodiester linkage between bases G2661 and A2662 (Endo and Wool, 1982). Several other cytotoxic molecules depurinate the adenine equivalent to A2660. These cytotoxins are widely distributed in the plant
kingdom (Feng et al. 1990) and are extremely poisonous (Hughes, 1979). The nucleotide sequence of this site is one of the most highly conserved in phylogeny and the secondary structure is a simple hairpin loop having an elongated stem and a large single stranded region (Figure 12). The tertiary structure is unknown, but apparently plays an important role in recognition by α-sarcin (Endo et al. 1990). The site has also been placed at the base of the L7/L12 stalk because of its association with EF-Tu and EF-G (Moazed et al. 1988).

The primary function of the α-sarcin site is to bind EF-Tu and EF-G, but more complicated functions are also indicated. Biological modification of the site results in the termination of protein synthesis through the inhibition of EF-Tu directed tRNA binding to the A/T site or through effects on EF-G GTP hydrolysis. Micro-injection experiments with α-sarcin, have demonstrated the absolute requirement for the sites integrity in vivo, similar results are obtained in cell free systems (Cundliffe, 0). A-sarcin and presumably the other cytotoxins hydrolyse rRNA at a specific point in the translation cycle (Endo et al. 1990).

1.9 Proposed problem

Studies on the structure and function of ribosomes using the technique of cDNA probing have become wide spread (White et al. 1988; Hill et al. 1988; Saxena, and Ackerman, 1990). The α-sarcin site has been investigated with this technique
Figure 12. Detailed view of *E. coli* α-sarcin site. Position cleaved by the cytotoxin is indicated.
but the RNA of ribosomes and subunits was found to be inaccessible (White et al. 1988). This is unusual because highly conserved functional sites of rRNA, like the α-sarcin site, are generally available to solvent and cDNA probes (Hill et al. 1986; Hill, and Tassanakajohn, 1987; Marconi, and Hill, 1988; Tapprich, and Hill, 1986; Marconi, and Hill, 1989; Marconi et al. 1990). Indeed the α-sarcin site is available to chemical probes, EF-Tu, EF-G and to the cytotoxin. All of this information indicates that the site should be exposed on the surface of the ribosome. However results from extensive experimentation clearly showed that under a variety of conditions the site was not available to cDNA probes (White et al. 1988). Considering the amount of information which suggested that the ribosome is a dynamic particle I decided to investigate the possibility that the α-sarcin site was transiently available. The objective was to demonstrate ribosomal dynamics by probing the molecule in all of its normal conformations. This was achieved for elongation cycle specific conformations by using an in vitro protein synthesis system. Once it was established that the α-sarcin site was available to cDNA probes during protein synthesis I could then try and isolate a portion or portions of the elongation cycle which contained an available α-sarcin site. The data would then be used to construct a model of the α-sarcin site which incorporated ribosomal dynamics.
2.1 Ribosome preparation

Tight couple 70S ribosomes were isolated from *E. coli* MRE 600. All reagents and tools were pre-cooled to 4°C and the entire procedure was performed as rapidly as possible. Twenty grams of cells were thawed and washed in cracking buffer (20 mM Tris-HCl, pH 7.4; 10-15 mM MgCl₂; 100 mM KCl, 6 mM 2-mercaptoethanol). The cell suspension was then centrifuged for 5 minutes at 5000 revolutions per minute (rpm). The pelleted cells were added to a large mortar encased by a -20°C freezer bag. Lysis of cells was performed in the 4°C coldroom by slow addition of 50 grams of baked alumina while grinding with a pestle. Typically lysis took twenty minutes. When lysis was complete, cracking buffer was added to dilute the grinding mix to a volume of about 60 ml. This was divided evenly between two centrifuge tubes and the alumina was cleared from the solution by centrifugation for 5 minutes at 5000 rpm. The supernatant (cell lysate) was saved and the pellet washed by suspension in cracking buffer and subjected to a second centrifugation, the resulting supernatant was combined with the first. In order to remove cellular debris and remaining alumina the cell lysate was clarified by centrifugation for 1 hour at 16,000 rpm (low speed). The supernatant from this low speed centrifugation is collected and the ribosomes pelleted from it by centrifugation at 60,000
rpm for 2.5 hours in a Beckman Ti70 rotor (high speed). The supernatant of the high speed centrifugation was sometimes saved as the S100 protein fraction. The ribosomal pellet was usually covered by a thick layer of chromosomal DNA which is removed manually, and the pellets washed several times with cracking buffer. Nucleases and extra-ribosomal proteins were dissociated from the ribosomes by suspending the pellet in SW buffer (20 mM Tris-HCl, pH 7.4; 10 mM MgCl$_2$; 500 mM NH$_4$Cl, 6 mM 2-mercaptoethanol). Low speed and high speed centrifugations were then repeated to isolate ribosomes as a pellet free of extra-ribosomal factors. The ribosomal pellet was prepared for zonal centrifugation by suspending in 10 to 20 ml of TC70 buffer (20 mM Tris-HCl, pH 7.4; 6 mM MgCl$_2$; 100 mM KCl, 6 mM 2-mercaptoethanol), after suspension one-tenth volume of fifty-percent sucrose is added. This solution was loaded into a Beckman Ti-14 rotor containing a 10-33 percent sucrose gradient and centrifuged for 3.5 hours at 47,000 rpm. The zonal gradient was fractionated into 5 ml portions and those fractions containing 70S ribosomes pooled. The ribosomes were then pelleted out of the sucrose solution by centrifugation for 6 hours at 60,000 rpm and the supernatant discarded. Ribosomes were then resuspended in TC70 buffer and residual sucrose removed by dialysis against 200-500 volumes of TC70 buffer for twenty-four hours, the buffer was replaced at twelve hours. Ribosomes were then divided into small aliquots and stored at -70°C, once thawed, ribosomes were not
used again. The quality of ribosomes was checked by *in vitro* protein synthesis, composite and polyacrylamide gel electrophoresis.

2.2 Milipore filtration

The binding of ligands to ribosomes was tested by incubating increasing amounts of radioactively labeled ligand with and without ribosomes in the appropriate buffer. When the incubation was complete ligand bound to ribosomes was separated from unbound ligand by passing the reactions through a Milipore 0.45 μM HAWP nitrocellulose filter. This was done by diluting a reaction to 1 ml with ice cold buffer and then quickly pipetting the entire volume to the center of a filter which had an applied vacuum. The filter was then washed two to three times with 900-950 μl of ice cold buffer, allowed to dry and then transferred to a scintillation vial. When all of the reactions in an experiment had been completed and the levels of radioactivity recorded by liquid scintillation, the percentage of ligand bound to ribosomes was calculated using the specific activity of the ligand.

2.3 Transfer RNA binding

*N*-acetyl-Phe-tRNA^phe^ (Mans Ehrenberg) was bound to the P/P and A/A sites of 70S ribosomes by incubating 25 pmol TC-70S ribosomes in 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM MgCl₂, 1 mM DTT, 12.5 μg of Poly (U), and 25-125 pmol of N-
acetyl-Phe-tRNA\textsuperscript{phe} in 50 \(\mu\)l total volume. The incubation was performed for 5 minutes at 37° C. Then 200 mM MgCl\(_2\) was added to obtain a final concentration of 20 mM MgCl\(_2\) before continuing incubation on ice for one hour. The level of binding was determined by Milipore filtration.

P site specific binding of N-acetyl-Phe-tRNA\textsuperscript{phe} was quantified in a manner analogous to that above except that Poly (U) mRNA was not included in the reaction, a situation which prohibits N-acetyl-Phe-tRNA\textsuperscript{phe} A/A site binding (Lill et al. 1984). Additionally the strength of tRNA binding to the P/P site is attenuated in reactions devoid of mRNA.

2.4 Binding of cDNA probes

All cDNA probes were synthesized on a Biosearch 8600 synthesizer using automated phosphoramidite chemistry. Deblocking and removal of the probes from the column was according to the manufacturer's protocol and purification was performed by reverse phase high performance liquid chromatography or NENprep 2000 column chromatography. A portion of all probes were radiolabeled with \(^{32}\)P and subsequently purified by NENprep chromatography. To assure quality, 10\(^{5}\)–10\(^{6}\) cpm of radiolabeled cDNA probe was electrophoresed on twenty percent polyacrilamide gels and visualized by autoradiography. Electrophoresis was at 40 mA at 4° C for long (thirty to forty minutes) and short (thirty second to ten minute) time periods, in order to check for
contaminating partial cDNA sequences and unincorporated ATP respectively.

Radiolabeled cDNA probes were assayed for ribosomal binding by incubating with ribosomes under various conditions, but typically containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 20 mM MgCl$_2$, 1 mM DTT, 25pmol TC-70S ribosomes or ribosomal subunits, and 125-500 pmol of radiolabeled cDNA at a specific activity of 500-2000 cpm/pmol. For certain experiments reactions may have also contained 12.5 µg Poly (U), and 25-125 pmol of N-acetyl-Phe-tRNA$^{phe}$. In order to efficiently bind N-acetyl-Phe-tRNA$^{phe}$ to the ribosomes, a five minute pre-incubation at 37° C containing 2 mM MgCl$_2$ was necessary, 200 mM MgCl$_2$ was added to obtain a final concentration of approximately 20 mM MgCl$_2$. The reactions were then incubated on ice for at least one hour. Ribosome bound cDNA was separated from unbound probe by Milipore filtration. Non-specific binding of cDNA to ribosomes was determined by incubating and quantifying probe binding to 30S subunits, which has only limited complementary sequences to the cDNA probes used in this study.

2.5 Gel electrophoresis

Five to ten percent polyacrylamide gels containing TBE (89 mM Tris-borate, pH 8.3; 1 mM EDTA) and 7M urea were used to analyze rRNA. Ethanol precipitated RNA samples were resuspended in tracking dye (7M urea, 0.025% bromphenol blue,
and 0.025% xylene cyanol), heated to 60°C, quick-cooled on ice and loaded into 12.5 cm X 13.5 cm X 1.5 mM slab gels. Electrophoresis was performed for various times at 15 mA and room temperature. RNA was visualized by staining with methylene blue. Samples were also separated on composite gels containing 0.5% agarose and 2.75% acrylamide. In these cases purified rRNA was resuspended in dye (0.025% bromphenol blue, 0.025% xylene cyanol, 10% sucrose) and gel electrophoresis was performed at 20°C with circulating TBE buffer. Composite gels were visualized with Stains All (Tm).

2.6 cDNA directed hydrolysis of 23S rRNA

The 23S rRNA isolated by phenol extraction of 50S ribosomal subunits was subjected to cDNA directed RNase H hydrolysis by incubating 40 pmol of RNA, 300-750 pmol cDNA, and 4 units of RNase H in buffer (20 mM - 50 mM Tris-HCl pH 7.5, 100 mM KCl, 10 mM MgCl₂, 1 mM DTT). Reactions were incubated at 37°C for 30 seconds to 30 minutes. The reactions were stopped by phenol extraction and the RNA recovered by ethanol precipitation. The purified RNA was then resuspended in tracking dye and separated on polyacrilamide or composite gels before visualization by staining.

2.7 cDNA directed hydrolysis of 70S ribosomes

To test cDNA directed hydrolysis at the α-sarcin site of TC-70S ribosomes, experiments were performed in the presence
of N-acetyl-Phe-tRNA\textsuperscript{phe}. The tRNA was bound specifically to the P/P site of 70S ribosomes or to both the A/A and P/P sites of the ribosomes in reactions very similar to those previously mentioned. P/P site specific binding was also achieved in a less rigorous manner by using low magnesium concentrations (Lill et al. 1984). Reactions contained fifty pmol of TC-70S ribosomes in 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM MgCl\textsubscript{2}, and 1 mM DTT in 50 \(\mu\)l. Additionally reactions may have contained 25 \(\mu\)g of Poly (U), 250 pmol of N-acetyl-Phe-tRNA\textsuperscript{phe}, 1000 pmol of cDNA probe and 4 units of RNase H. After addition of magnesium, reactions were incubated for another 25 minutes at 37\(^\circ\) C. The reactions were stopped by phenol extraction which was then repeated and the RNA recovered by ethanol precipitation. The RNA was then separated on five percent polyacrylamide gels or on composite gels and visualized.

2.8 Protein synthesis

Protein synthesis was performed according to the protocol of Traub et al. 1979 or Ehrenberg, and Kurland, 1988. In experiments, where we wished to identify synthesis dependent cDNA directed hydrolysis of rRNA, several modifications were made to these systems. First, reactions may have contained, in addition to the normal components, a twenty fold molar excess of cDNA oligomer to ribosomes, RNase H (Wako, 4 units), or 1 \(\mu\)g of \(\alpha\)-sarcin (Ira Wool). Additionally, reactions used
in cDNA-directed hydrolysis experiments were not precipitated with trichloacetic acid after the elongation incubation. Instead, in order to isolate the RNA of ribosomes which had been synthesizing proteins, three volumes of phenol and two volumes of buffer were added to each reaction. This mixture was then agitated and centrifuged before removing the aqueous layer. The phenol extraction was then repeated on the aqueous phase and RNA was precipitated from it by the addition of three volumes of ethanol, incubation for 15 minutes at -70°C, and centrifugation at maximum rpm in an Eppendorf microfuge at 4°C. The ethanol was carefully aspirated off and the resulting RNA resuspended in the appropriate tracking dye and separated on five percent polyacrylamide gels or on composite gels.
Chapter 3. Results

3.1 Alpha-sarcin site dynamics

The α-sarcin site of intact ribosomes has previously been shown to be inaccessible to cDNA probes or to cDNA probes and RNase H (White et al. 1988; Endo et al. 1990). The conservation and important role of the site during translational elongation made it unlikely that the site was unavailable to solvent. Experiments were therefore designed to address the possibility that the α-sarcin site was sequestered, in some undefined manner, on the ribosomes used in previous reports. Initial experiments were based on the assumption that all of the conformations normally available to the ribosome would be present, at least transiently, during protein synthesis.

To test this hypothesis cDNA probes and RNase H were included in an in vitro protein synthesis reaction and the rRNA integrity was compared to that obtained from in vitro protein synthesis reactions not having additional components. Figure 13 indicates that a rRNA fragment of the appropriate size was generated by inclusion of cDNA probes and RNase H.

3.2 Alpha-sarcin site structure in isolated rRNA

In the absence of ribosomal proteins and other factors rRNA is generally considered to be in a non-native form. Certain portions of rRNA may retain a local conformation which
Figure 13. cDNA directed hydrolysis of E. coli α-sarcin site during protein synthesis. In vitro protein synthesis using 40 pmol of 70S ribosomes was performed according to the procedure of Traub et al. (1979). 6 units of RNase H and 639 pmol of cDNA were added with Poly(U) after the first incubation. Ribosomal RNA was isolated by phenol extraction and separated on polyacrylamide gels as described in the text. Lane 1: In vitro protein synthesis reaction; Lane 2: In vitro protein synthesis reaction plus cDNA complimentary to bases 2654-2667 of 23S rRNA; Lane 3: In vitro protein synthesis reaction plus RNase H and cDNA complimentary to bases 2654-2667 of 23S rRNA; Lane 4: In vitro protein synthesis reaction plus 0.5 micrograms of alpha sarcin. Lane 5: 40 pmol of 70S ribosomes incubated for 15 min at 37°C (50mM Tris-HCl, pH 7.6, 50mM KCl) in the presence of 0.5 micrograms of alpha sarcin.
is stable when proteins are not present. In an attempt to indicate if this is possible at the α-sarcin site, cDNA directed RNase H hydrolysis was performed on protein free 23S rRNA. As indicated in Figure 14 the α-sarcin site is structured in isolated 23S rRNA. This is demonstrated by the inability of DNA complementary to bases (2653-2661) to cause hydrolysis in the presence of RNase H. Conversely cDNA directed at bases (2653-2667) and (2659-2667) are capable of hydrolysis in the presence of RNase H, see figure 14.

3.3 Ribosomal tRNA binding and α-sarcin site structure

The ribosome contains three fundamental binding sites for tRNA the A, P, and E sites (Figure 11). Previously it has been shown that the α-sarcin site is not accessible to cDNA probes and RNase H when the ribosome is occupied by deacylated tRNA, similar conclusions were reached in these studies (Figure 15). The acylated tRNA analogue N-acetyl-Phe-tRNA^phe preferentially binds to the P/P site of 70S ribosomes under conditions of low magnesium or in the absence of messenger RNA. Conversely N-acetyl-Phe-tRNA^phe can be bound to both the A/A and P/P sites of 70S ribosomes in the presence of higher magnesium concentrations and messenger RNA. N-acetyl-Phe-tRNA^phe also binds more tightly to the P/P site under these conditions. Figure 16 indicates the binding of N-acetyl-Phe-tRNA^phe to 70S ribosomes for some of the conditions used in these studies and is in general accord with the data found in
Figure 14. cDNA directed hydrolysis of *E. coli* α-sarcin site of 23S rRNA. Five percent polyacrylamide gel of RNA isolated from molecules which were subjected to cDNA directed RNase H hydrolysis for 30 minutes at 37° C. Reactions containing RNA instead of ribosomes were incubated for 1 minute. 40 microliter reactions where appropriate contained 40 pmol of 23S rRNA or 70S ribosomes, 750 pmol of cDNA, 4 units of RNase H in 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 100 mM KCl, and 1 mM DTT. RNA was isolated as described in the text, dissolved in tracking dye and electrophoresed. Lane 1: 70S ribosomes; Lane 2: 23S rRNA and cDNA 23S(2653-2667); Lane 3: 23S rRNA and cDNA 23S(2653-2661); Lane 4: 23S rRNA and cDNA 23S(2659-2667); Lane 5: 70S ribosomes and cDNA 23S(2653-2667); Lane 6: 70S ribosomes and cDNA 23S(2653-2661); Lane 7: 70S ribosomes and cDNA 23S(2659-2667). The α-sarcin and 5S labels indicate RNA fragments migrating at those positions.
Figure 15. Composite gel of RNA isolated from ribosomes subjected to cDNA directed RNase H hydrolysis with deacetylated tRNA present in the P and E or A and P sites. 50 microliter reactions where appropriate, contained 50 pmol of 23S rRNA, 48 pmol of 70S ribosomes, 250 pmol of tRNA^Phe, 25 micrograms of Poly(U), 1000 pmol of cDNA, and 4 units of RNase H in 20 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 100 mM KCl, and 1 mM DTT. Reactions containing ribosomes were incubated for 5 minutes in 2 mM Mg++ before adjusting the concentration to 18 mM with concentrated MgCl₂ and then continuing the 37°C incubation for 25 minutes. Reactions containing RNA were incubated for 1 minute at 37°C. Purified RNA was obtained as described in the text, dissolved in tracking dye and electrophoresed. Lane 1: RNA size markers of the indicated length; Lane 2: 23S rRNA and cDNA 23S(2653-2661); Lane 3: 70S ribosomes, tRNA^Phe, and cDNA 23S(2653-2661); Lane 4: 70S ribosomes, tRNA^Phe, Poly(U) and cDNA 23S(2653-2661). The α-sarcin label indicates RNA fragments migrating at the correct position.
Figure 16. Binding of N-acetyl-Phe-tRNA\textsuperscript{Phe} to the P and to the A and P sites of 70S ribosomes. Reactions containing 25 pmol of 70S ribosomes and 25 to 250 pmol of (\textsuperscript{14}C) N-acetyl-Phe-tRNA\textsuperscript{Phe} were used to isolate P site binding. Reactions directed at A and P site binding also included 10 micrograms of Poly(U). Controls did not contain ribosomes. All reactions were incubated for 5 minutes at 37\degree C in 50 microliters of a buffer containing Tris-HCl, pH 7.5, 2 mM MgCl\textsubscript{2}, 100 mM KCl and 1 mM DTT. Reactions containing ribosomes were then brought to a final MgCl\textsubscript{2} concentration of 16.5 mM using concentrated MgCl\textsubscript{2} and incubated for an additional 25 minutes. All reactions were then incubated on ice for an hour. Filter binding assays were performed as described in the text.
the literature (Lill et al. 1984).

Initial experiments suggested that the α-sarcin site could be hydrolyzed by cDNA and RNase H when the ribosome was occupied by acylated tRNA. To identify which portion or portions of the elongation cycle were responsible for cDNA directed RNase H hydrolysis, combinations of N-acetyl-Phe-tRNA^{phe} and Poly (U) were used to isolate tRNA binding to the P/P or to the A/A and P/P sites of the ribosome. Figure 17 indicates that binding of N-acetyl-Phe-tRNA^{phe} to the A/A and P/P sites of 70S ribosomes alters the structure of the α-sarcin site so that it is available for hydrolysis. Additionally the evidence suggest that tRNA present on the ribosome in the A/P and P/E site may also be available to cDNA (Figure 17). To quantify the level of cDNA bound to ribosomes when the A/A and P/P sites were occupied by N-acetyl-Phe-tRNA^{phe} Milipore filtration with radiolabeled cDNA was performed. Using this technique we were unable to demonstrate that cDNA became bound to ribosomes.

3.4 Enzymatic activities associated with purified ribosomes

During the course of this study, it was found that deoxyribonuclease and other enzymatic activities were associated with our ribosome preparations. A general procedure, see the materials and methods section, which often results in ribosomes free of enzymatic activities, that alter cDNA, was developed. Attempts to modify the cDNA and inhibit
Figure 17. Composite gel of RNA isolated from ribosomes subjected to cDNA directed RNase H hydrolysis with acylated tRNA present in the P or A and P sites. 50 microliter reactions where appropriate, contained 50 pmol of 23S rRNA, 48 pmol of 70S ribosomes, 250 pmol N-acetyl-Phe-tRNA^{Phe}, 1000 pmol Phe-tRNA^{Phe}, 25 micrograms Poly(U), 1000 pmol of cDNA, and 4 units of RNase H in 20 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 100 mM KCl, and 1 mM DTT. Reactions containing ribosomes were incubated for 5 minutes in 2 mM MgCl₂ before adjusting the concentration to 18 mM with concentrated MgCl₂. After incubation RNA was isolated as described in the text, dissolved in tracking dye and electrophoresed. Lane 1: 23S rRNA and cDNA 23S(2653-2667); Lane 2: 70S ribosomes and cDNA 23S(2653-2667); Lane 3: 70S ribosomes, N-acetyl-Phe-tRNA^{Phe}, and cDNA 23S(2653-2667); Lane 4: 70S ribosomes, N-acetyl-Phe-tRNA^{Phe}, Poly(U) and cDNA 23S(2653-2667); Lane 5: 70S ribosomes, Phe-tRNA^{Phe}, Poly(U) and cDNA 23S(2653-2667)
the enzymatic activities or to directly inhibit the activities, proved ineffectual. These included, a 3' ribonucleotide on the cDNA, addition of excess DNA to reactions, and NaF treatment. One reason for these failures is that this type of approach is typically aimed at inhibiting a specific enzymatic activity. While investigating this problem evidence for several types of deoxyribonuclease, and a phosphatase were found. A 0.5M NH₄Cl wash was eventually used to make ribosomes without measurable contaminating activities. Though these ribosomes are more highly purified and thus less "native", they have been found to perform better in in vitro protein synthesis reactions.

A discussion of some points in the purification scheme is necessary to indicate certain pitfalls and possible avenues for improvement. Thorough removal of the DNA layer which pellets above the ribosomes during the high speed centrifugation is crucial. All of it must be removed, even at a significant cost in ribosome yield. The remaining ribosome pellet should be washed several times with buffer, in order to solvate remaining DNA and the upper layer of ribosomes. The NH₄Cl wash and following steps should be performed in the maximum allowable volume, which is usually limited by the rotor used for centrifugation. All ribosomes should be purified by centrifugation through sucrose, this is normally done when purifying TC70S or 50S and 30S. An improvement in this method could be made by combining the NH₄Cl wash and
sucrose purification during the first or second high speed spin. None of these additional precautions is a panacea. Every ribosome preparation must be individually checked for deoxyribonuclease and phosphatase activities, with radiolabeled DNA. Some of the DNA modifying activities were found to behave in a significantly different manner depending on the buffer used. It is therefore necessary to check ribosome preparations for DNA modification in the specific buffer for which experiments are planned.
Chapter 4. Discussion

This study was initiated in order to solve the paradox which existed for the α-sarcin site of large subunit rRNA. Several lines of evidence indicate that the site is available to solvent and large macromolecules in 70S ribosomes. At the same time exhaustive work showed that the site was completely inaccessible to cDNA probes (White et al. 1988). This situation might arise if the α-sarcin site were transiently available during the dynamic aspects of protein synthesis.

To test this possibility cDNA was used to probe ribosomes which were actively synthesizing protein. An intermediate stage of the elongation cycle was also identified which contained an α-sarcin site accessible to cDNA and RNase H. This was done by testing several complexes, by RNase H and filter binding assays, which are similar to intermediate steps in the elongation cycle, for their ability to bind cDNA probes. It is clear that the availability of the α-sarcin site to cDNA probes is altered by structural changes within the ribosome produced when acylated tRNA is present in the A/A and P/P site.

The structure of the α-sarcin site was investigated in protein free 23S rRNA. The data indicate that the site contains a structure which is unaccessible to cDNA on the 5' side of the loop. A similar cDNA which is complementary to the 3' side of the α-sarcin loop is capable of directing RNase
H hydrolysis, see figure 14. The DNA complementary to the entire single stranded region of the α-sarcin site can also cause hydrolysis of the site in the presence of RNase H. It is unclear if this is due to binding to the 3' side of the loop or destabilization of structure and binding to the entire α-sarcin loop. However α-sarcin, which recognizes the structure of the α-sarcin site, is capable of recognizing an RNA oligomer which contains the single stranded loop and a portion of the stem (Endo et al. 1990). It is also important that double stranded RNA oligomers with single stranded bases extending from a 3' end are significantly more stable than those with single stranded bases extending from a 5' end (Turner et al. 1987; Jaeger et al. 1990). The conclusion emanating from this work is that the effect of base stacking on RNA stability is more forcefully exerted in the 5' to 3' direction than in the 3' to 5' direction. This would produce an α-sarcin loop stabilized on the 5' side which is in agreement with the cDNA directed hydrolysis data. Therefore, at the resolution of cDNA and α-sarcin binding, it is possible that the structure of RNA in the α-sarcin loop is determined solely by the sequence of its constituent nucleotides and not by ribosomal proteins.

In vitro protein synthesis was used to produce most of the conformations normal to ribosomes. The availability of the α-sarcin site was determined in this system by incubating some reactions with cDNA probe and RNase H. The production of
an appropriately-sized rRNA fragment demonstrated that the ribosome has at least two conformations, having an α-sarcin site either available or unavailable to cDNA. These experiments did not define the exact stage(s) in protein synthesis which contained the structure sensitive to cDNA and RNase H. However the information provided was sufficient to design experiments which could be used to produce the modified structure by including the necessary constituents.

Analogues of elongation cycle structures were generated by using combinations of Mg^{2+}, N-acetyl-Phe-tRNA^{p}he, Phe-tRNA^{p}he, tRNA^{p}he, Poly (U) and 70S ribosomes. The availability of the α-sarcin site was then tested by demonstrating sensitivity to cDNA and RNase H. Occupation of the ribosome by acylated tRNA is required to produce the rRNA fragment characteristic of an accessible α-sarcin site. This indicates that the ribosome has structures which are unique when occupied by acylated tRNA as compared to deacylated tRNA. Though not investigated here, this information has significant impact on previous work which demonstrated that ppGpp synthesis by the ribosome is performed when it is occupied by deacylated tRNA.

It was not possible to unequivocally demonstrate that ribosomes with acylated tRNA in the P/P site are capable of binding cDNA probes. Indications that this may indeed occur are present but are not substantiated with hard data. Hydrolysis at the α-sarcin site with cDNA and RNase H when
only the P/P site was occupied by N-acetyl-Phe-tRNA\(^{\text{phe}}\) did occur. The magnitude of this hydrolysis was very low and not consistently reproducible. The hydrolysis could have been produced by two mechanisms. In these experiments N-acetyl-Phe-tRNA\(^{\text{phe}}\) may temporarily occupy the A/A site upon initially binding the ribosome and then move into the unoccupied P/P site. N-acetyl-Phe-tRNA\(^{\text{phe}}\) may also have a very limited and transient affinity for the A/A site of ribosomes when they do not contain mRNA. These situations would be undetectable by standard filter binding assays but could result in a small amount of cDNA directed RNase H hydrolysis. It is not possible to establish a reasonable conclusion from the experiments performed here.

Hydrolysis of the \(\alpha\)-sarcin site is greatly enhanced when tRNA is present in both the A/A and the P/P sites of 70S ribosomes. An attempt to quantify the number of ribosomes which produced available \(\alpha\)-sarcin sites was made. Unexpectedly, it was not possible to bind radiolabeled cDNA probes to ribosomes capable of producing cDNA-directed RNase H fragments. This phenomenon, although troublesome, can be explained in a variety of ways consistent with the available data. The RNase H experiments are performed at 37° C and the filter binding assays at 4° C for technical reasons. The available structure may only form at higher temperatures and thus, for thermodynamic reasons, is never available in filter binding assays. Alternatively the available structure may be
transient and exist on a time scale amenable to investigation by enzymatic catalysis but not resolvable by filter binding. At the present time there is no indication which would favor one explanation over another.

Qualitatively the hydrolysis of the α-sarcin site by cDNA directed RNase H is not as complete with elongation cycle analogues as was the hydrolysis obtained during in vitro protein synthesis. Each ribosome in the protein synthesis experiments undergoes the binding of acylated tRNA to the A/A site several times during the process of elongation. It may therefore be the case that cDNA has several opportunities to encounter the available α-sarcin site structure in protein synthesis reactions. In the reactions with N-acetyl-Phe-tRNA\(^{\text{phe}}\) occupying both the P/P and A/A sites of ribosomes the structure would only be formed a single time. This explanation would require that the α-sarcin site is not available to cDNA when N-acetyl-Phe-tRNA\(^{\text{phe}}\) actually occupies the A/A site but rather that the process of A/A site occupation contains a transient structure capable of binding the cDNA.

A more likely possibility is that cDNA binds the α-sarcin site when the acylated tRNAs are present in the P/E and A/P sites of the hybrid tRNA model (Moazed, and Noller, 1989). This situation would occur several times per ribosome during protein synthesis. On the other hand this could occur in the experiments using elongation analogues only when Phe-tRNA\(^{\text{phe}}\),
a contaminant of N-acetyl-Phe-tRNA^phe preparations, occupied the A/A site. Peptidyl transferase could then occur and the P/E, A/P structure capable of binding cDNA would then form. Additionally N-acetyl-Phe-tRNA^phe present in the A/A site may not be totally restricted from forming ribosomal conformations similar to the one equivalent to P/E, A/P bound ribosomes simply because it cannot undergo peptidyl transferase. Experiments with Phe-tRNA^phe occupying programmed 70S ribosomes were performed to try and identify if P/E, A/P bound ribosomes were capable of greater cDNA directed RNase H hydrolysis. Though incomplete, the evidence indicates that P/E, A/P bound ribosomes are not significantly more available to cDNA than P/P, A/A ribosomes. Further experiments will be necessary in order to solve the differential sensitivity of in vitro synthesis and elongation analogues to cDNA hydrolysis.

To explain the subtle relationships which can be used to generate a plausible mechanism for these results it is most useful to suggest a model which incorporates the data presented here as well as that available in the literature. The model which best fits all of the data is one in which there is a conformational change. This conformational change is caused by the binding of acylated tRNA to the A/A site of 70S ribosomes. Although the availability of the α-sarcin site increases upon aatRNA binding evidence suggests that the structure of the rRNA in this region remains relatively unaltered (Moazed et al. 1988). Thus the α-sarcin fragment is
generated by cDNA and RNase H because the conformational change allows cDNA to access the α-sarcin site not because rRNA structure within the α-sarcin site has been changed.

It is possible to suggest that the ribosome has always been competent to bind cDNA and that aatRNA allows RNase H access. However the inability of radiolabeled cDNA to bind free ribosomes indicates that this is probably not so. Radiolabeled cDNA does contain an extra phosphate at the 5' end, but it seems unlikely that this would inhibit binding. In either case, a ribosomal conformational change is necessary and it is most likely that this event increases the ability of cDNA to bind the ribosome.

The conformational change exposes the α-sarcin site, but is probably not responsible for altering its structure. It has been previously demonstrated that chemical modification of the α-sarcin site is the same with and without N-acetyl-Phe-tRNA^phe^ present at the P/P and A/A site (Moazed et al. 1988). If the local structure of the α-sarcin site changed when aatRNA was bound to the ribosome several criteria would have to be met. Transfer RNA-free ribosomes would expose a specific set of α-sarcin site bases to chemical modification. Under these same conditions the local structure of the α-sarcin site would not be altered by a complementary 15 base cDNA probe. Then, when ribosomes were occupied by an acylated tRNA, a conformational change would occur within the α-sarcin site. This change would retain the same chemical modification
pattern but would now be capable of binding cDNA probes. This situation seems doubtful and it is more likely that the ribosomes exist in two conformations that do not directly alter the \( \alpha \)-sarcin site structure.

Further evidence which indicates that the conformational change involves the availability of the \( \alpha \)-sarcin site and not its structure comes from experiments on protein free rRNA and RNA oligomers which imitate the site. Both \( \alpha \)-sarcin (Endo et al. 1990) and cDNA probes (Figure 14) are capable of cleaving at least one of these RNAs. Because the \( \alpha \)-sarcin site analogues are hairpin loops \( \alpha \)-sarcin must recognize the tertiary structure of the RNA and not only the proper sequence of nucleotides. Additionally \( \alpha \)-sarcin can only cleave ribosomes when acylated tRNA\(^{Phe}\) is present in the P/P and A/A site (Endo et al. 1990). In order for these criteria to be met it would be necessary for the RNA hairpin loops to form the structure equivalent to that formed when N-acetyl-Phe-tRNA\(^{Phe}\) occupies the P/P and A/A sites of ribosomes. This is possible, however, but it provides another constraint which, when combined with the chemical probing data, makes it unlikely that the \( \alpha \)-sarcin site has multiple structures. It is probably the case that tRNA-free ribosomes have a structure in which the \( \alpha \)-sarcin site is available to solvent and other small molecules such as chemical probes, but not available to larger cDNAs. Thus when acylated tRNA is bound to the A/A
site of ribosomes, the resulting conformational change allows large molecules access to the α-sarcin site.

Although I have referred to cDNA binding to the ribosome when N-acetyl-Phe-tRNA\textsuperscript{phe} is present in the A/A site this study did not eliminate the possibility that the binding and thus conformational change is formed during the process of tRNA binding. The experiments also do not rule out that the cDNA binding actually occurs when the ribosome is occupied by P/E and P/A site bound tRNA. If this is the case a conformational change within the α-sarcin site is still possible.
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