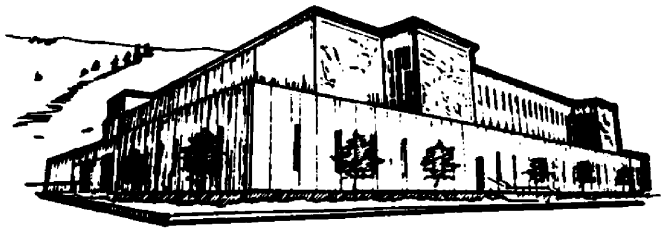
1990

Probing for the binding site of 4.5S RNA on Escherichia coli ribosomes using complementary DNA oligomers

Van R. Reese
The University of Montana



Probing for the Binding Site of 4.5S RNA on *Escherichia coli* Ribosomes Using Complementary DNA Oligomers

By

Van R. Reese

B.S., Brigham Young University, 1986

Presented in partial fulfillment of the requirements

for the degree of

Master of Science

University of Montana

1990

Approved by

Walter E. Hill

Chairman, Board of Examiners

R C Murray

Dean, Graduate School

Feb. 20, 1990

Date

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346

Reese, Van R., M.S., February 1990 Microbiology

Probing for the Binding Site of 4.5S RNA on Escherichia coli Ribosomes Using Complementary DNA Oligomers.
(70 pp.)

Director: Walter E. Hill Walter E. Hill

For the ribosome to carry out its functions, many different accessory factors are needed. These consist of a variety of proteins and nucleic acids. One of these essential factors in E. coli is the 4.5S RNA. Although it is known that 4.5S RNA interacts with the ribosome, the binding site has not yet been determined. This work is the first to present evidence for such a site.

This work has involved the use of complementary DNA oligomers to probe for the 4.5S RNA binding site on ribosomal RNA. Probe/ribosome interactions were quantified through nitrocellulose membrane filtration techniques. Specificity of probe hybridization was demonstrated by digesting the rRNA in RNA/DNA heteroduplexes with RNase H. A sequence search was conducted to determine possible binding sites and compared to the fragments obtained with RNase H digestion.

The results of this study indicate two binding sites which are overlapping and are at 435-440 and 440-444 of 23S rRNA; of further interest is that both sites are complementary to a single site at residues 44-49 of 4.5S RNA.

ACKNOWLEDGEMENTS

I wish to thank Dr. Walter Hill for his encouragement, support and much patience during the course of this work. I wish to thank, also, my coworkers for many informative discussions and insights. I acknowledge with gratitude the other researchers studying 4.5S RNA for their insights and especially Dr. M.J. Fournier for also supplying 4.5S RNA.

I am grateful to my parents for their love and support in my endeavors. Most importantly, I want to thank Kris who gave me the courage to begin this venture and without whose patience, love and understanding I surely could not have finished.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	=	adenine
ATP	=	adenosine 5'-triphosphate
C	=	cytosine
cDNA	=	complementary DNA
Ci	=	Curie(s)
CIAP	=	calf intestinal alkaline phosphatase
cm	=	centimeter
cpm	=	counts per minute
ddH_2O	=	double-distilled H_2O
DNA	=	deoxyribonucleic acid
DTT	=	dithiothreitol
EDTA	=	ethylenediamine tetraacetic acid
EF-G	=	elongation factor G
EF-Tu	=	elongation factor Tu
FBA	=	filter binding assay
g	=	gram(s)
G	=	guanine
GTP	=	guanosine 5'-triphosphate
HEPES	=	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HPLC	=	high-performance liquid chromatography
hr	=	hour(s)
M	=	molar
mA	=	milliamperes
min	=	minute(s)
mm	=	millimeter
mM	=	millimolar
mmol	=	millimole(s)
mRNA	=	messenger RNA
pmol	=	picomole(s)
PNK	=	polynucleotide kinase
RNA	=	ribonucleic acid
rpm	=	revolutions per minute
rRNA	=	ribosomal RNA
scRNA	=	small cytoplasmic RNA
SRP	=	signal recognition particle
T	=	thymine
TBE	=	tris, boric acid, EDTA
TC70S	=	tight-couple 70S ribosomes
TEA	=	triethylamine
tris	=	tris[Hydroxymethyl]aminomethane
tRNA	=	transfer RNA
U	=	uracil
(v/v)	=	volume to volume
(w/v)	=	weight to volume
μl	=	microliters

CHAPTER I

INTRODUCTION

The Ribosome

Perhaps more than many other biological processes, protein biosynthesis is a very complicated operation consisting of the precise interaction of many components. In all cells, protein biosynthesis is orchestrated by ribonucleoprotein complexes called ribosomes.

Because they are so closely tied to many of the cellular processes, ribosomes have been studied quite intensively for more than 30 years. Although ribosomes from a variety of organisms have been studied, by far the most extensive studies have focused on the ribosomes of the bacterium Escherichia coli. The E. coli ribosome is roughly 35% protein and 65% RNA and has a sedimentation coefficient of 70S. Physical studies in solution have shown the 70S ribosome to have a molecular mass of approximately 2.7 X 10^6 Daltons and dimensions equivalent to an ellipsoid with axes of 170Å x 230Å x 250Å. (37). At reduced Mg^{2+} concentration (about 1.5 mM) the 70S ribosome dissociates into two subunits, a large (50S) subunit and a small (30S) subunit.

The 30S subunit has a molecular weight of 9.0 X 10^5 Daltons. It contains one 16S rRNA having 1542 nucleotides of which nine bases are methylated (19) and 21 proteins, ranging in size from 8500 Daltons to 61,000 Daltons (106). Physical

studies have shown that in solution the 30S subunit has an asymmetric shape with approximate dimensions of 55Å x 220Å x 220Å (40).

The 50S subunit has a molecular weight of 1.8 X 10^6 Daltons. It contains two RNA molecules, a 5S rRNA of 120 nucleotides (16) and a 23S rRNA having 2904 nucleotides of which ten bases are methylated, three are pseudouridine residues and one base is a ribothymidine (11). There are 32 proteins ranging from 5400 Daltons to 24,500 Daltons (106). Solution studies have shown that the 50S subunit is also asymmetric and is a rather prolate ellipsoidal structure with dimensions of roughly 115Å x 230Å x 230Å (40).

Electron microscopy has made it possible to visualize 70S ribosomes and 50S and 30S subunits directly. This has given several three-dimensional structures which vary slightly from the model obtained through solution studies (45,47,48,89,104). Although there are a certain amount of differences among the models, they all share the same general form and composition; this is shown in Figure 1. The upper one-third of the 30S subunit is referred to as the "head" and is separated from the lower two-thirds, or base, by a neck-like indentation. A region referred to as the "platform" extends from the base of the subunit to form a cleft between it and the head. The model for the 50S molecule is also asymmetrical, consisting of a central protuberance and two protrusions (referred to as the "L7/L12 stalk" and the "L1

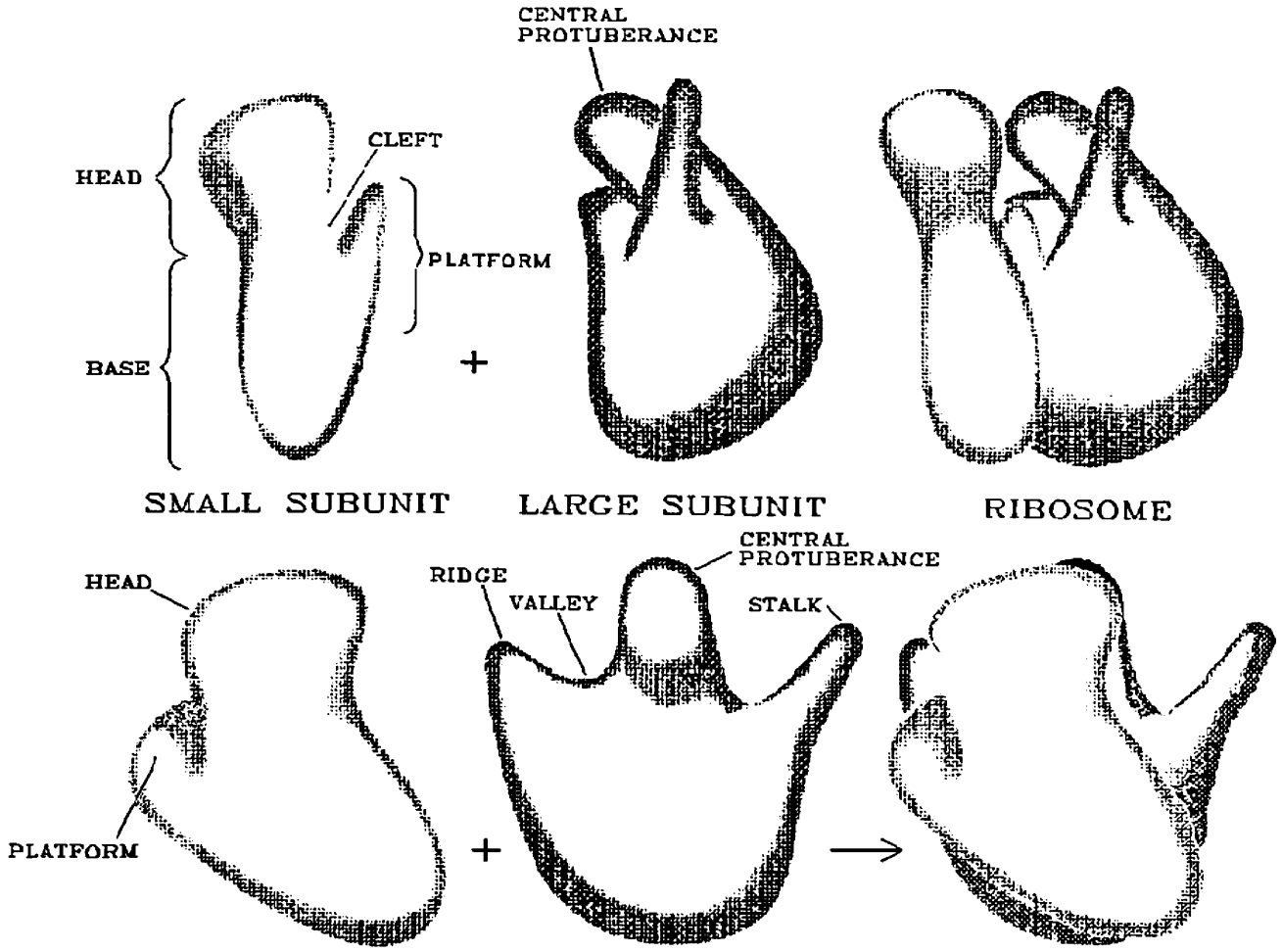


Figure 1: Three-dimensional model of the E. coli ribosome and ribosomal subunits. This figure represents the consensus model of the E. coli ribosome obtained from electron microscopy. From Oakes et al., (1986a).

shoulder") approximately 50 degrees to either side of the central protuberance extending from a roughly hemispherical body. In the 70S ribosome, the small subunit is positioned asymmetrically on the large subunit, as shown in Figure 1. The platform of the small subunit contacts the large subunit, so the partition between the head and body of the small subunit is aligned with the notch of the large subunit. The overall length of the ribosome is about 250Å (40).

Many of the separate components of the ribosome are well-characterized and although there is some consensus concerning the general form of the ribosome and its subunits, exactly how these components are arranged, remains to be elucidated. Some success in placing the proteins on the subunits have been made using immunoelectron microscopy (49,90,91), protein-RNA crosslinking (110), and protein-protein crosslinking (1,82,100). The most informative results about protein positions relative to each other have been obtained using small angle neutron scattering (59). Using neutron scattering data, it has been possible to propose a model for the spatial arrangement of all twenty-one of the proteins in the 30S subunit (17,18). While the neutron scattering data are less complete for the 50S subunit, some success has been seen and work is progressing (62,66). At present, the distances between thirteen 50S subunit proteins have been determined.

The Ribosomal RNAs

Only one-third of the mass of the ribosome consists of proteins; the other two-thirds of the ribosomal mass is contributed by RNA. Complete primary and secondary structures for *E. coli* ribosomal RNAs have been determined (53,63,108). The current secondary structure models for the rRNAs closely agree with one another with only slight deviations. The secondary structures of the rRNAs are divided into structural regions or domains which are conserved among all species.

The 16S rRNA molecule is subdivided into three major structural domains, and one minor domain, by three sets of long-range base-paired interactions (Fig. 2). The 5'domain (residues 26-557); the central domain (residues 564-912); and the 3' major domain (residues 926-1391) and the 3' minor domain (residues 1392-1542). Within each domain, the structure is organized into a series of helices, separated by various interior loops and bulges.

The 23S rRNA is organized by long-range base-pair interactions into six structural domains containing over 100 helices (Fig. 3a & 3b): domain I (residues 16-524); domain II (residues 579-1261); domain III (residues 1295-1645); domain IV (residues 1648-2009); domain V (residues 2043-2625); and domain VI (residues 2630-2882). The structural organization within the domains is similar to that of 16S rRNA. About 52% of the residues exist in base-paired structures. The 5' and 3' terminal sequences are also base-paired, giving the whole the form of a closed loop.

Although the primary and secondary structures of the ribosomal RNAs have been elucidated with some degree of confidence, the three-dimensional organization of the rRNAs within their respective subunits is considerably less clear. Studies with protein-free rRNA have given conflicting results; under certain ionic conditions, the rRNA molecules are able to organize into compact structures having the same approximate shape of ribosomal subunits (27,103), while under

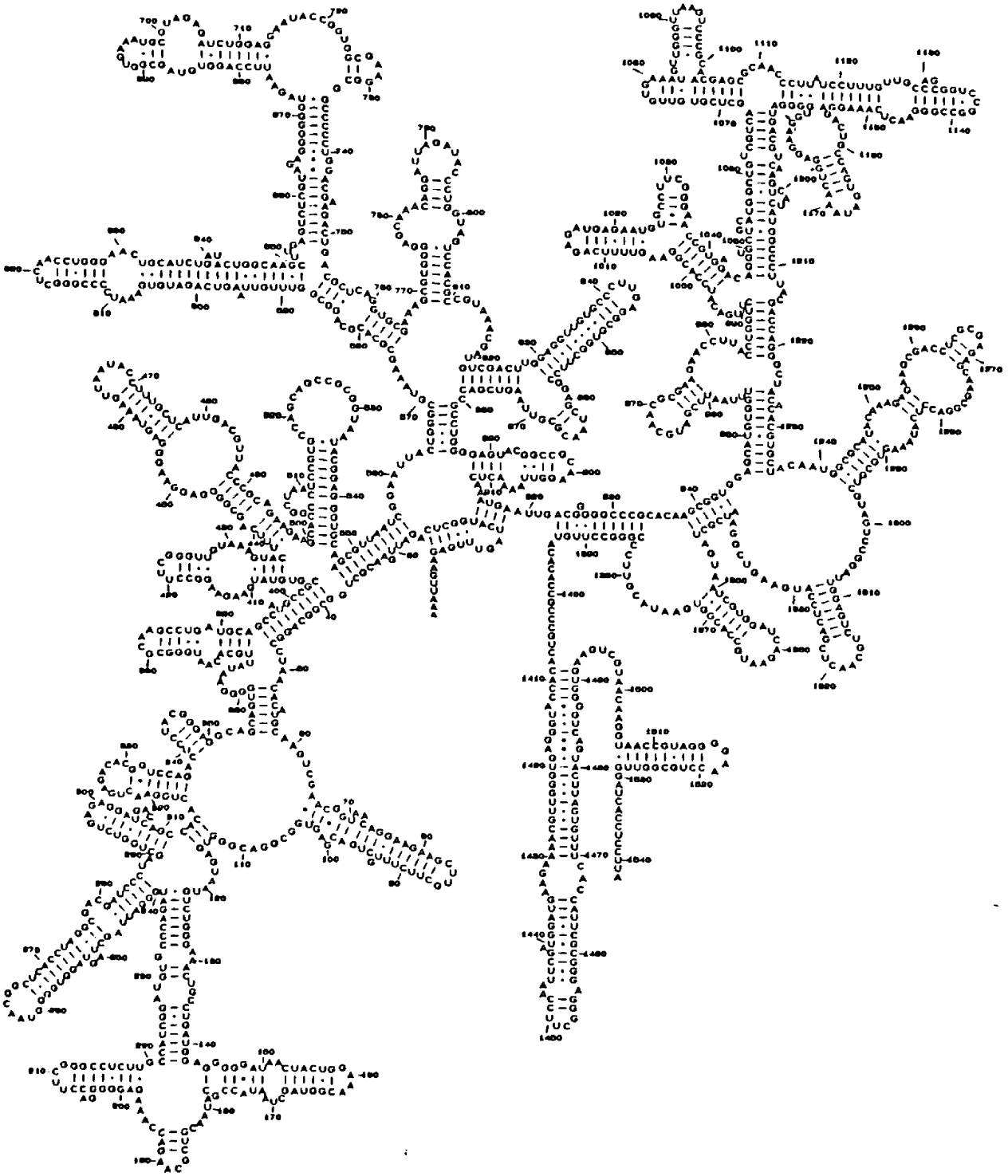


Figure 2: Secondary structure of E. coli 16S rRNA (Noller et al, 1986)

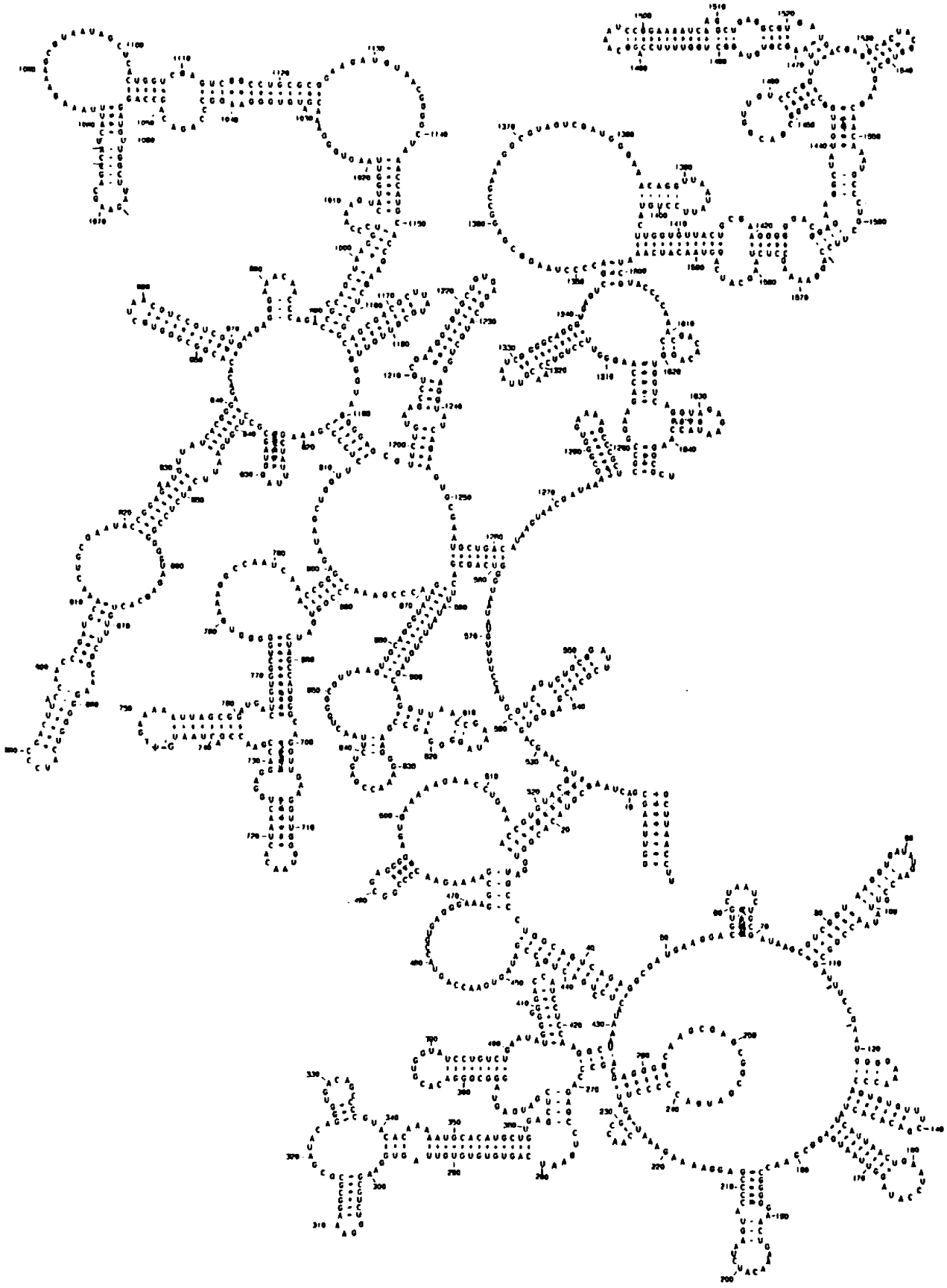


Figure 3a: Secondary structure of E. coli 23S rRNA (Noller et al, 1986).

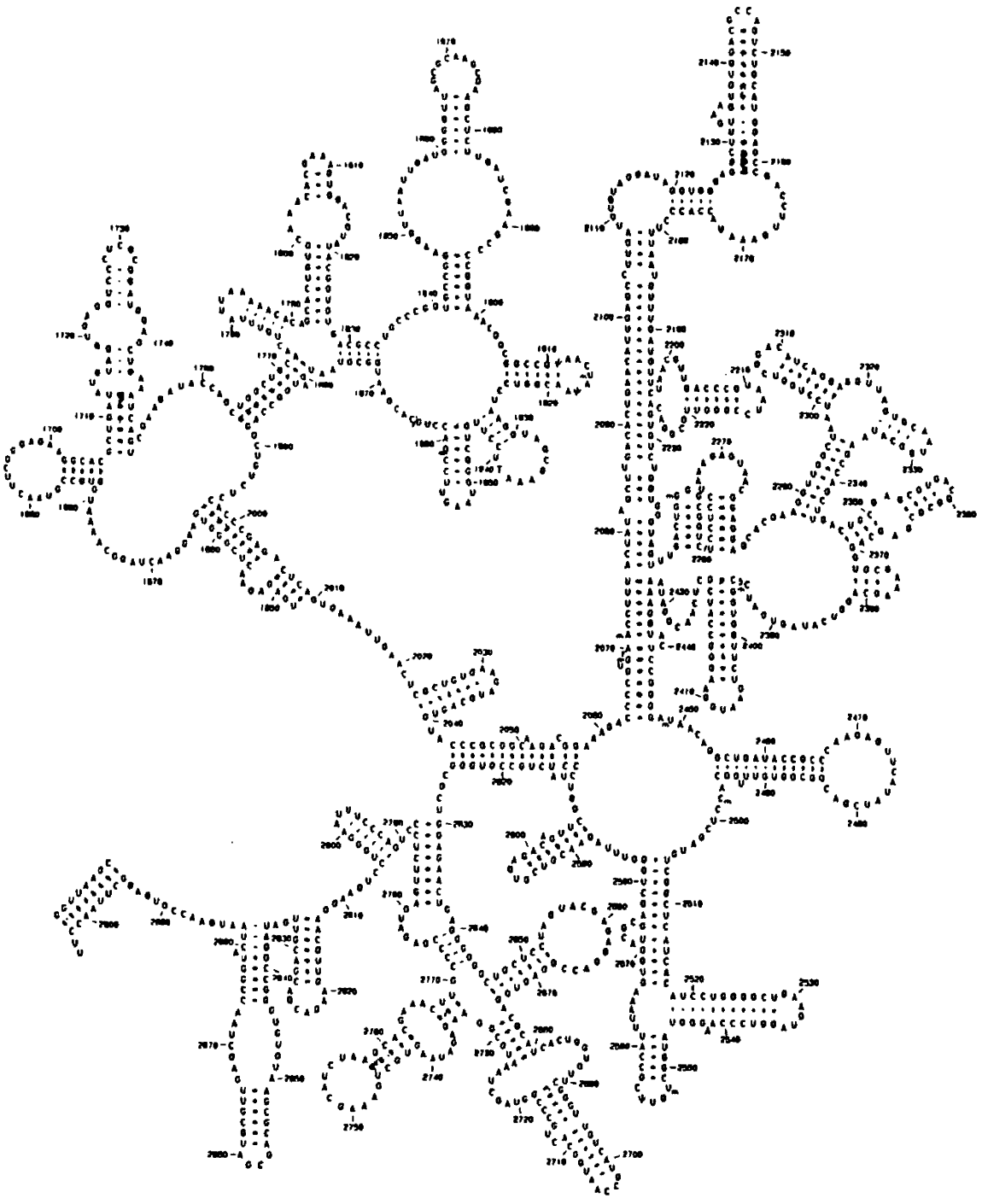


Figure 3b: Secondary structure of E. coli 23S rRNA (continued from 3a).

other conditions, rRNAs alone cannot attain a high degree of tertiary structure (8,94,95). In association with certain ribosomal proteins, however, the rRNA molecules are able to acquire a significant degree of folding (8,102). The evidence indicates that, it might be possible for the free RNA to form tertiary interactions in solution, but rRNA in the free state is quite a bit less ordered than rRNA in the subunit. Also, any tertiary structure probably depends upon association of specific structural proteins (8).

Specific tertiary interactions of rRNA within the subunits have been determined using a variety of techniques. Irradiation of subunits by ultraviolet light (88) or chemical crosslinkers (28,88) have been used to induce intra-RNA crosslinks in rRNA. Ribosomal RNA regions which are exposed to the surface of subunits have been localized using hybridization electron microscopy (67,68) and by localization of N_6,N_6-dimethyladenosine residues by electron microscopy of ribosome-antibody complexes (71). Ribonucleoprotein fragments of subunits prepared by limited nuclease digestion have been used to characterize protein-RNA and noncovalent RNA-RNA interactions for protein content and rRNA sequence (84,85).

Preliminary tertiary structure models for the rRNAs based on secondary structure, intramolecular RNA crosslinking results, protein-RNA interactions, and the locations of proteins within the subunits have been proposed (12,28,87).

These models, though quite informative, are far from complete as yet.

Ribosomal RNA Functions

After it was initially determined that the site of protein biosynthesis was on the ribonucleoprotein complexes, research efforts focused on determining the structural properties of the ribosomes. Perhaps because of the general consensus that proteins were the only molecules having any enzymatic activity, it was originally thought that all ribosomal functions were carried out by the ribosomal proteins. This suggested that the RNA portion of the ribosome acted merely as a scaffolding for the proteins to carry out their functions; tRNA and mRNA were merely molecules to be acted upon by the ribosomes and were essentially "inactive" so far as translation was concerned (107). Subsequently, it was revealed that no enzymatic activity could be assigned to any of the ribosomal proteins; researchers then started looking at the rRNA more closely. Interest in rRNA was especially high after Woese (107) and Noller and Woese (65) showed phylogenetic conservation.

Recent studies indicate that the RNA molecules are not just scaffolding, but also play an important role in ribosomal function (63,99). Among the various functions of the rRNAs are those of specific protein binding (109,112), mRNA binding (3,79,86), tRNA binding (26,35,55,69,73,78,113), antibiotic resistance (22,56,80) as well as subunit-subunit interactions

(36,98) translation factor binding and probably a multitude of other functions (63,105).

Upon analysis of functionally active rRNA sequences, certain important features become apparent. First, there is a high degree of phylogenetic conservation in those regions of ribosomal RNA sequences (22,63,107); second, structural features of active sequences are conserved (56,63); last, they are often exposed on the surface of the subunit (63).

With the advent of DNA synthesizers which can synthesize large fragments quickly and in relatively high yields, the use of cDNA hybridization as a research technique has greatly expanded. DNA probes have advantages over other techniques such as nuclease digestion or chemical modification because they can be targeted to specific rRNA sequences and the interactions are reversible.

Complementary oligodeoxyribonucleotides have been used successfully to probe RNA secondary structure (41,50) and to locate or identify rRNA sequences exposed on the surface of ribosomal subunits (55,68). Complementary DNA probes have also been used to aid in identification of rRNA sequences involved in specific ribosomal functions such as mRNA binding (3,97,101), subunit-subunit association (98) and tRNA binding (60,77)

Small RNAs

RNA is an exceptionally versatile macromolecule which is demonstrated by its ability to assume various conformations

and by its chemical reactivity. In prokaryotes, several small RNAs which are associated with a variety of functions have been discovered (reviewed in Inouye & Delihas, 1988). Recently, it has been found that some RNAs are able to display catalytic activities previously ascribed to proteins (20,31). Some have been shown to be associated with regulatory functions such as control of transcription (70), translation (51), transposition (81) and plasmid copy number (46) and plasmid transfer (24). Other small RNAs are involved in synthetic functions such as DNA replication (44), peptidoglycan synthesis (74), RNA processing (31) and phage DNA packaging (32).

One of the most interesting of the small RNAs which have recently been found is the 4.5S RNA of Escherichia coli. The 4.5S RNA is a small, stable RNA that has been shown to be essential for cell growth. Although a specific function has yet to be shown, it has been implicated in the initiation of protein synthesis (10) and in EF-G functioning (13,15).

Current Knowledge About 4.5S RNA

Griffin was the first to discover and sequence 4.5S RNA (29,30). Little additional work was done until 1984 when 4.5S RNA was sequenced and studied further (42). It was found that 4.5S RNA is a very stable molecule with a melting temperature of from 79°C to nearly 89°C (9). Cloning and sequencing studies (14) on the gene which codes for 4.5S RNA (ffs) showed

that it maps near lon at about 10 minutes on the E. coli gene map.

In wild-type E. coli, 4.5S RNA comprises approximately 2% of the total small RNA population (42), approximately the same level as a single species of tRNA. This represents about 3,000 molecules of 4.5S RNA per cell (61). Normally, about 1,000 molecules of 4.5S RNA per cell are required to maintain cell growth (15).

The sequence and secondary structure which Fournier and his colleagues suggest is shown in Figure 4; it was derived by utilizing secondary base pairings that maximize the free energy of folding (6). The molecule they suggest contains 114 nucleotides in a helix with

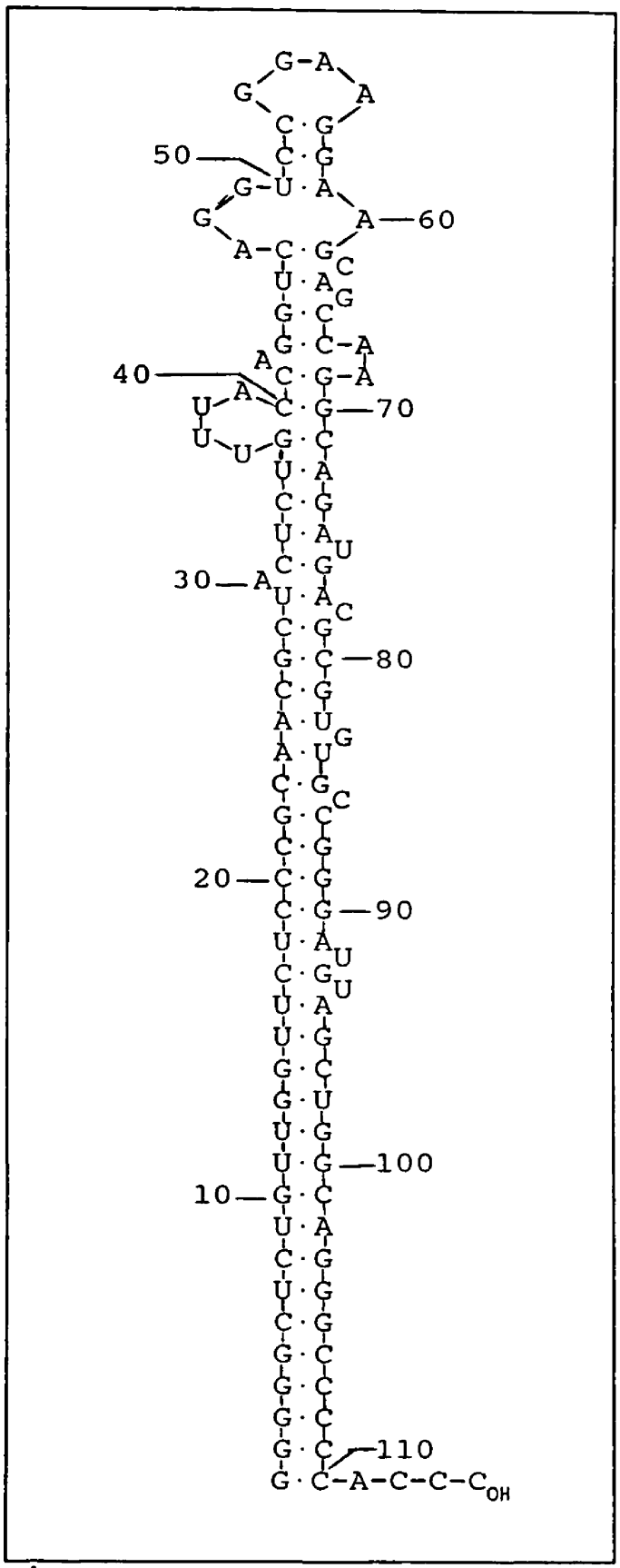


Figure 4: The proposed structure of 4.5S RNA (Hsu et al, 1984).

a length of 12.5 to 16.0 nm and a diameter of 3.7 to 2.6 nm (9) with a M.W. of 13,319.

Going from the denatured state to the base-paired form shown, 4.5S RNA has a calculated change in free-energy of -60 kcal/mol. In this form, 75% of the 114 bases are involved in 43 secondary base-pairs. The G+C content is 64% (9).

Like tRNA, the biosynthesis of 4.5S RNA is stringently controlled (43) and is processed by RNase P, a transfer RNA processing enzyme (7). This led researchers to theorize that 4.5S RNA might be involved in protein synthesis.

Brown and Fournier (14) were able to demonstrate the essentiality of 4.5S RNA by examining the recombination behavior of substitution mutations of the 4.5S RNA structural gene (*ffs*) generated *in vitro*. They showed that substitution mutants of *ffs* could not maintain cell viability except in the presence of an intact copy of *ffs*. They then attempted to determine the function of 4.5S RNA in the cell. To do this, they used a strain of *E. coli* in which *ffs* was placed under the control of a *lac* operon. This allowed them to study the effects of depleting the cell of 4.5S RNA simply by withdrawing the *lac* operon inducer, isopropyl-β-D-thiogalactoside (IPTG). The normal chromosomal copy of the *ffs* gene was inactivated by partially replacing the coding region with a kanamycin determinant. Following the removal of *lac* inducer, a coordinate decrease in DNA synthesis and growth rate was observed; however, the rate of protein synthesis fell

much more rapidly. From these analyses they were able to confirm that the ffs gene is essential for cell viability and that interruption of 4.5S RNA synthesis leads to eventual disruption of protein synthesis activity.

More recently, Bourgaize and Fournier have attempted to further characterize the physiological changes which take place when the bacterial cell is depleted of 4.5S RNA (10). They also utilized a strain of E. coli in which the synthesis of 4.5S RNA is under the control of IPTG. As shown in the previous study, a decline in protein synthesis was seen preceding a decline in DNA synthesis and growth rate by about 15-20 minutes. Extracts were prepared at several time points following removal of inducer and analyzed for translational activity. It was observed that the 4.5S RNA depleted extracts were able to maintain elongation, and that there was no apparent accumulation of incomplete proteins. In addition, further analyses of the 4.5S RNA depleted cell fractions revealed a lack of initiation-competent ribosomes. This information, together with the previous observation (42) that the loop region of the 4.5S RNA has two overlapping sequences similar to the Shine-Dalgarno sequence on mRNA, led them to conclude that 4.5S RNA is involved in initiation of translation.

Other recent studies have focused on a 22-base sequence which includes the loop region of the molecule mentioned above. This sequence consists of residues G44 through C65 of

4.5S RNA. This 22 base sequence is highly conserved and has been found in small cytoplasmic RNAs (scRNA) of Bacillus subtilis, Pseudomonas aeruginosa, and Thermus thermophilia (92,93) as well as 4.5S RNA of E. coli. It has been shown that the scRNA of B. subtilis, and the 4.5S RNAs from P. aeruginosa and T. thermophilia can function to replace 4.5S RNA in a strain of E. coli lacking the 4.5S RNA gene (92). In addition, it has been observed (72) that one arm of the eukaryotic 7S SRP RNA contains the putatively conserved 22 base sequence. This observation suggests the possibility of 4.5S RNA playing a role in the secretory pathway. Another possibility is that 7S RNA has a function not previously attributed to it.

Current research of Brown has focused on the role of 4.5S RNA in the function of protein synthesis elongation factor G (EF-G). As Brown points out, "Often the function of a cellular component can be inferred from the known components with which it interacts". Since 4.5S RNA is known to be essential, it can be assumed that it must interact with some other component in carrying out its function. Using a strain of E. coli which is under the control of the lac operon, Brown looked for inducer-independent suppressors which would restore viability by coding for cellular components with an increased affinity for 4.5S RNA (15). He was able to show that mutants mapping to fus, the structural gene for EF-G, altered the intracellular distribution of 4.5S RNA so that it sedimented at 70S or greater in a sucrose gradient. This corresponds to the

sedimentation velocity of ribosomes and polysomes. The results obtained show that 4.5S RNA physically associates with the ribosome in the performance of its essential function, and that this association is mediated by EF-G.

In a more recent work, Brown looked at the time of action of 4.5S RNA in E. coli translation (13). He utilized a new class of suppressor mutants which reduced the requirement for 4.5S RNA in translation by increasing the intracellular concentration of uncharged tRNA. Suppression might occur by prolonging the period in which translating ribosomes have translocated but not yet released the uncharged tRNA, indicating that this is the point at which 4.5S RNA enters translation. It was shown that the release of 4.5S RNA from polysomes was affected by antibiotics which inhibit protein synthesis. From the antibiotic-sensitivity observed, Brown concluded that 4.5S RNA exits the ribosome following translocation and just prior to release of EF-G.

One of the most interesting parts of this latest study is the model proposed by Brown in which he suggests that 4.5S RNA functions as part of the EF-G binding site on the ribosome following translocation. Two lines of evidence indicate that EF-G associates with a region of 23S rRNA surrounding nucleotide 1068. First, Skold has shown direct crosslinking of EF-G to a 27 nucleotide fragment of 23S RNA going from nucleotide 1055 to 1081 (83). Footprinting studies (57) have given additional support for EF-G binding to this portion of

23S RNA with their finding that EF-G protects nucleotides 1067 and 1069 of 23S RNA from dimethyl sulfate in 70S ribosomes. With this in mind, it is striking that the decanucleotide sequence (GAAGCAGCCA) from 1068 to 1077 of 23S RNA is identical to the decanucleotide sequence from 58 to 67 of mature 4.5S RNA (42). With the evidence indicating a functional relationship between 4.5S RNA and EF-G, and the evidence localizing the time of action of 4.5S RNA to post-translocation along with the sequence identity, Brown proposed the following model. Prior to translocation, the EF-G binding site on the ribosome includes the 1068 domain of 23S RNA. Following translocation, 4.5S RNA replaces this portion of 23S RNA on EF-G to either stabilize or stimulate a productive transition to occur in the post-translocation complex (13).

As one test for his model, Brown utilized plasmids encoding site-specific mutations at either nucleotide 1067 or 2661 of 23S RNA to test for their ability to suppress for low levels of 4.5S RNA. Nucleotide 2661 was used as a control because it also was implicated in EF-G binding by footprinting studies (57) but is distinct from nucleotide 1067. The model described above predicts that a pyrimidine (instead of the normal purine) at position 1067 will bind EF-G less effectively and suppress the requirement for 4.5S RNA to displace it. The results showed that pyrimidines at position 1067 greatly reduce the requirement for 4.5S RNA; however,

the mutation at 2661 does not reduce the requirement for 4.5S RNA.

Research Objectives

This study has attempted to answer two basic questions related to 4.5S RNA function. These questions are, first, does 4.5S RNA bind to the ribosome *in vitro*, and second, what are the interactions between 4.5S RNA and the ribosome? These questions were answered using labeled 4.5S RNA and DNA probes in conjunction with filter binding techniques and using unlabeled probes in RNase H digestion assays.

Although it has been shown by Brown that 4.5S RNA does associate with the ribosome in the performance of its function (15), previous studies (10) had concluded that it did not bind. From further discussions with the researchers, they had determined that 4.5S RNA does bind to the ribosome (Fournier, personal communication). It would seem reasonable that 4.5S RNA would interact with the ribosome, if only to properly position it to carry out its essential function. In any case, it is essential to show that 4.5S RNA binds to the subunits. To answer the first question I proposed to label 4.5S RNA at the 3' end and incubate it with ribosomes and subunits.

The main goal of this study has been to answer the second question which pertains to elucidating an interaction between 4.5S RNA and the ribosome. From what is known so far, it can be assumed with some certainty that 4.5S RNA does bind to ribosomes. Knowledge of the binding site for 4.5S RNA would

give one more clue, perhaps, as to how this intriguing molecule functions. To answer this question, I proposed to use cDNA oligomers which are identical to a specific region of the 4.5S RNA molecule and assay the binding of these DNA probes to ribosomes and subunits. The use of DNA oligomers to study the interactions of specific sequences of RNA, is a method commonly used in this laboratory (55,98) and in others (2,3). Since the 22 bases from nucleotide 44 to 65 have been found to be conserved and, therefore, implicated in 4.5S RNA function, I will start by using a DNA probe identical to this region to carry out a competition assay in which 4.5S RNA will be used to compete with the 22 nucleotide DNA probe for a binding site. While it is quite likely that the 22-base DNA probe does not behave in the same manner as 4.5S RNA, it would seem reasonable that base-pairing interactions involving the conserved apex region of 4.5S RNA can be studied using the DNA probe. A competition assay could be used to confirm this assumption; competition would infer that both molecules are binding to the same site. RNase H will be used with the 22-nucleotide DNA oligomer to clip the RNA at the sites of DNA hybridization; analysis of these clips will reveal possible binding sites. By the use of shorter probes (6-8 nucleotides) it should be possible to better define the binding site.

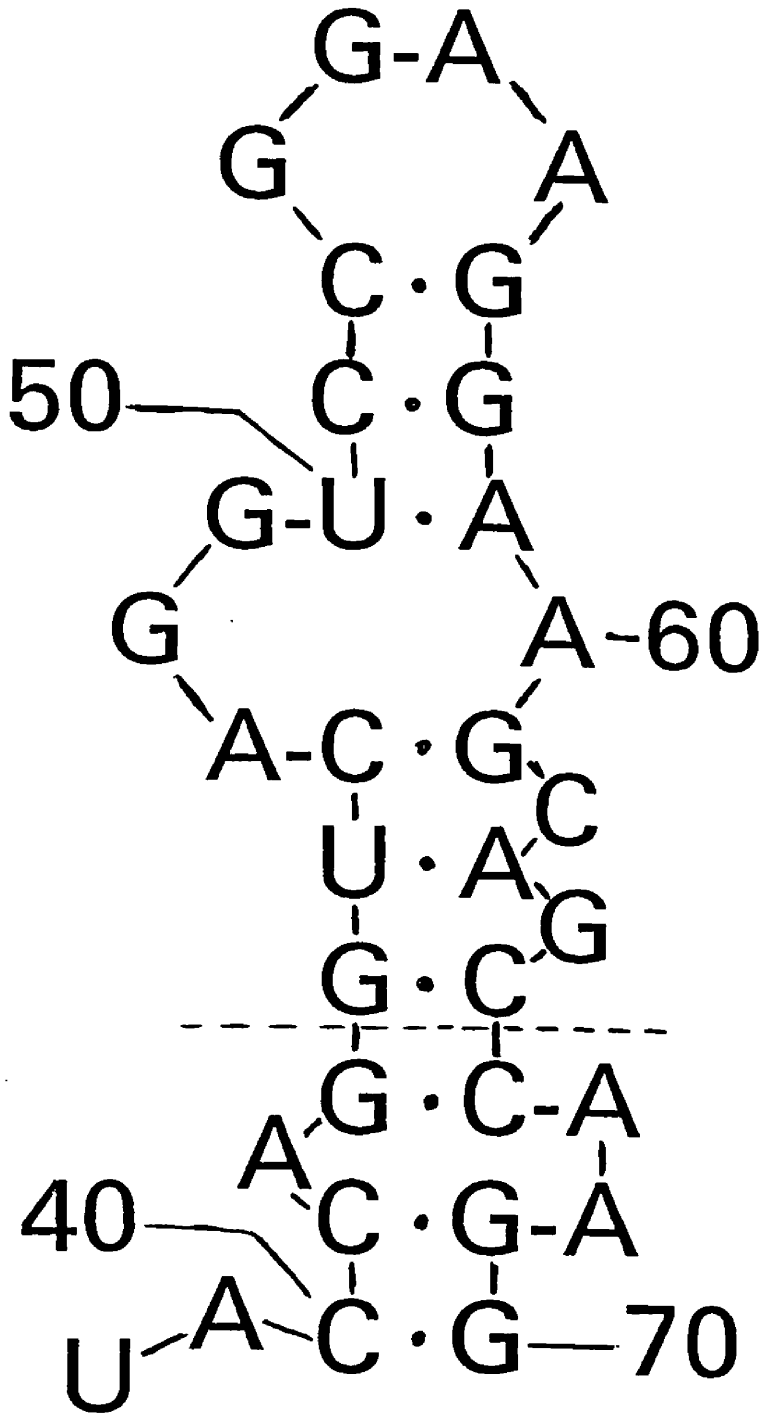


Figure 5: The hairpin-loop region of 4.5S RNA showing the conserved 22-base region from G44 to C65 (39, 68)

CHAPTER II

MATERIALS AND METHODS

Preparation of Ribosomes and Ribosomal Subunits

Ribosomes were isolated from E. coli RNase I deficient strain MRE 600 obtained from Grain Processing Co. (Muscatine, IA). The cells were grown to early log phase, quick cooled during harvesting and stored at -70°C. Essentially, the method of Hill et al (37) was followed in isolating the ribosomes. Typically, 100 g of frozen cells were thawed in 100 ml of buffer A (10 mM Tris-HCl, pH 7.4; 70 mM KCl; 10 mM $MgCl_2$) and kept on ice. The cells were pelleted at 10,000 rpm for 20 minutes in a Sorvall SS-34 rotor to wash the cells. The cells were disrupted using either a Gifford-Wood Mini-Mill (22,000 rpm set at approximately 1.5 mm) with a slurry of 0.25-0.30 mm glass beads, cells and buffer A to a final volume of 125 ml or, more recently, by hand grinding using a mortar and pestle on a slurry of alumina, cells and buffer A (2.5:1 (w/w) alumina : cells). The disruption lasted for about 50 min, maintaining a temperature of 4°C. The alumina or glass beads were removed by centrifugation or settling respectively, following which the supernatants were centrifuged 1 hr 30 min. in the Sorvall SS-34 at 15,000 rpm (low-speed spin) to remove cell debris and remaining glass beads or alumina. The supernatant was then spun at 60,000 rpm in a Beckman Ti 70 rotor for 2.5 hr (high-speed spin) and the pellet resuspended

overnight in buffer A. The sample was then given another low- and high-speed centrifugation and the final pellet resuspended in 30-50 buffer (10 mM Tris-HCl, pH 7.4; 100 mM KCl; 1.5 mM $MgCl_2$) or TC70 buffer (20 mM Tris-HCl, pH 7.4; 100 mM KCl; 6 mM $MgCl_2$) depending upon whether ribosomal subunits or tight-couple 70S (TC70S) ribosomes were desired, respectively. Yields were typically better for the alumina grinding than for the Mini-Mill disruptions producing 1.6 g ribosomes/100 g cells and 1.2 g/100 g cells respectively. Ribosomes were stored at

-70°C until needed.

Purification of 30S and 50S subunits was accomplished by sucrose-gradient zonal centrifugation as described by Tam & Hill (96). One gram of ribosomal subunits made 5% (w/v) in 0.1% diethylpyrocarbonate (DEP)-treated sucrose in 30-50 buffer was loaded into a Beckman Ti 15 rotor spinning at 2,000 rpm. A 10-34% sucrose gradient was pumped (International Equipment Co./Gradient Pump) into the rotor displacing the sample to the rotor core. The zonal was centrifuged at 31,000 rpm for 14.5 hr. The gradient was displaced by pumping H_2O into the center of the rotor, and ten milliliter fractions were collected and monitored (Gilson collector/Chromatronix Model 220 detector). Fractions containing 30S and 50S subunits were pooled separately and centrifuged at 60,000 rpm for 6 hr. to pellet. The pellets were resuspended in 30-50 buffer and stored at -70°C.

Using a modification of the method described by Hapke and Noll (33) for the preparation of tight-couple 70S ribosomes, 70S ribosomes resuspended in TC70 buffer were separated from 50S and 30S using zonal centrifugation as previously described with DEP-treated sucrose in TC70 buffer. The purified TC70S ribosomes were pelleted at 47,000 rpm for 5 hr, resuspended in TC70 buffer and stored at -70°C.

Preparation of salt-washed subunits was carried out as follows. Unwashed subunits were incubated on ice for 30-45 min. in 1M NH_4Ac 30-50 buffer (1.5 mM $MgCl_2$; 100 mM KCl; 1M NH_4Ac; 10 mM Tris-HCl, pH 7.4) with occasional vortexing. Salt-washed subunits were pelleted in a Beckman TLA 100.2 rotor at 100,000 rpm for 1 hr., resuspended in activation buffer (15 mM $MgCl_2$; 150 mM KCl; 10 mM Tris-HCl, pH 7.4; 2 mM DTT), and stored at -70°C.

Activation of 30S subunits as described by Moazed et al (111) and Zamir et al (58) was done by adjusting the sample buffer to 15 mM $MgCl_2$, 150 mM KCl, and 10 mM Tris-HCl (pH 7.4) and 2 mM DTT (1x activation buffer), heating the sample to 37°C for 15-20 min and then placing the sample immediately on ice.

Although great effort was put forth to standardize the isolation of ribosomes and subunits, there are undoubtedly minor differences present from one preparation to another. This might produce slight variations in results from one experiment to another, but these fluctuations should be small.

Preparation of DNA Probes

DNA probes were synthesized on a Biosearch Model 8600 microprocessor controlled DNA synthesizer using β-cyanoethyl-phosphoramidite chemistry. Synthesis was carried out as outlined in the Model 8600 instruction manual using derivatized controlled pore glass supports. To obtain optimum yields the column was removed from the synthesizer following the coupling of the last monomer; the oligomers were manually cleaved from the solid support by incubating the column in concentrated NH_4OH for 2 hours at room temperature. The benzoyl blocking groups were removed by incubating in a sealed ampule with NH_4OH at 55°C for a minimum of 7 hrs. The 5'-dimethoxytritylated (5'-DMT) probes were dried under vacuum and resuspended in 100 μl ddH_2O.

Reverse-phase high performance liquid chromatography (RP-HPLC) using a Gilson HPLC Gradient System with a 25 cm, 5 micron octadodecyl silane column (Column Engineering) was utilized to purify tritylated oligomers from the untritylated failure sequences. The mobile phase was a gradient of 100% Buffer A (10 mM TEA-OAc, pH 7.3) to 70.8% Buffer B (50% Buffer A: 50% Baker HPLC grade acetonitrile). The length of the gradient varied from 20 min. (for shorter probes) to 45 min. (for probes ≥10 nucleotides) at a flow rate of 1 ml/min. Purified 5'-DMT probes were evaporated to dryness, resuspended in 1 ml of 80% acetic acid and incubated for 1 hour at room

temperature to detritylate. 1 ml of ddH_2O was added and the probes were extracted four times with 2 ml portions of diethyl ether. The sample was evaporated to dryness, resuspended in 100 μl ddH_2O, and purified by gradient RP-HPLC as previously described. The purified oligomers were dried under vacuum, resuspended in 200 μl ddH_2O and stored at -70°C.

5'-Labeling of Probes

Purified DNA probes were 5'-end labeled using T4 polynucleotide kinase (PNK, United States Biochemical) and [γ-^{32}P] ATP (New England Nuclear) essentially following the procedure described by Chaconas and Van de Sande (21) with some modification. The labeling reactions contained 35 pmol probe (non-dephosphorylated), 35 pmol [γ-^{32}P] ATP (3000 Ci/mmol), and 4 units of PNK in 30 μl of buffer containing 10 mM DTT; 51.5 mM Tris-HCl, pH 8.8; 5 mM $MgCl_2$ and 3.3 mM $ZnCl_2$. Reactions were incubated for 1 to 1.5 hrs. at 37°C and stopped by addition of 5 μl of 200 mM EDTA. The labeled probes were purified on NENSORB 20 columns (New England Nuclear) as outlined in the NENSORB handbook. The purified, labeled probes were dried down and rehydrated in 50 μl of ddH_2O. Typical recovery was at a specific activity of 1.5×10^6 cpm/μl.

Preparation of 4.5S RNA

M.J. Fournier of the University of Massachusetts kindly provided 4.5S RNA for this study. The 4.5S RNA was isolated from a strain of *E. coli* harboring multiple copies of a plasmid (pKK247-2) containing the *ffs* gene. Isolation and

purification was performed in that lab essentially as described by Hsu et al (42). This involved isolation of 4.5S RNA from crude E. coli tRNA using gel filtration chromatography and polyacrylamide gel electrophoresis. Using conditions developed for separation of tRNA and 5S RNA (76), 200 mg of crude tRNA dissolved in about 1 ml of 1 M NaCl was fractionated on a Sephadex G-100 column (180 cm x 2 cm). The column was eluted with 1 M NaCl at a flow rate of about 1 ml/min; 5 ml fractions were collected and the elution profile was determined by measuring absorbance at 260 nm.

Fractions enriched in 4.5S RNA were pooled and the RNA recovered by ethanol precipitation. The RNA was further purified by 2 cycles of gel electrophoresis in 10% (w/v) polyacrylamide gel slabs (19:1) in a TBE (Tris-borate/EDTA) buffer system. RNA bands were detected by staining a border strip with 0.04% methylene blue, and the 4.5S RNA was recovered by extracting excised and mashed gel pieces with 2x SSC (SSC is 15 mM sodium citrate; 150 mM NaCl, pH 7.0). 4.5S RNA prepared in this manner proved to be quite homogeneous when analyzed by electrophoresis on a 10% polyacrylamide gel containing 7 M urea, giving a single band.

3'-Labeling of 4.5S RNA

In order to assay the binding of 4.5S RNA to the ribosome, it was essential that either the 5'- or the 3'-end of the molecule be labeled. The 5'-end of 4.5S RNA is putatively base-paired and probably inaccessible for 5'-end

labeling. However, the 3'-end is quite accessible and lends itself well to labeling. The procedure outlined by D'Alessio (23) was followed using T4 RNA ligase (Pharmacia) and [5'-^{32}P]pCp (New England Nuclear).

The RNA ligase reaction ligates 5' phosphate groups to 3' hydroxyl groups and is capable of forming intramolecular or intermolecular bonds which compete with the labeling reaction. To avoid this problem, the RNA was first dephosphorylated. This was done by incubating 25 pmol 4.5S RNA with 0.1 units of calf intestinal alkaline phosphatase (CIAP, Boehringer Mannheim) in 100 μl of 100 mM Tris-HCl, pH 8.0 for 30 min. at 37°C. The RNA was extracted with an equal volume of phenol:chloroform (1:1, v/v) by vigorous vortexing (the phenol was saturated with 0.1 M Tris-HCl, pH 8.0). The aqueous layer was removed and placed in a clean tube. The tube containing phenol:chloroform was back-extracted with 50 μl 10 mM Tris-HCl, pH 8.3 and the aqueous phase was combined with the aqueous phase from the first extraction. The pooled aqueous phases were extracted with another 100 μl of phenol:chloroform with vigorous vortexing. The upper (aqueous) phase was transferred to a clean microcentrifuge tube and 500 μl diethyl ether were added to remove the residual phenol and vortexed. The upper (ether) phase was discarded and the ether extraction was repeated. The 4.5S RNA solution was placed on ice and ethanol precipitated by addition of 50 μl 2 M sodium acetate (pH 5.5) and 500 μl of cold ethanol. After mixing

thoroughly, the solution was chilled at -70°C for 30 min and centrifuged for 5 min at 4°C in a microcentrifuge. The ethanol was removed very carefully to avoid disturbing the pellet after which 500 μl cold ethanol was carefully layered onto the pellet and chilled at -70°C for 10 min. The RNA was centrifuged for 1 min at 4°C and the ethanol removed. The tube was placed under vacuum for several minutes to remove traces of ethanol and the 4.5S RNA was dissolved in 10 μl sterile distilled water. The dephosphorylated 4.5S RNA was stored frozen at -70°C until needed.

To carry out the 3'-end labeling reaction, 10 μl dephosphorylated RNA (25 pmol, prepared above), 3 μl 10x ligase buffer (0.5 M HEPES-NaOH, pH 7.5; 0.1 M $MgCl_2$ and 33 mM DTT), 3 μl dimethylsulphoxide (DMSO), 3 μl 33 μM ATP (Na^+; pH 6-7) and 6 μl ddH_2O were mixed in a siliconized, sterile 1.5 ml microcentrifuge tube and placed on ice. Behind a Perspex shield, 10 μl (100 μCi) of [5'-^{32}P]pCp (3000Ci/mmol) was transferred to another 1.5 ml microcentrifuge tube. The tube was covered with Parafilm\ and several holes were punched in it with a needle. The tube was placed under a vacuum until all the ethanol was evaporated and returned to ice. The entire RNA mix was transferred to the tube containing the dried [5'-^{32}P]pCp and vortexed briefly. The reaction was initiated by the addition of 5 μl T4 RNA ligase (1-2 μg protein;2-4 units), vortexing and incubating at 4°C for 6 hours. The tube was returned to ice and the reaction terminated with 25 μl cold

4 M ammonium acetate (an inhibitor of T4 RNA ligase). The RNA was precipitated by addition of 10 ul of phenol-extracted tRNA as carrier (1 mg/ml) and 180 ul of cold ethanol. The tube was chilled for 20 min at -70°C and centrifuged for 5 min at 4° to pellet the RNA. The supernatant was carefully removed and discarded. The RNA was dissolved in 50 μl 0.5 M sodium acetate (pH 5.5) and vortexed. To precipitate again, 150 μl of cold ethanol were added, mixed, chilled on dry ice and centrifuged as above. The supernatant was discarded into the radioactive waste and 500 μl of cold ethanol was carefully layered onto the pellet, but not mixed or vortexed. The sample was chilled 5 min on dry ice and centrifuged 1 min at 4°C in the microcentrifuge. The ethanol was carefully removed and discarded. The tube was covered with Parafilm\, several holes were punched in it with a needle and it was placed under a vacuum for several minutes to evaporate residual ethanol. The RNA was purified using a Nensorb 20 column as outlined in the NENSORB handbook accompanying the columns. The labeled fraction from the column was dried under vacuum and resuspended in about 50 μl ddH_2O. Typical yields were about 3.3×10^6 cpm/μl.

Filter Binding Assays

Filter binding assays were used to determine whether or not probe and 4.5S RNA would bind to subunits or TC70S ribosomes. These assays were carried out by incubating a series of reaction mixtures containing 10 pmol 50S or 30S

subunits or TC70S ribosomes and increasing amounts of 5'-^{32}P end-labeled probe or 3'-^{32}P end-labeled 4.5S RNA in 20 μl of activation buffer (15 mM $MgCl_2$, 150 mM KCl, and 10 mM Tris-HCl (pH 7.4) and 2 mM DTT). The incubation was at 4°C for 4 or 12 hrs depending on the experiment being done. Blanks containing equivalent amounts of radiolabeled probe or 4.5S RNA and no subunits were used as controls in these assays. Following incubation, the reaction mixtures were brought up to 1 ml with wash buffer (activation buffer without DTT) and spotted onto Millipore HAWP 0.45 μm nitrocellulose filters and washed twice with 1-ml aliquots of wash buffer. The radioactivity retained on the filters was determined on a Packard Tri-Carb\ Model 1500 liquid scintillation analyzer. The amount of probe or 4.5S RNA binding to the subunits was determined by subtracting the radioactivity retained on the blank filters from that retained on the corresponding reaction filters containing subunits.

Competition Assays

A competition assay was set up to see if the 22 nucleotide probe binds to the same site on the subunits and ribosomes as the 4.5S RNA. The conditions used were similar to those in the filter binding assays; a series of reaction mixtures were set up containing 10 pmol of subunits or TC70S ribosomes, 200 pmol 5'-^{32}P end-labeled probe and increasing amounts of unlabeled 4.5S RNA in 30 μl of activation buffer. The incubation was for 12 hrs at 4°C. Again, blank samples

containing equivalent amounts of radiolabeled probe and no subunits were prepared as controls in these assays. Following incubation, the reaction mixtures were diluted to 1 ml with 970 μl wash buffer and spotted onto Millipore HAWP 0.45 μm nitrocellulose filters and washed twice with 1 ml aliquots of binding buffer. The radioactivity retained on the filters was determined by liquid scintillation. The amount of probe binding to the subunits was determined by subtracting the radioactivity retained on the blank filters from that retained on the corresponding reaction filters containing subunits.

RNase H Assays

To determine the exact site or sites of cDNA probe base-pairing, the probe-subunit complex was incubated with RNAse H under conditions similar to those described by Donis-Keller (25) and the digestion products analyzed by electrophoresis. Reactions containing about 50-80 pmol of subunits or protein-free RNA, 200-1000 pmol cDNA probe and 2-4 units of RNase H (Waco Pure Chemical Industries) in 25-50 μl of RNase H buffer containing 40 mM Tris-HCl, pH 7.9; 60 mM KCl; 10mM $MgCl_2$ and 1 mM DTT were incubated at 4°C for 1 to 20 hr.

The ribosomal RNA digestion products were analyzed by gel electrophoresis. The RNA from subunits was prepared by extracting once each with buffer equilibrated phenol, phenol:chloroform (1:1, v/v) and chloroform. The aqueous layer was extracted twice with 1 vol. of diethyl ether to remove residual phenol and chloroform. The RNA was precipitated with

2.5 vol 100% ethanol and about 10 μl of 2 M sodium acetate at -70°C for 30 min and pelleted by centrifuging for 10 min at 14,000 rpm in a microcentrifuge. The ethanol was carefully removed and another aliquot of ethanol was layered onto the pellet and incubated at -70°C for 10 min. This was spun as before and the ethanol again removed. The RNA pellets were evaporated to dryness and resuspended in 20 μl tracking dye consisting of 7 M urea, 18% ficoll, 0.025% xylene cyanol and 0.025% bromophenol blue and electrophoresed in a 5% polyacrylamide gel (25 cm x 13.5 cm x 1.5 mm) containing 7 M urea and TBE at 12 mA for 7 hours at room temperature. RNA size markers (Bethesda Research Laboratories) were prepared according to the manufacturers protocol and run with the RNase H digestion reactions and controls. RNA products were stained in the gel with methylene blue.

CHAPTER III

RESULTS

Synthesis and Purification of cDNA Probes

Using the sequence and structure of 4.5S RNA proposed by Hsu et al (1984) and the 22 nucleotide conserved sequence in the hairpin-loop region proposed by Poritz et al (1988), several probes which are identical to certain segments of the conserved region were synthesized. The probe sequences and their area of identity are shown in Table I and Figure 6. Most of the probes made are identical to regions in the conserved sequence which have been implicated in ribosomal function or are identical to a region of ribosomal RNA which has been implicated in function. These probes were assigned codes based on the first 5'-end nucleotide and the length of the sequence. For example, probe C52/6 (5'-dCGGAAG) starts at residue 52 of 4.5S RNA, which is a "C" and is six nucleotides long.

Labeling of Probes and 4.5S RNA

Probes were labeled at the 5'-end with Γ-^{32}P-ATP and polynucleotide kinase while 4.5S RNA was dephosphorylated and labeled at the 3'-end using [5'-^{32}P] pCp and RNA ligase. After purification on NENSORB columns, preparations typically yielded specific activities of 1-3 x 10^6 cpm/pmol. Before using, the labeled probes and 4.5S RNA were normally diluted with unlabeled probe or 4.5S RNA to specific activities of 750-1000 cpm/pmol. This was done to reduce the amount of

Table I: Probes Used in These Studys

PROBE	SEQUENCE	REGION OF IDENTITY ON 4.5S RNA
G44/6	5'-dGTCAGG	44-49
C46/6	5'-dCAGGTC	46-51
C52/6	5'-dCGGAAG	52-57
G58/10	5'-dGAAGCAGCCA	58-67
A47/10	5'-dAGGTCCGGAA	47-56
G44/22	5'-GTCAGGTCCGGAAGGAAGCAGC	44-65

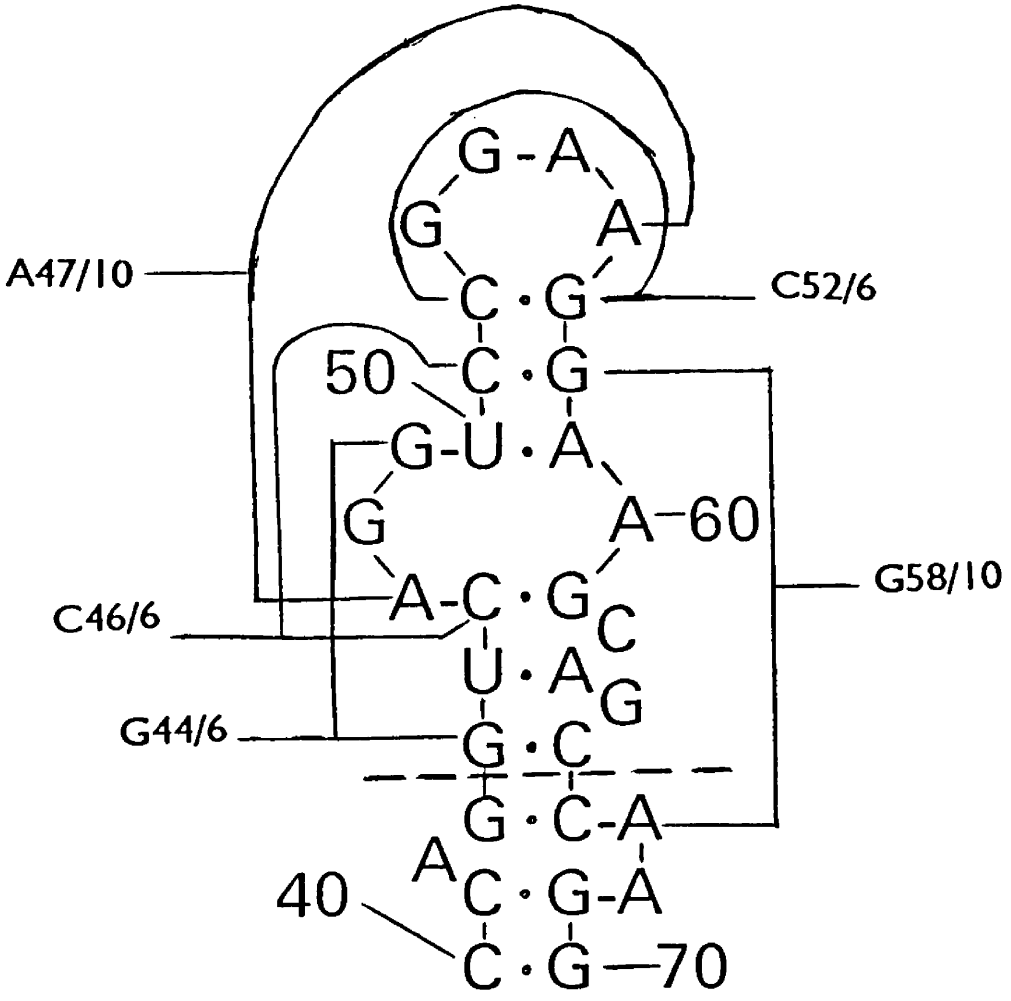


Figure 6: The apex region of 4.5S RNA showing the regions identical to the probes used in this study.

labeled sample used and to standardize the specific activities for each experiment. Some probe preparations were checked for homogeneity using polyacrylamide gel electrophoresis and autoradiography.

4.5S RNA Binding to Subunits and TC70S Ribosomes

Initially, to check binding of 4.5S RNA to subunits, subunits or TC70S ribosomes were incubated with increasing amounts of 3'-end labeled 4.5S RNA in activation buffer (15 mM $MgCl_2$; 150 mM KCl; 10 mM Tris-HCl, pH 7.4; 2 mM DTT) for approximately 12 hrs at 4°C. The percentage of bound 4.5S RNA was assayed by nitrocellulose membrane filtration techniques. The "percent binding" values for the 4.5S RNA were very close for 50S and 30S subunits at about 42% (Fig.7) and a little higher for TC70S ribosomes at about 60% (Fig.8). All points were performed in triplicate. The shape of the curves suggests that the binding of 4.5S RNA is going to saturation, but is there even at a ratio of 40:1. The data reported for 30S subunits used in these experiments is for non-activated subunits. In a separate experiment it was observed that activation of 30S subunits did not seem to have any effect on binding of 4.5S RNA.

4.5S RNA / G44/22 Probe Competition Experiments

To determine if 4.5S RNA can bind and displace a probe which is identical to the 22-base conserved region, competition experiments on 30S and 50S subunits and TC70S ribosomes were conducted. These experiments were conducted

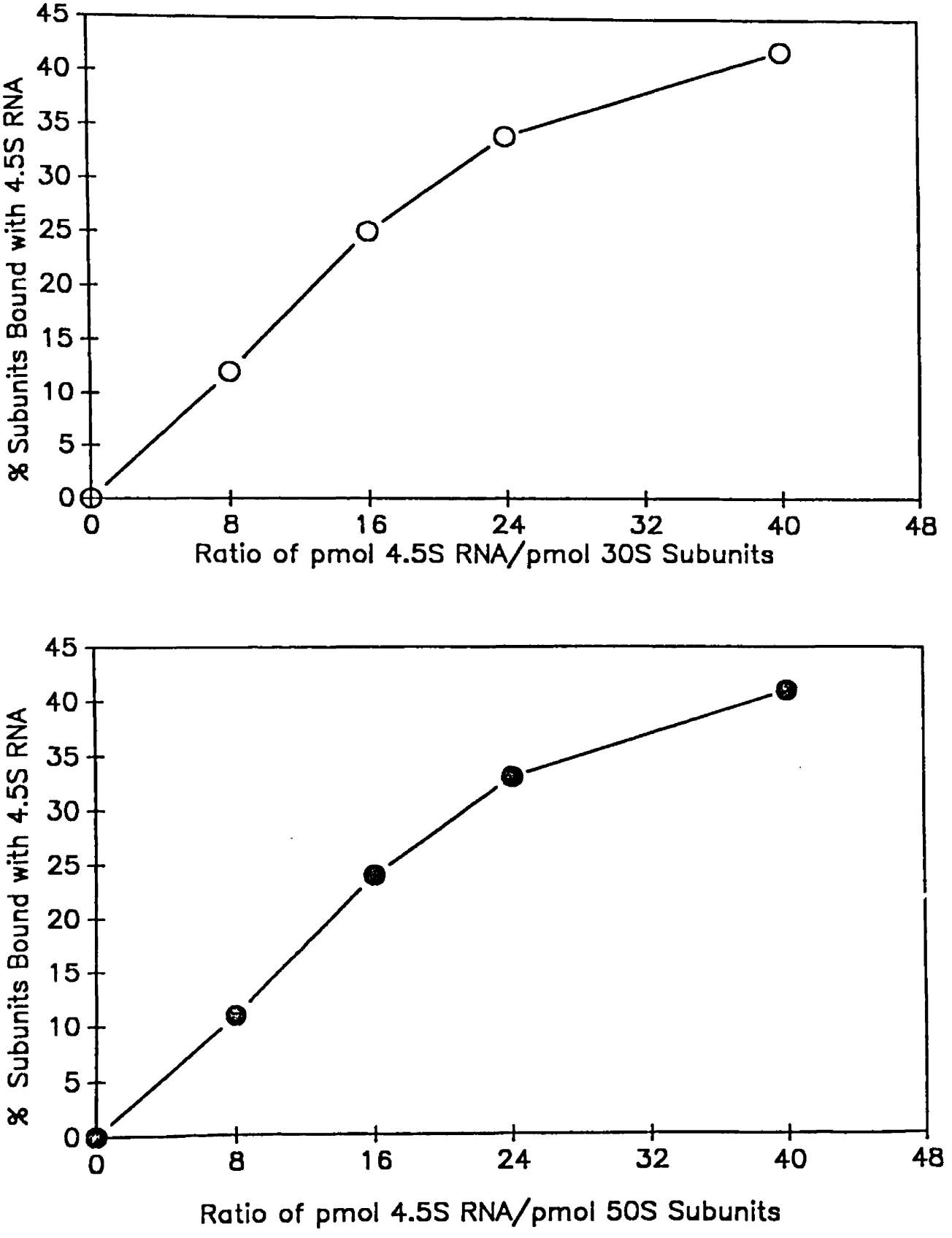


Figure 7: Binding of 4.5S RNA to 30S (top) and 50S (bottom) subunits *in vitro*.

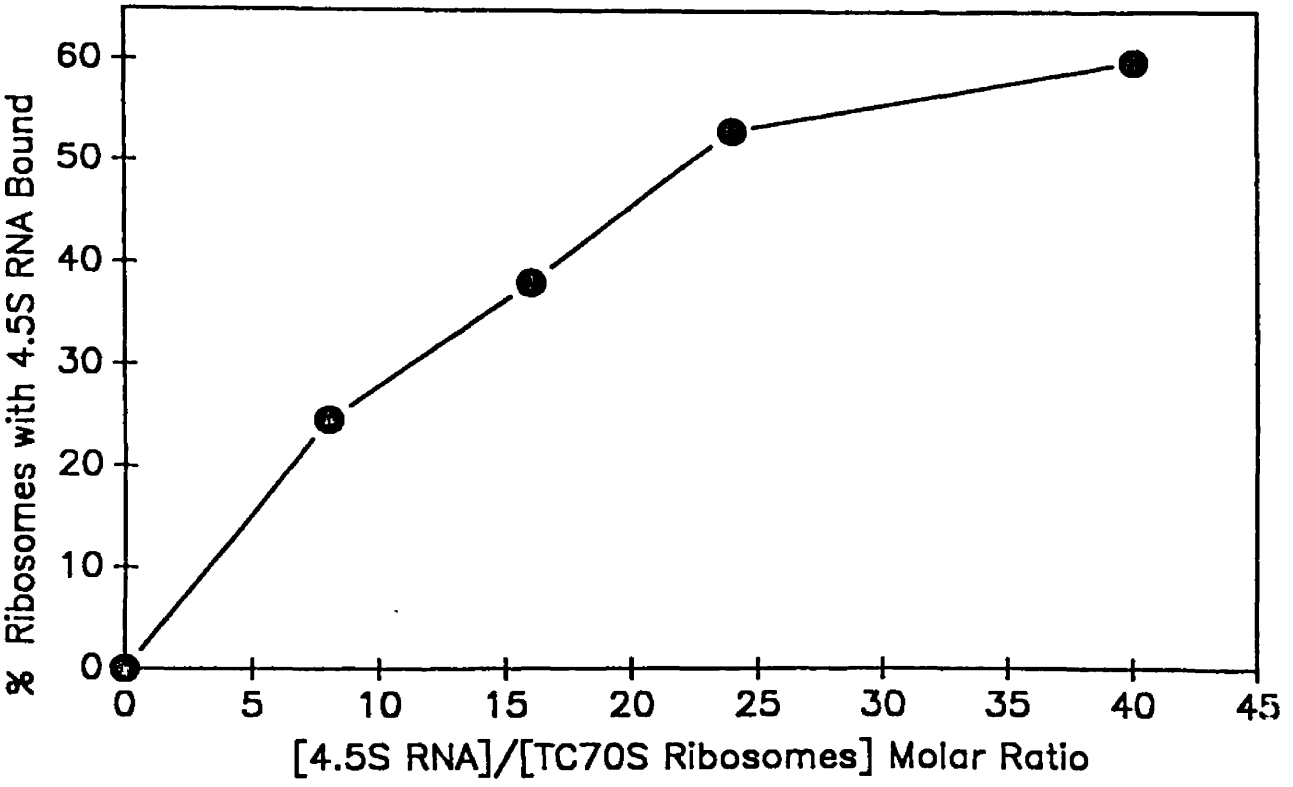


Figure 8: Binding of 4.5S RNA to TC70S ribosomes.

under probe hybridization conditions; for each experiment, 10 pmol of subunits or ribosomes and 200 pmol of the 22 nucleotide probe (G44) were incubated in activation buffer for 12 hrs at 4°C. The level of binding of the G44 probe was quantified through nitrocellulose filtration and liquid scintillation analysis.

The data for the competition assays on 50S subunits and TC70S ribosomes are shown in Figure 9. The percent binding of G44/22 in the absence of 4.5S RNA shows good binding at about 54% for 50S subunits and 65% for TC70S ribosomes. As the level of 4.5S RNA increases the percent binding declines quite rapidly at 10:1 ratio of 4.5S RNA to ribosomes for TC70S ribosomes and 20:1 for 50S subunits. Competitive binding experiments on 30S subunits showed no competition between G44/22 and 4.5S RNA (data not shown).

Sequence Search

In order to determine which regions of the ribosomal RNA are complementary to parts of the 22-base conserved sequence of 4.5S RNA, a sequence search was carried out on ribosomal RNA looking at five bases at a time. Five nucleotides represent the minimum number of bases which need to be available for hybridization in order to detect binding in a binding assay; whereas, RNase H can detect a four base hybrid (25). The results of the sequence search on 16S and 23S rRNA are shown in Figures 10, 11a and 11b.

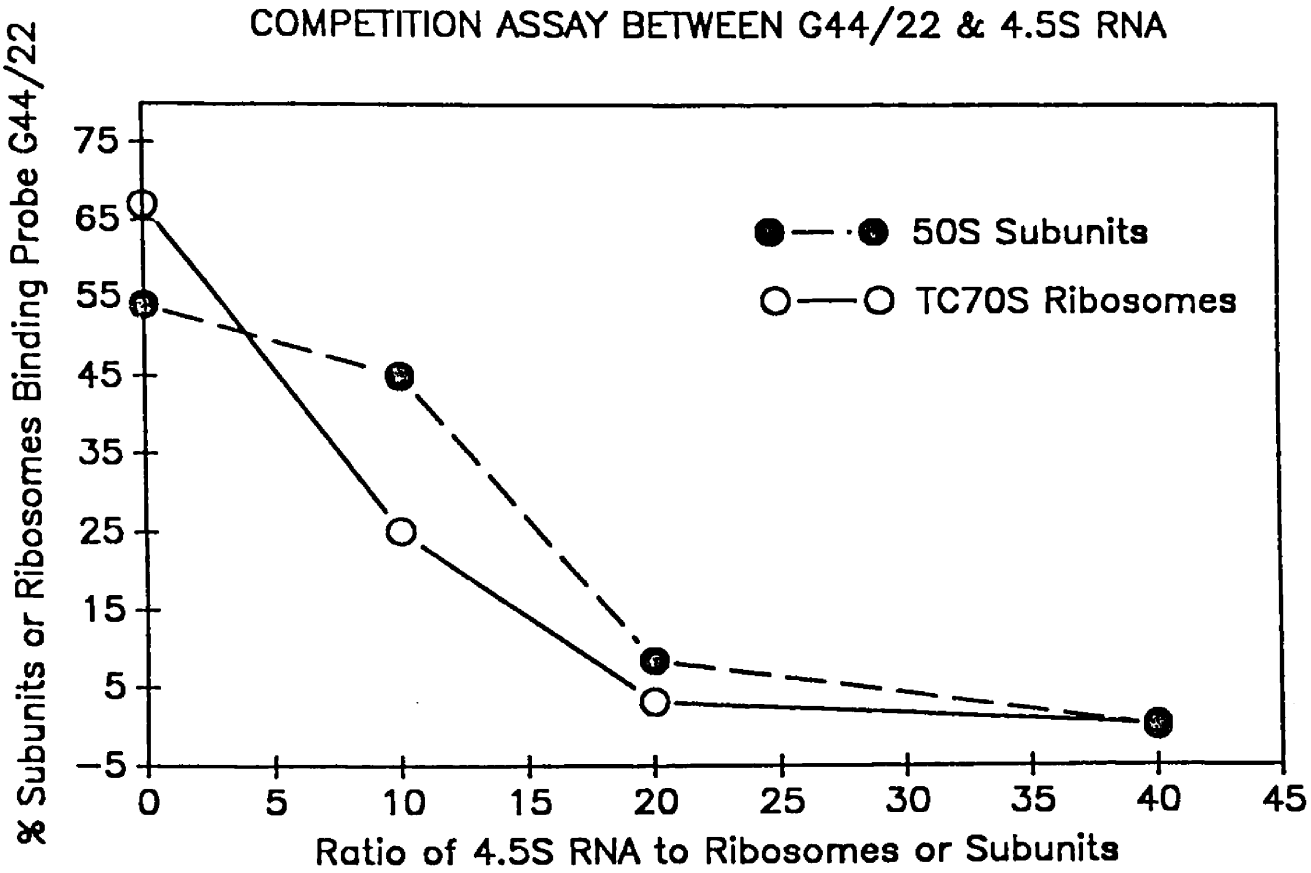


Figure 9: Competition experiments on 50S subunits and TC70S ribosomes. Increasing amounts of 4.5S RNA were added to reactions having a constant amount of subunits (or ribosomes) and probe G44/22.

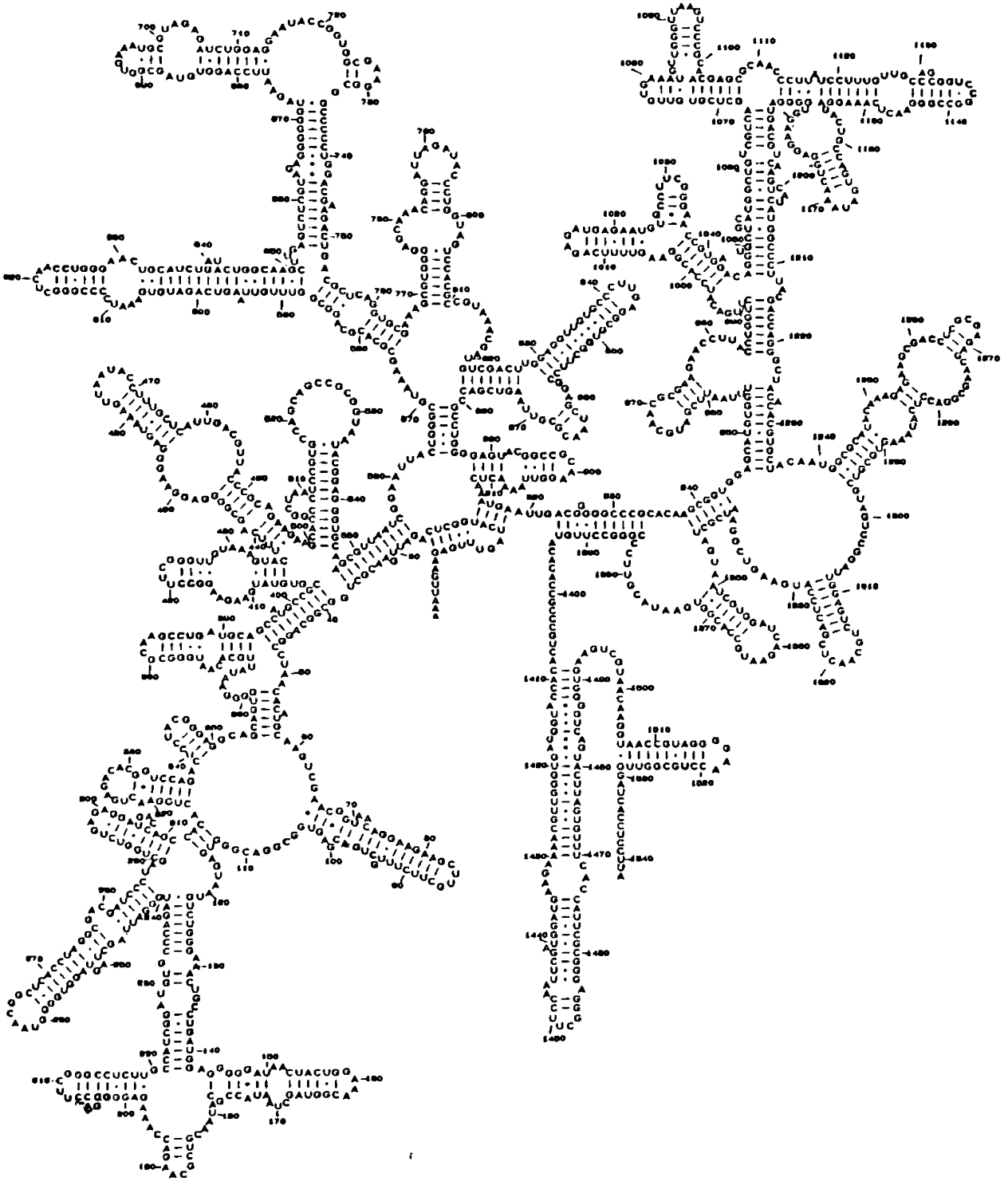


Figure 10: The secondary structure of 16S rRNA showing the regions which are compementary to probe G44/22.

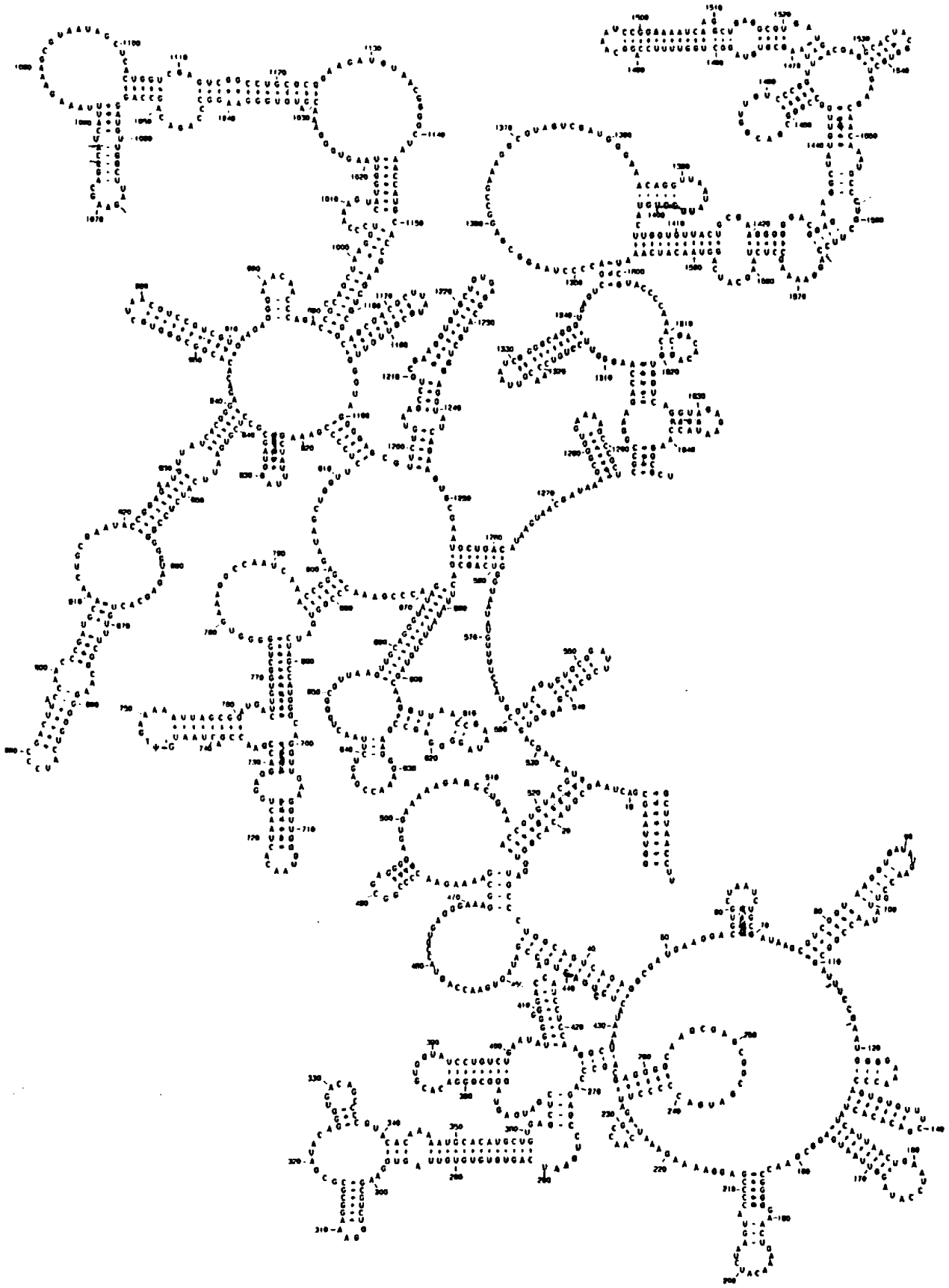


Figure 11a: The secondary structure of 23S rRNA showing the regions which are complementary to probe G44/22.

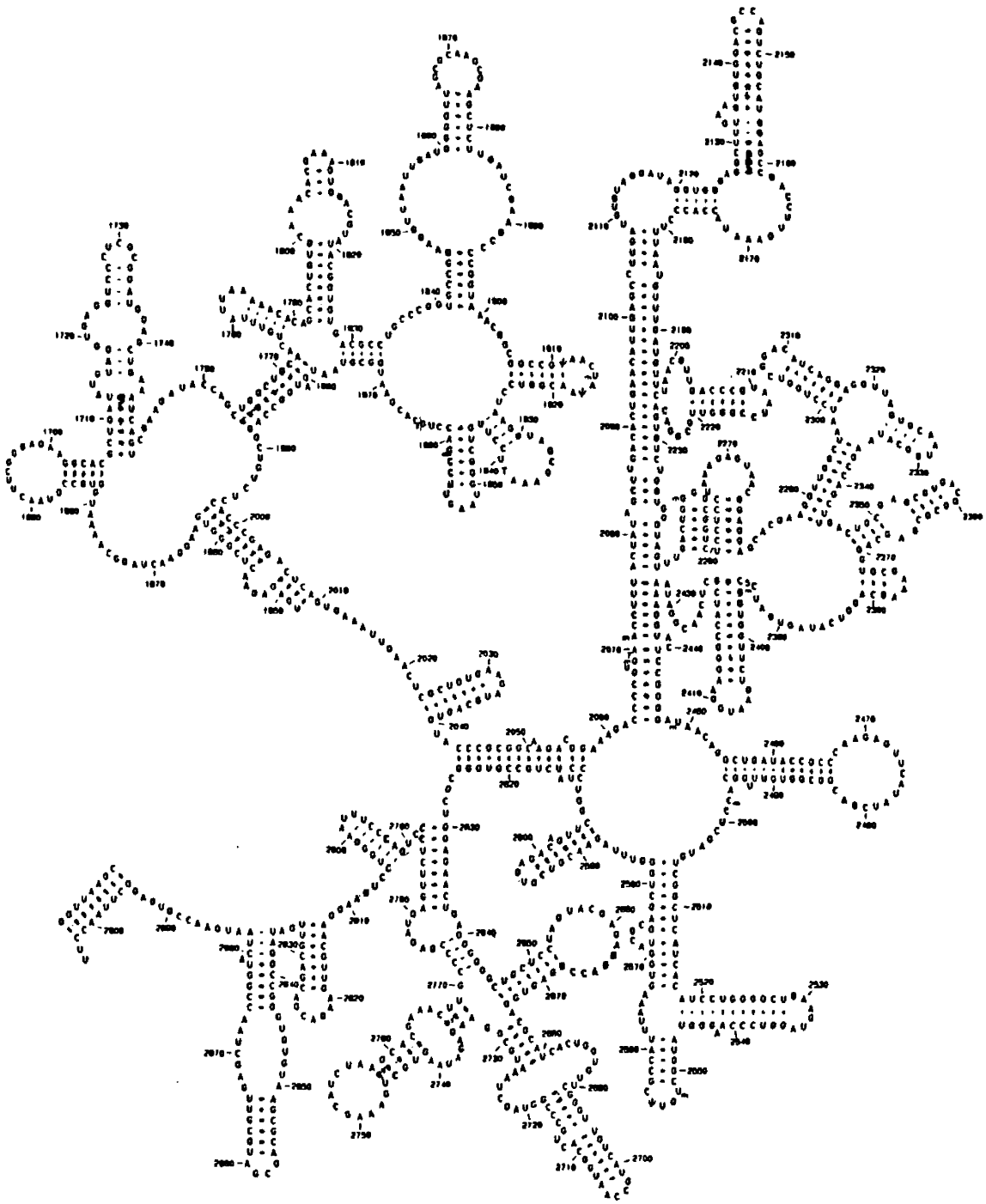


Figure 11b: Continued from Figure 11a.

Probe Binding Experiments

The filter binding assay (FBA, 75,98) was used to examine the availability of possible 4.5S RNA binding sites *in situ*. An assay such as this provides a method to separate the unbound DNA oligomers from the ribosomal particle-probe complexes thus making it possible to determine the amount of bound probe left on the filter. The FBA furnishes both qualitative and quantitative information about complex formation between radiolabeled DNA probe and the ribosomal particle.

Based on the lack of evidence for the 4.5S RNA competing with G44/22 probe on 30S subunits, most of the assays were carried out only on 50S subunits. Similar to 4.5S RNA binding experiments, the probe was labeled with ^{32}P at one end (the 5'-end) and increasing concentrations of labeled probe were incubated with 10 pmol of subunits in activation buffer at 4°C for 4 to 12 hours. The results of the filter binding assays are shown in figures 12 and 13. Figure 12 shows the binding curves obtained for probe G44/6 hybridization to 30S and 50S subunits. The shape of the curves would indicate that G44/6 does bind to 50S subunits but not to 30S subunits. Figure 13 shows the binding data obtained from hybridization experiments on 50S subunits with probes C46/6, C52/6, and G58/6. It might appear that C46/6 binds well, but the shape of the curves indicates that binding in all cases is low or non-specific.

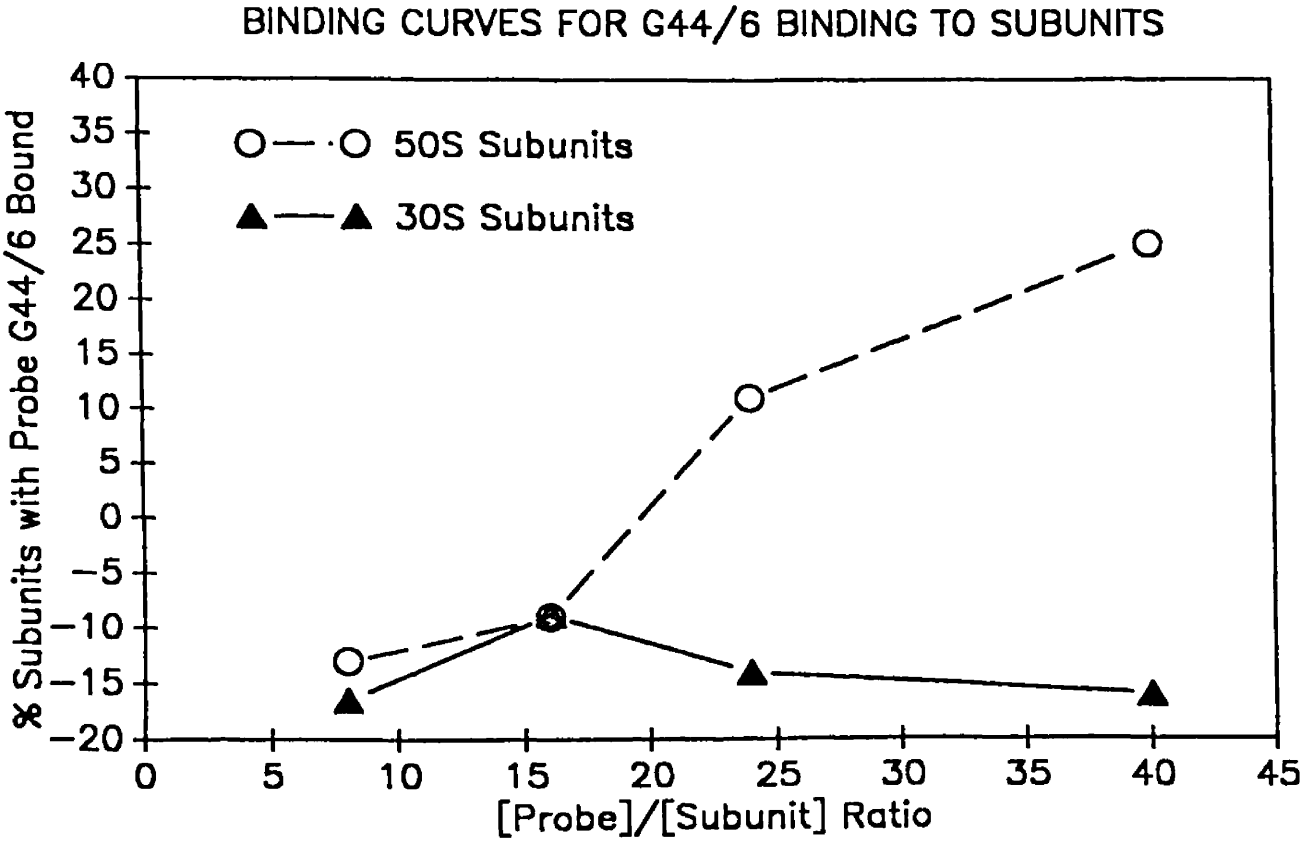


Figure 12: Binding data for probe G44/6 binding to 30S and 50S subunits. Incubation for both was for 4 hours at 4°C.

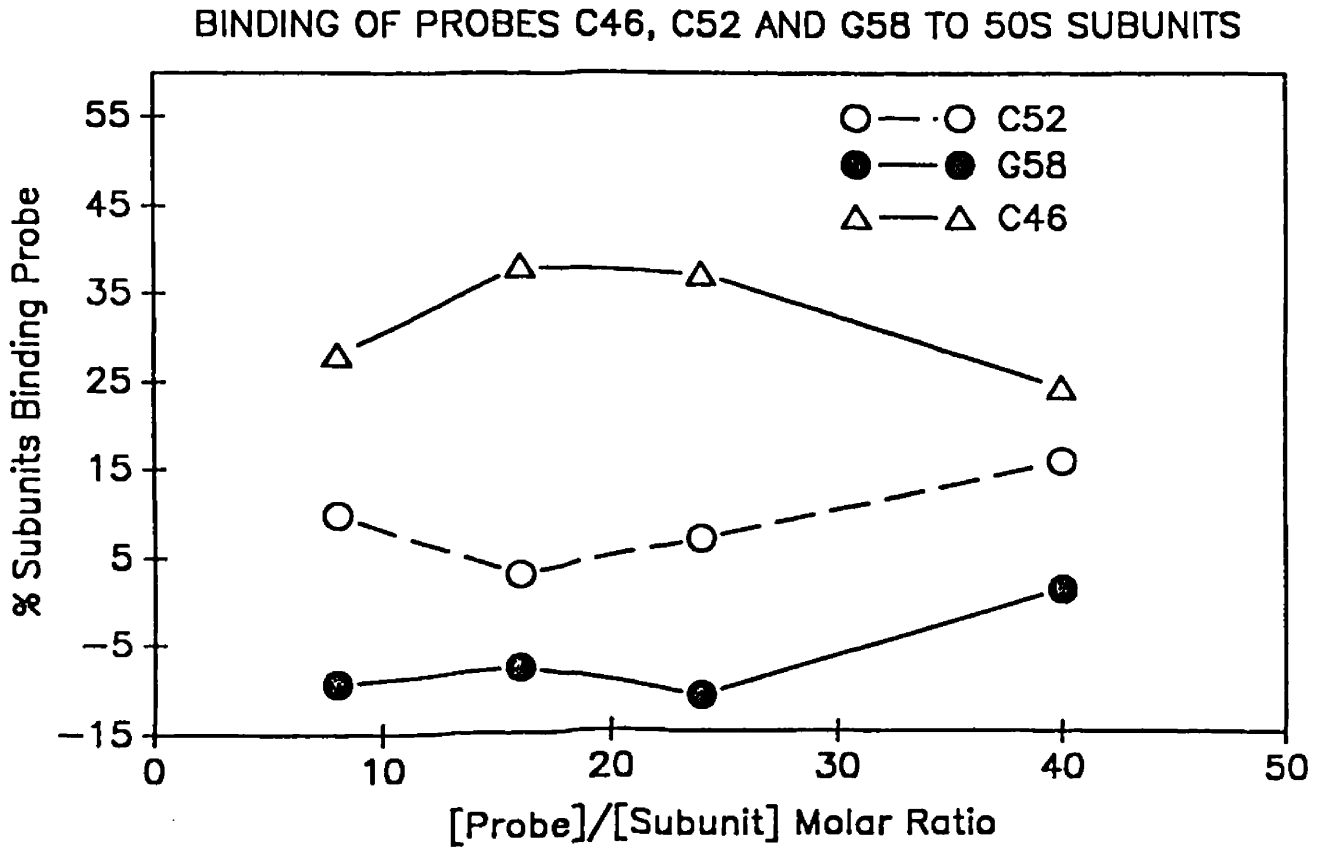


Figure 13: Binding data for probes C46, C52 and G58 on 50S subunits. Probes were incubated for 4 (G58) or 12 (C46 and C52) hours at 4°C.

It was noted that 4.5S RNA contains a six base palindromic sequence going from U50 to A55. This region is contained in probe A47/10 and makes it mostly self-complementary. As a result, the data were uninterpretable and it was determined that probe binding techniques would not be useful in studying this region.

RNase H Digestion Experiments

Some of the most interesting data was obtained from RNase H digestion experiments. RNase H assays were used to show specific binding of a probe to a site on ribosomal RNA. This involved incubating DNA probe with the 50S subunits or naked 23S RNA and treating the complexes with RNase H. The endoribonuclease RNase H digests the RNA in a DNA-RNA hybrid (5,34). The specificity of ribonuclease H reactions have previously been utilized to identify DNA oligomer-RNA hybrids (4,25,39,52,55,98). The lengths of the resulting fragments provide an indication of the specificity of binding which can be certified by sequencing the fragment. This assay is also useful for demonstrating that probe interactions with the subunit *in situ* are due to DNA:RNA interactions.

The procedure involved combining probe and subunits or RNA in a ratio of about 6:1 in RNase H buffer. Two units of Rnase H were added to the reactions and controls and they were incubated at 4°C for 45 minutes (23S rRNA) or 4-20 hours (50S subunits). The probe:subunit (or RNA) ratio used here might seem very low compared to the ratios used in the filter

Table II: Using the results of the sequence search in Figure 11, regions within 23S RNA which have at least four-base complementarity were identified for each probe.

Probe	Complementary regions of 23S RNA
G44/6	274-278, 435-440, 440-444, 509-513, 1257-1261, 1398-1402, 2788-2793, 2805-2809
C46/6	274-277, 411-414, 435-438, 508-512, 728-732, 1398-1402, 1959-1964, 2162-2166, 2663-2667, 2788-2791, 2805-2808
C52/6	113-117, 852-855, 1497-1500, 1561-1564, 1955-1959, 2213-2216, 2393-2396, 2441-2445
G58/10	1157-1161, 1558-1565, 1767-1771, 2645-2650

binding assay, but Marconi has shown (54) that a probe:subunit ratio of 2:1 gave clips nearly as well as a ratio of 150:1. Figure 14 shows the results of incubating 50S subunits and naked 23S rRNA with RNase H in the presence of probe G44. As can be seen, RNase H was unable to produce a clip on 50S subunits but was able to produce a very good clip on 23S RNA which ran between the RNA markers at 400 and 530 bases. Both salt-washed and non-salt-washed 50S subunits were tested as well as variations in incubation times from 4-20 hours but no clips were seen. The band produced from 23S RNA is thought to be approximately 440 nucleotides long. To further define the region responsible for producing the clip, the shorter probes used in the filter binding assays were examined for complementarity at sites which would yield RNase H digestion

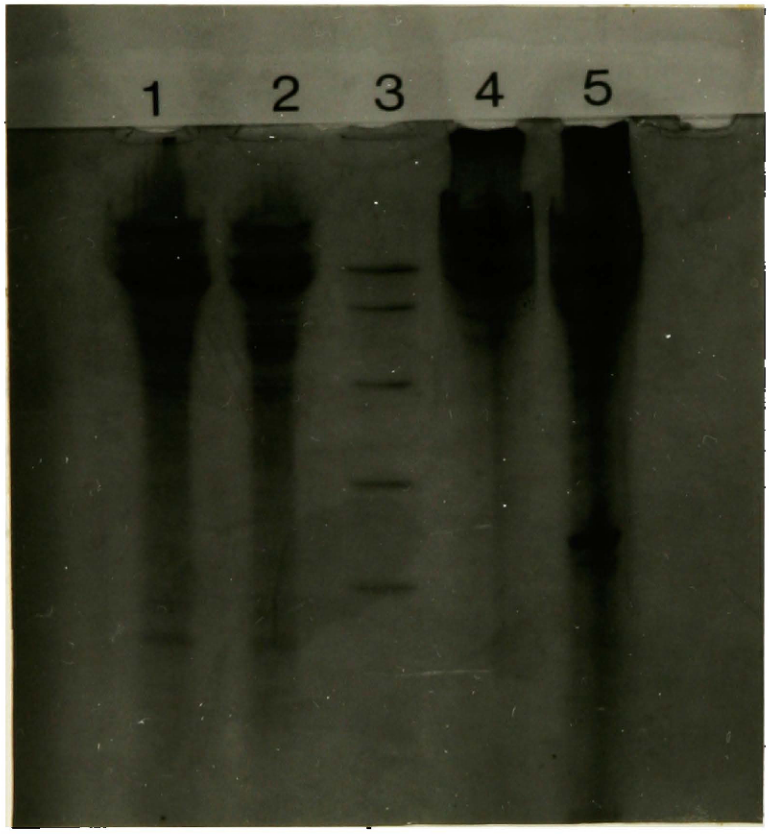


Figure 14: RNase H digestion of 50S subunits and 23S RNA. Lane 1: Control 50S subunits; Lane 2: 50S subunits with probe G44/22 and RNase H; Lane 3: RNA size markers; Lane 4: Control 23S RNA; Lane 5: 23S RNA with probe G44/22 and RNase H.

fragments of 430-460 residues (see Table II). It was discovered that 23S rRNA contains three sites that could yield the appropriate size of fragment. Two of the sites are complementary to probe G44/6 and are end to end at 435-440 and 440-444 respectivly. The other site is complementary to probe C52 and is at 2441-2445; this would yield a 459 base fragment. An RNase H digestion performed with probes G44/6 and C52 showed that G44/6 gave clips equal to those given by G44/22, but the reaction with C52 yielded no observable clipping. Figure 15 shows the results of RNase H digestion in the presence of probes G44/22, G44/6 and C52. It is apparent from this data that probe C52 is not involved in any interactions with 23S RNA. Probe G44/6, however, seems to be involved in interacting with 23S RNA at bases 435-440 or 440-444 or both.

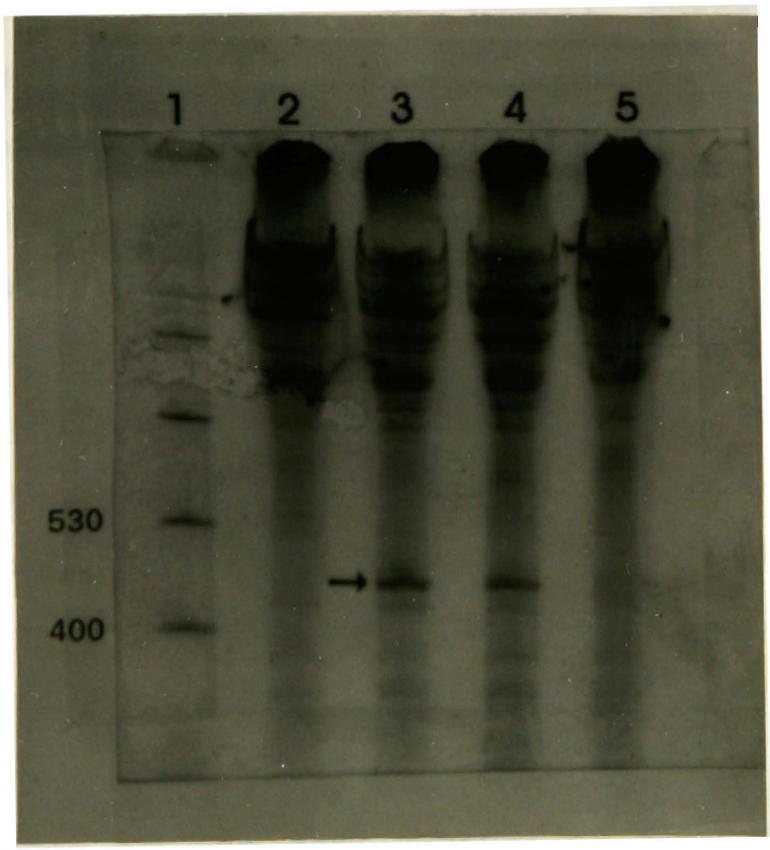


Figure 15: RNase H digestion on 23S RNA. Lane 1: RNA size markers; Lane 2: Control 23S RNA; Lane 3: 23S RNA with G44/22; Lane 4: 23S RNA with G44/6; Lane 5: 23S RNA with C52.

CHAPTER IV

DISCUSSION

The results of this study give evidence for a binding site for 4.5S RNA on the E. coli ribosome. Data from filter binding assays using 3'-end labeled 4.5S RNA indicate that 4.5S RNA binds to both subunits and TC70S ribosomes in vitro. The binding appears to be nearly equal in all assays indicating that perhaps one region of 4.5S RNA binds to 50S subunits and a different region binds to 30S subunits. This theory was supported by the data from the competition assays; 4.5S RNA was able to successfully compete with the 22-base probe on 50S subunits and TC70S ribosomes but not on 30S subunits. Further studies on the conserved regions on the stem of 4.5S RNA might reveal a binding site on 30S subunits as well. Through the use of filter binding techniques and RNase H assays it was possible to propose two possible binding sites for 4.5S RNA on the 50S subunit. These binding sites are at 435-440 and 440-444. It is fascinating that both sites are complementary to the same region of 4.5S RNA (bases 44-49, Figure 4) and are overlapping. From the secondary structure proposed by Noller et al (60), it can be seen that these sites overlap in a long helical region with one bulged base at each site (Figure 11). It has been suggested (64) that some of the proposed helical regions might alternately form and break during the ribosome cycle creating helical "switches". This

suggests that 4.5S RNA could be involved in a switching mechanism of some type.

Taken with the evidence presented by Brown (1987, 1989) for 4.5S RNA being involved in EF-G function and his suggestion of a possible conformational change, a model can be proposed. Prior to translocation 4.5S RNA might be bound to one of the 440 sites which favors or even stabilizes the EF-G interaction with 23S RNA. Translocation would produce a conformational shift, 4.5S RNA would bind to the alternate 440 site which destabilizes EF-G interactions with 23S RNA and allows binding to the putative EF-G site on 4.5S RNA. The 4.5S RNA then leaves, further destabilizing EF-G and, causes EF-G to leave as Brown has suggested.

Prior to this time there was no evidence for 4.5S RNA binding to ribosomes in vitro. Therefore, the first part of this study focused on determining if 4.5S RNA would bind to subunits and ribosomes under in vitro conditions. To answer this question, a series of filter binding assays using labeled 4.5S RNA were carried out on 30S and 50S subunits as well as TC70S ribosomes. It was shown that 4.5S RNA does bind to both subunits and ribosomes in vitro. While the evidence is not strong for binding to a single site on each subunit, it does show binding to a limited number of sites.

Competition experiments were carried out on 30S and 50S subunits and TC70S ribosomes to determine the ability of 4.5S RNA to displace a probe having a sequence identical to the

conserved 22-base hairpin region. The results showed that competition takes place on 50S subunits and 70S subunits but not on 30S subunits. This was somewhat surprising at first since 4.5S RNA did bind to 30S subunits and it was believed that the hairpin region was involved in binding (42). Later studies (72,92) indicated that there are conserved regions on the stem of 4.5S RNA as well. It is possible that these sites are involved in binding to the 30S subunit to, perhaps, stabilize the molecule. Since only the hairpin region was studied in this work, such interactions could only be speculated upon. However, if the proposed model is correct it is highly unlikely since the Tm for the stem is very high.

Filter binding studies on 50S subunits were carried out using short probes which covered certain regions of the conserved 22-base hairpin. The data for most of the probes showed a constant level of binding as the amount of probe increased, indicative of non-specific binding. Probe G44/6, however, gave a curve more indicative of specific binding; as the probe:subunit ratio was increased, the percent binding values also increased and the curve starts to flatten as the binding sites are saturated at the higher values.

The results of these filter binding studies are important because, from the sequence of probe G44/6, the general area of 4.5S RNA involved in interactions with the ribosome can be localized to bases 44-49. From analysis of the 4.5S RNA sequence and possible interaction sites on ribosomal RNA, Hsu

et al (1984) and Struck et al (1988a) have suggested other areas of 4.5S RNA (the Shine-Dalgarno-like region and bases 46-51, respectively) to be involved in binding to ribosomes. But we have no evidence of such binding. Our results would preclude the binding of any of the 4.5S RNA loop region to a complementary site on 16S rRNA.

The 50S subunit is a different story. Struck et al (1988a) and Brown (1989) have implicated bases 58-67 of 4.5S RNA in EF-G binding. Filter binding assays using a probe identical to that region indicate that bases 58-67 are not involved in binding to ribosomes and, indeed, could be accessible for the binding of EF-G. This was interesting because it showed that the site interacting with the ribosome and the site which interacts with EF-G are two separate sites. In addition, the filter binding data using probe G44/6 places the ribosome interaction site on the side of the hairpin-loop opposite the proposed EF-G binding site making it convenient to propose a model in which 4.5S RNA binds to the ribosome and to EF-G at the same time.

The filter binding results from this study and the conclusions suggested by Brown (13) and Struck et al (92) were further supported by the results of the RNase H digestion assays. As with the filter binding assays, the data indicate that the region identical to probe G44/6 (44-49) is involved in binding 4.5S RNA to the ribosomes. These data go further to identify two sites on the 23S rRNA which are involved in

binding 4.5S RNA to the ribosome. These sites overlap at base 440 of 23S rRNA. It is important to note that these cleavages are produced only in the presence of probe which binds specifically; neither probe alone nor RNase H alone will produce cleavages. These data together with the suggestion of Noller et al (61) for helical switch regions and the data presented by Brown (12) for 4.5S RNA involvement in EF-G function are the basis for the model suggested earlier in this discussion.

There are a few shortcomings to the RNase H technique. In the introduction it was mentioned that ribosomal RNA has several domains involved in activity. The ribosome must protect those regions from RNases, yet allow them to carry out the reactions needed for proper functioning. There are at least two ways this can be accomplished. First, the RNA might be base-paired, making it fairly resistant to degradation. This might, in some circumstances, make it difficult to bind probe to that sequence. However, probes might be able to disrupt base-pairing in areas involved in ribosomal function. A second method of protection could be achieved by sandwiching the RNA in valleys between ribosomal proteins. This would allow nucleic acids to interact but ribonucleases (which are too large to fit in the crevice) would be stericly hindered from interacting with the ribosomal RNA. RNase H digestion at the target site might vary also due to alterations in the conformation of the ribosome caused by

binding of ribosome-associated factors at or near the target site. It is possible that the binding of the DNA probe itself might cause a steric shift of a protein or other factor and shield the duplex region from RNase H digestion. This would explain why RNase H is unable to produce a clip on 50S subunits incubated with G44/22 probe. The results of the filter binding assays were further supported by the RNase H digestion data.

While the evidence is very solid for identifying the area of probe hybridization as the 440 region of 23S rRNA, it is not possible to determine if one or both sites are involved. Generally speaking, RNase H can clip anywhere along the duplex region resulting in a nested set of fragments. The gel system used here is unable to separate such fragments.

Small DNA oligomers have been used quite successfully in this lab (39,55,98) and others (3,50,68) to probe ribosomal RNA for structure and function. The use of cDNA oligomers offers several advantages over other commonly published techniques such as chemical modification, cross-linking, and enzymatic treatment. One of the primary advantages to this technique is the ability to select a specific target site. Target site distinction is usually not provided by the other established techniques mentioned above. Another valuable advantage to the use of DNA probes is their reversibility. This makes it possible to study the binding of various molecules which interact with ribosomes. Another advantage of

DNA probing methods is that the experimental conditions under which they are performed in this lab are accepted as being within the physiological conditions necessary for maintenance of the ribosome; again, this is not always the case with other methods.

The use of DNA oligomers in this study differs only slightly from how they are commonly used in this lab. Normally, the target site is known to some extent and cDNA oligomers are constructed to probe that site completely. For the work described here, the target site is not known; in fact, it was not completely known that there is a binding site for 4.5S RNA on the ribosome. This necessitated a broad, "fishing expedition" approach to isolate the general region on both 4.5S RNA and ribosomal RNA involved in binding.

The work presented here is the first known study on defining a binding site for 4.5S RNA on the E. coli ribosome. Further work needs to be done to confirm these findings. Sequencing the RNase H fragments might produce further evidence of specificity and might be able show if one or both of the proposed sites are involved in probe binding. Again, however, the fragments obtained from RNase H digestion form a nested set and very exacting measures are required to gain any useful information from sequencing (38). Future studies might involve use of site-specific mutagenesis on both 4.5S RNA and 23S RNA or further probing to more closely define the binding site; also, work needs to be done to look at the other

conserved areas on the helix of 4.5S RNA. Other areas of future studies could focus on continuing the interesting work on EF-G/4.5S RNA function. Mutation studies could yield some very interesting information on this relationship. Fluorescence studies might also be used to determine distance between 4.5S RNA and EF-G. Some interesting work on tertiary interactions is being done and it should be interesting to see how the 440 region and the 1067 region (EF-G binding region) relate in 23S RNA. In summary, until this time there has been no direct data relating 4.5S RNA to EF-G and a specific site on ribosomal RNA. It is believed that the work presented here will help reduce the "enigma" that has sometimes been associated with 4.5S RNA and be beneficial in future studies of this fascinating small RNA.

1. **Acharya, A.S., P.B. Moore, and F.M. Richards.** 1973. Cross-linking of Elongation Factor EF-G to the 50S Ribosomal Subunit of Escherichia coli. Biochemistry **12**:3108-3113.

2. **Agarwal, K.L., J. Brunstedt, and B.E. Noyes.** 1981. A General Method for Detection and Characterization of an mRNA using an Oligonucleotide Probe. J. Biol. Chem. **256**:1023-1028.

3. **Backendorf, C., G.P. Overbeek, J.H. Van Boom, G. Van Der Marel, G. Veeneman, and J. Van Duin.** 1980. Role of 16S RNA in Ribosome Messenger Recognition. Eur.J.Biochem. **110**:599-604.

4. **Backendorf, C., C.J.C. Ravensbergen, J. Vanderplas, J.H. Van Boom, and J. Van Duin.** 1981. Basepairing Peotential of the 3' Terminus of 16S rRNA: Dependance on the Functional State of the 30S Subunit and the Presence of Protein. Nucl.Acids Res. **9**:1425-1444.

5. **Berkower, I., J. Leis, and J. Hurwitz.** 1973. Isolation and Characterization of an Wndonuclease from Escherichia coli Specific for Ribonucleic Acid in Ribonucleic Acid-Deoxyribonucleic Acid Hybrid Structures. J.Biol.Chem. **248**:5914-5921.

6. **Borer, P.N., B. Dengler, I. Tinoco Jr., and O.C. Uhlenbeck.** 1974. Stability of Ribonucleic acid Double-stranded Helices. J.Mol.Biol. **86**:843-853.

7. **Bothwell, A.L.M., R.L. Garber, and S. Altman.** 1976. Nucleotide Sequence and in Vitro Processing of a Precursor Molecule to Escherichia coli 4.5S RNA. J. Biol. Chem. **251**:7709-7716.

8. **Boublik, M., N. Robakis, and W. Hellmann.** 1982. Electron Microscopic Study of Free and Ribosome Bound rRNAs from E. coli. Eur.J.Cell Biol. **27**:177-184.

9. **Bourgaize, D.B., C. Farrell, K.H. Langley, and M.J. Fournier.** 1984. Physical properties of the E. coli 4.5S RNA results suggest a hairpin helix of unusual thermal stability. NAR **12(4)**:2019-2034.

10. **Bourgaize, D.B., and M.J. Fournier.** 1987. Initiation of translation is impaired in E. coli cells deficient in 4.5S RNA. Nature **325**:281-284.

11. **Branlant, C., A. Krol, M.A. Machatt, J. Poyet, J.P. Ebel, K. Edwards, and H. Kossel.** 1981. Primary and Secondary Structures of Escherichia coli MRE 600 23S ribosomal RNA. Comparison with Models of Secondary Structure for Maize Chloroplast 23S rRNA and for Large Portions of Mouse and Human 16 S Mitochondrial rRNAs. Nucl.Acids Res. **9**:4303-4324.

12. **Brimacombe, R., J. Atmadja, W. Stiege, and D. Schuler.** 1988. A Detailed Model of the Three-Dimensional Structure ofEscherichia coli 16 S Ribosomal RNA in situ in the 30 S Subunit. J. Mol. Biol. **199**:115-136.

13. **Brown, S.** 1989. Time of action of 4.5S RNA inEscherichia coli translation. J. Mol. Biol. **209(1)**:79-90.

14. **Brown, S., and M.J. Fournier.** 1984. The 4.5S RNA Gene ofEscherichia coli is Essential for Cell Growth. J. Mol. Biol. **178**:533-550.

15. **Brown, Stanley** 1987. Mutations in the Gene for EF-G Reduce the Requirment for 4.5S RNA in the Growth of E. coli. CELL **49**:825-833.

16. **Brownlee, G.G., F. Sanger, and B.G. Barrell.** 1968. The sequence of 5S ribosomal ribonucleic acid. J.Mol.Biol. **34**:379-412.

17. **Capel, M.S., D.M. Engelman, B.R. Freeborn, M. Kjeldgaard, J.A. Langer, V. Ramakrishnan, D.G. Schindler, D.K. Schneider, B.P. Schoenborn, I.-Y. Sillers, S. Yabuki, and P.B. Moore.** 1987. A Complete Mapping of the Proteins in the Small Ribosomal Subunit ofEscherichia coli. Science **238**:1403-1406.

18. **Capel, M.S., M. Kjeldgaard, D.M. Engelman, and P.B. Moore.** 1988. Positions of S2,S13,S16,S17,S19 and S21 in the 30 S Ribosomal Subunit ofEscherichia coli. J. Mol. Biol. **200**:65-87.

19. **Carbon, P., C. Ehresmann, B. Ehresmann, and J. Ebel.** 1979. The Complete Nucleotide Sequence of the Ribosomal 16-S RNA fromEscherichia coli Experimental Details and Cistron Heterogeneities. Eur.J.Biochem. **100**:399-410.

20. **Cech, T.R.** 1985. Mechanism of self-splicing of the ribosomal RNA precursor of Tetrahymena. Proc.Robert A.Welch Found.Conf.Chem.Res. **29**:345-349.

21. **Chaconas, G., and J.H. van de Sande.** 1980. 5'-32P Labeling of RNA and DNA Restriction Fragments. Methods in Enzymology **65**:75-88.

22. **Cundliffe, E.** 1986. Involvment of Specific Portions of Ribosomal RNA in Defined Ribosomal Functions: A Study Utilizing Antibiotics, p.586-604. In B. Hardesty, and G. Kramer (eds.), Structure, Function, and Genetics of Ribosomes. Springer-Verlag, New York.

23. **D'Alessio, J.M.** 1982. RNA Sequencing, p.173-197. In D. Rickwood, and B.D. Hames (eds.), Gel electrophoresis of nucleic acids a practical approach. IRL Press, Oxford/Washington DC.

24. **Dempsey, W.B.** 1987. Transcript analysis of the Plasmid R100 traJ and finP Genes. Mol. Gen. Genet. **209**:533-544.

25. **Donis-Keller, H.** 1979. Site Specific Enzymatic Cleavage of. Nucl.Acids Res. **7**:179-192.

26. **Douthwaite, S., R.A. Garrett, and R. Wagner.** 1983. Comparison of E. coli tRNA(phe) in the free state, in the ternary complex and in the ribosomal A and P sites by chemical probing. Eur.J.Biochem. **131**:261-269.

27. **Dunn, J.M., and K. Wong.** 1979. Structure and Stability of the Reconstituted Intermediate Particles Involved in the In Vitro Self-assembly of the 30S Ribosomal Subunit. Biochemistry **18**:4380-4385.

28. **Expert-Bezancon, A., and P. Wollenzien.** 1985. Three-dimensional arrangement of theEscherichia coli 16S ribosomal RNA. J.Molecular Biology **184**:53-66.

29. **Griffin, B.E.** 1971. Separation of ^{32}P-labelled Ribonucleic Acid Components. The Use of Polyethylenimine-cellulose (TLC) as a Second Dimension in Separating Oligoribonucleotides of '4.5S' and 5S from E. coli. FEBS Letters **15(3)**:165-168.

30. **Griffin, B.E.** 1975. Studies and sequences ofEscherichia coli 4.5S RNA. J.Biol.Chem. **250(14)**:5426-5437.

31. **Guerrier-Takada, C., K. Gardiner, T. Marsh, N. Pace, and S. Altman.** 1983. The RNA Moiety of Ribonuclease P Is the Catalytic Subunit of the Enzyme. CELL **35**:849-857.

32. **Guo, P.X., S. Erickson, and D. Anderson.** 1987. A Small Viral RNA is Required for in vitro Packaging of bacteriophage phi-29 DNA. Science **236**:690-694.

33. **Hapke, B., and H. Noll.** 1976. Structural Dynamics of Bacterial Ribosomes: Classification of Ribosomes by Subunit Association. J.Mol.Biol. **105**:97-109.

34. **Hausen, P., and A. Stein.** 1970. Ribonuclease H: An Enzyme Degrading the RNA Moiety of DNA-RNA Hybrids. Eur.J.Biochem. **14**:278-283.

35. **Helk, B., and M. Sprinzl.** 1985. Interaction of Unfolded tRNA with the 3'-terminal region of E. coli 16S Ribosomal RNA. Nucl.Acids Res. **13**:6283-6298.

36. **Herr, W., M.N. Champman, and H.F. Noller.** 1979. Mechanism of Ribosomal Subunit Association: Discrimination of Specific Sites in 16 S RNA Essential for Association Activity. J.Mol.Biol. **130**:433-449.

37. **Hill, W.E., G.P. Rosssetti, and K.E. Van Holde.** 1969. Physical Studies of Ribosomes fromEscherichia coli. J.Mol.Biol. **44**:263-277.

38. **Hill, W.E., W.E. Tapprich, and A. Tassanakajohn.** 1986. Probing ribosomal structure and function, p.233-252. In B. Hardesty, and G. Kramer (eds.), Structure, Function and Genetics of Ribosomes. Springer-Verlag, New York.

39. **Hill, W.E., and A. Tassanakajohn.** 1987. Probing Ribosome Structure using Short Oligodeoxyribonucleotides: The Question of Resolution. Biochimie **69**:1071-1080.

40. **Hill, W.E., J.D. Thompson, and J.W. Anderegg.** 1969. X-Ray Scattering Study of Ribosomes from Escherichia coli. J.Mol.Biol. **44**:89-102.

41. **Hogan, J.J., R.R. Gutell, and H.F. Noller.** 1984. Probing the conformation of 18S rRNA in yeast 40S ribosomal subunits with kethoxal. Biochemistry **23**:3322-3330.

42. **Hsu, L.M., J. Zagorski, and M.J. Fournier.** 1984. Cloning and sequence analysis of theEscherichia coli 4.5S RNA gene. J.Mol.Biol. **178**:509-531.

43. **Ikemura, T., and J. Dahlberg.** 1973. Small Ribonucleic Acids of Escherichia coli. II. Noncoordinate Accumulation During Stringent Control. J. Biol. Chem. **248**:5033-5041.

44. **Itoh, T., and J. Tomizawa.** 1980. Formation of an RNA Primer for Initiation of Replication of ColE1 DNA by Ribonuclease H. Proc.Natl.Acad.Sci. **77**:2450-2454.

45. **Kastner, B., M. Stoffler-Meilick, and G. Stoffler.** 1981. Arrangement of the Subunits in the Ribosomal of Escherichia coli: Demonstration by Immunoelectron Microscopy. Proc.Natl.Acad.Sci. **78**:6652-6656.

46. **Kim, K.H., and R.J. Meyer.** 1986. Copy-number of Broad Host-Range Plasmid R1162 Is Regulated by ;a Small RNA. Nucl.Acids Res. **14**:8027-8046.

47. **Lake, J.** 1976. Ribosome Structure Determined by Electron Microscopy of Escherichia coli Small Subunits, Large Subunits and Monomeric Ribosomes. J.Mol.Biol. **105**:131-159.

48. **Lake, J.A.** 1982. Ribosomal Subunit Orientations Determined in the Monomeric Ribosome by Single and by Double-Labeling Immune Electron Microscopy. J.Mol.Biol. **161**:89-106.

49. **Lake, J.A., and W.A. Strycharz.** 1981. Ribosomal Proteins L1,L17 and L27 from Escherichia coli Localized at Single Sites on the Large Subunit by Immune Electron Microscopy. J.Mol.Biol. **153**:979-992.

50. **Lewis, J.B., and P. Doty.** 1977. Identification of the Single-Strand regions inEscherichia coli 5S RNA, Native and A Forms, by the Binding of Oligonucleotides. Biochemistry **16**:5016-5025.

51. **Liao, S.M., T.H. Wu, C.H. Chiang, M.M. Susskind, and W.R. McClure.** 1987. Control of Gene-expression in Bacteriophage P22 by a Small Antisense RNA. 1, Characterization in vitro of the PSAR Promoter and the SAR RNA Transcript. Genes Dev. **1**:197-203.

52. **Lorenz, S., R.K. Hartmann, N. PIEL, N. Ulbrich, and V.A. Erdmann.** 1987. Structural analysis of 5S rRNA, 5S rRNA-protein complexes and ribosomes employing RNase H and d(GTTCGG). European Journal of Biochemistry **163**:239-246.

53. **Maly, P., and R. Brimacombe.** 1983. Defined Secondary Structures Models for the 16S and 23S Ribosomal RNA of Escherichia coli. Nucl.Acids Res. **11**:7263-7286.

54. **Marconi, R.T.** 1989. Probing the Peptidyltransferase Center of theEscherichia coli Ribosome with cDNA Oligomers. Ph.D. Thesis, University of Montana

55. **Marconi, R.T., and W.E. Hill.** 1988. Identification of defined sequences in domain V of E. coli 23S rRNA in the 50S subunit accessibile for hybridization with complementary oligodeoxyribonucleotides. Nucl.Acids Res. **16**:1603-1615.

56. **Moazed, D., and H.F. Noller.** 1987. Interaction of Antibiotics with Functional Sites in 16S Ribosomal RNA. Nature **327**:389-394.

57. **Moazed, D., J.M. Robertson, and H.F. Noller.** 1988. Interaction of elongation factors EF-G and EF-Tu with a conserved loop in 23S RNA. Nature **334**:362-364.

58. **Moazed, D., B. Van Stolk, S. Douthwaite, and H.F. Noller.** 1986. Interconversion of active and inactive 30S ribosomal subunits is accompanied by a conformational change in the decoding region of 16S RNA. JMB **191**:483-493.

59. **Moore, P.B., M. Capel, M. Kjeldgaard, and D.M. Engelman.** 1986. A 19 protein map of the 30S ribosomal subunit ofEscherichia coli, p.87-100. In B. Hardesty, and G. Kramer (eds.), Structure, Function and Genetics of Ribosomes. Springer-Verlag, New York.

60. **Nagase, T., S. Ishii, and F. Imamoto.** 1988. Differential transcriptional control of the two $tRNA^{Met}_{f}$ genes of Escherichia coli K-12. Gene **67**:49-57.

61. **Neidhardt, F.C.** 1987. E. coli and S. typhimurium, Cellular and Molecular Biology, p.3-6. ASM Press, Washington D.C..

62. **Nierhaus, K.H., R. Lietzke, V. Nowotny, H. Schultze, P. Wurmbach, and R.P. May.** 1983. Shapes and Distances Between Components within the Large (50S) Subunit of E. coli Ribosomes. PHYSICA B&C **120**:426-435.

63. **Noller, H.F.** 1984. The structure of ribosomal RNA. Annu.Rev.Biochem. **53**:119-162.

64. **Noller, H.F., M. Asire, A. Barta, S. Douthwaite, T. Goldstein, R.R. Gutell, D. Moazed, J. Normanly, J.B. Prince, S. Stern, K. Triman, S. Turner, B. Van Stolk, V. Wheaton, B. Weiser, and C.R. Woese.** 1986. Studies on the Structure and Function of Ribosomal RNA, p.143-163. In B. Hardesty, and G. Kramer (eds.), Structure, Function and Genetics of Ribosomes. Springer-Verlag, New York.

65. **Noller, H.F., and C. Woese.** 1981. Secondary Structure of 16S Ribosomal RNA. Science **212**:403-411.

66. **Nowotny, V., R.P. May, and K.H. Nierhaus.** 1986. Neutron-scattering analysis of structural and functional aspects of the ribosome: the strategy of the glassy ribosome, p.101-111. In B. Hardesty, and G. Kramer (eds.), Structure, Function and Genetics of Ribosomes. Springer-Verlag, New York.

67. **Oakes, M., M. Clark, E. Henderson, and J.A. Lake.** 1986. DNA Hybridization Electron Microscopy: Ribsomal RNA Nucleotides 1392-1407 are Exposed in the Cleft of the Small Subunit. Proc.Natl.Acad.Sci.USA **83**:275-279.

68. **Oakes, M., E. Henderson, A. Scheinman, M. Clark, and J. Lake.** 1986. Ribosome Structure, Function and Evolution: Mapping Ribosomal RNA, Proteins, and Functional Sites in Three Dimensions, p.47-67. In B. Hardesty, and G. Kramer (eds.), Structure, Function and Genetics of Ribosomes. Springer-Verlag, New York, NY.

69. **Ofengand, J., R. Liou, J. Kohut III, I. Schwartz, and R.A. Zimmermann.** 1979. Covalent Cross-linking of Transfer Ribonucleic Acid to the Ribosomal P Site. Mechanism and Site of Reaction in Transfer Ribonucleic Acid. Biochemistry **18**:4322-4332.

70. **Okamoto, K., and M. Freundlich.** 1986. Mechanism for the Autogeous Control of the CRP Operon - Transcriptional Inhibition by A Divergent RNA Transcript. Proc.Natl.Acad.Sci. **83**:5000-5004.

71. **Politz, S.M., and D.G. Glitz.** 1977. Ribosome Structure: Localization of N(6), N(6)-dimethyladenosine by electron micrscopy of a ribosome-antibody complex. Proc.Natl.Acad.Sci. **74**:1468-1472.

72. **Poritz, M.A., K. Strub, and P. Walter.** 1988. Human SRP RNA and E. coli 4.5S RNA contain a highly homologous structural domain. CELL **55**:4-6.

73. **Prince, J.B., B.H. Taylor, D.L. Thurlow, J. Ofengand, and R. Zimmermann.** 1982. Covalent crosslinking of tRNA-val to 16S RNA at the ribosomal P site: identification of the crossliked residues. Proc.Natl Acad.Sci.USA **79**:5450-5454.

74. **Roberts, R.J.** 1974. Staphylococcal Transfer Ribonucleic Acids. J.Biol.Chem. **249**:4787-4796.

75. **Rose, S.I.I.I., P. Lowary, and O. Uhlenbeck.** 1983. Binding of Yeast tRNA-phe Anticodon Arm to E. coli 30S Ribosomes. J.Mol.Biol. **167**:103-117.

76. **Schleich, T., and J. Goldstein.** 1966. Gel Filtration Heterogeneity of Escherichia coli Soluble RNA. J.Mol.Biol. **15**:136-146.

77. **Schmitt, M., U. Nanderschied, A. Kyriatsoulis, U. Brinkmann, and H.G. Gassen.** 1980. Tetranucleotides as Effectors for the Binding of Initiator tRNA toEscherichia coli Ribosomes. Eur.J.Biochem. **109**:291-299.

78. **Schwartz, I., and J. Ofengand.** 1978. Photochemical Cross-linking of Unmodified Acetylvalyl-tRNA to 16S RNA at the Ribosomal P Site. Biochemistry **17**:2524-2530.

79. **Shine, J., and L. Dalgarno.** 1974. The 3'-Terminal Sequence of Escherichia coli 16S Ribosomal RNA: Complementarity to Nonsense Triplets and Ribosome Binding Sites. Proc.Natl.Acad.Sci. **71**:1342-1346.

80. **Sigmund, C.D., M. Ettayebi, and E.A. Morgan.** 1984. Antibiotic resistance mutations in 16 S and 23 S ribosomal RNA genes ofEscherichia coli. Nucl.Acids Res. **12**:4653-4663.

81. **Simons, R.W., and N. Kleckner.** 1983. Translational Control of IS10 Transposition. CELL **34**:683-689.

82. **Skold, S.E.** 1982. Chemical Cross-linking of Elongation Factor G to Both Subunits of the 70S Ribosomes from Escherichia coli. Eur.J.Biochem. **127**:225-229.

83. **Skold, S.E.** 1983. Chemical Cross-linking of Elongation Factor G to the 23S RNA in 70S Ribosomes from Escherichia coli. Nucl.Acids Res. **11**:4923-4932.

84. **Spitnik-Elson, P., D. Elson, S. Avital, and R. Abramowitz.** 1982. A Ribonucleoprotein Fragment of the 30 S Ribosome of E. coli Containing Two Contiguous Domains of the 16 S RNA. Nucl.Acids Res. **10**:4483-4492.

85. **Spitnik-Elson, P., D. Elson, S. Avital, and R. Abramowitz.** 1985. Longe Range RNA-RNA Interactions in the 30S Ribosomal Subunit of E coli. Nucl.Acids Res. **13**:4719-4738.

86. **Steitz, J.A., and K. Jakes.** 1975. How Ribosomes Select Initiatior Regions in mRNA: Base Pair Formation Between the 3'Terminus of 16S rRNA and the mRNA During Initiation of Protein Synthesis inEscherichia coli. Proc.Natl.Acad.Sci.USA **72**:4734-4738.

87. **Stern, S., B. Weiser, and H.F. Noller.** 1988. Model for the three-dimensional folding of 16 S ribosomal RNA. J.Mol.Biol. **204**:447-481.

88. **Stiege, W., C. Zwieb, and R. Brimacombe.** 1982. Precise Localization of Three Intra-RNA Cross-links in 23S RNA, and One in 5S RNA, Induced by Treatment of Escherichia coli 50S Ribosomal Subunits with bis-(2-chloroethyl)-metylamine. Nucl.Acids Res. **10**:7211-7299.

89. **Stoffler, G., and M. Stoffler-Meilicke.** 1984. Immunoelectron Microscopy of Ribosomes, p.303-330. In D.M. Engelman, C. Cantor, and T.D. Pollard (eds.), Annual Reviews of Biophysics and Bioengineering. , Palo Alto.

90. **Stoffler-Meilicke, M., M. Noah, and G. Stoffler.** 1983. Localization of Eight Ribosomal Proteins on the Surface of the 50S Subunit from Escherichia coli. Proc.Natl.Acad.Sci. **80**:6780-6784.

91. **Stoffler-Meilicke, M., G. Stoffler, O.W. Odom, G. Kramer, and B. Hardesty.** 1981. Localization of 3' ends of %sand 23S

rRNAs in reconsituted subunits ofEscherichia coli ribosomes. Biochemistry **78**:5538-5542.

92. **Struck, J.C.R., R.A. Lempicki, H.Y. Toschka, V.A. Erdmann, and M.J. Fournier.** 1988. E. coli 4.5S RNA can be Complemented by Heterologous Bacterial RNA Genes. (UnPub)

93. **Struck, J.C.R., D.W. Vogel, N. Ulbrich, and V.A. Erdmann.** 1988. The Bacillus subtilis scRNA is related to the 4.5S RNA fromEscherichia coli. Nucl.Acids Res. **16(6)**:2719.

94. **Tam, M.F., J.A. Dodd, and W.E. Hill.** 1981. Physical Characteristics of 23 S rRNA from the 50 s Ribosomal Subunit ofEscherichia coli. FEBS Letters **130**:217-220.

95. **Tam, M.F., J.A. Dodd, and W.E. Hill.** 1981. Physical Characterisitic of 16S rRNA under Reconstitution Conditions. J.Biol.Chem. **256**:6430-6434.

96. **Tam, M.F., and W.E. Hill.** 1981. Physical Characteristics of the Reconstitution Intermediates (RI30 and RI30*) from the 30S Ribosomal Subunit ofEscherichia coli. Biochemistry **20**:6480-6484.

97. **Taniguchi, T., and C. weissmann.** 1978. Inhibition of QB RNA 70S ribosome initiation complex formation by an oligonucleotide complementary to the 3' terminal region of E. coli 16S ribosomal RNA. Nature (London) **275**:770-772.

98. **Tapprich, W.E., and W.E. Hill.** 1986. Involvement of bases 787-795 of Escherichia coli 16S ribosomal RNA in ribosomal subunit association. Proc.Natl.Acad.Sci.U.S.A. **83**:556-560.

99. **Toschka, H.Y., P. Hopfl, W. Ludwig, K.H. Schleifer, N. Ulbrich, and V.A. Erdmann.** 1988. Complete nucleotide sequence of a 16S ribosomal RNA gene from Pseudomonas aeruginosa. Nucl.Acids Res. **16(5)**:2348-2348.

100. **Traut, R., J. Lambert, G. Boileau, and J. Kenny.** 1980. Protein Topography of Escherichia coli Ribosomal Subunits as Inferred from Protein Crosslinking, p.89-110. In G. Chambliss, G. Craven, L. Kahan, and M. Nomura (eds.), Ribosomes: Structure, Function and Genetics. University Park Press, Baltimore.

101. **Van Duin, J., C.J.C. Ravensbergen, and J. Doornbos.** 1984. "Basepairing of oligonucleotides to the 3' end of 16S ribosomal RNA is not stabilized by ribosomal proteins.". Nucl.Acids Res. **12(12)**:5079-5086.

102. **Vasiliev, V.D., V.E. Koteliansky, and G.V. Rezapkin.** 1977. The Complex of 16S RNA with Proteins S4, S7, S8, S15

Retains the Main Morphological Features of the 30 S Ribosomal Subparticle. FEBS Letters **79**:170-174.

103. **Vasiliev, V.D., O.M. Salinonova, and V.E. Koteliansky.** 1978. Specific Self Packing of the Ribosomal 16S RNA. FEBS Letters **95**:273-276.

104. **Vasiliev, V.D., O.M. Selinonova, and S.N. Ryazantsev.** 1983. Structure of the Escherichia coli 50S Ribosomal Subunit. J. Mol. Biol. **171**:561-569.

105. **Wickstrom, E.** 1983. Nuclease mapping of the secondary structure of the 49-nucleotide 3' terminal cloacin fragment of Escherichia coli 16S RNA and its interactions with initiation factor 3. Nucl.Acids Res. **11**:2035-2052.

106. **Wittman-Liebold, B.** 1986. , p.326-361. In B. Hardesty, and G. Kramer (eds.), Structure, Function, and Genetics of Ribosomes. Springer-Verlag, New York.

107. **Woese, C.R.** 1980. Just So Stories and Rube Goldberg Machines: Speculations on the Origin of the Protein Synthetic Machinery, p.357-373. In G. Chambliss, G.R. Craven, J. Davies, and K. Davis (eds.), Ribosomes: Structure ,Function & Genetics. University Park Press, Baltimore.

108. **Woese, C.R., R. Gutell, R. Gupta, and H.F. Noller.** 1983. Detailed Analysis of the Higher-order Structure of 16S-Like Ribosomal Ribonucleic Acids. Microbiology Reviews **47**:621-669.

109. **Wower, I., and R. Brimacombe.** 1983. The Localization of Multiple Sites on 16S RNA Which are Crosslinked to Proteins S7 and S8 in E. coli 30S Ribosomal Subunits by Treatment with 2-Iminothiolane. Nucl.Acids Res. **11**:1419-1437.

110. **Wower, I., J. Wower, M. Meinke, and R. Brimacombe.** 1981. The use of 2-iminothiolane as an RNA-proteiun cross-linking agent in E. coli ribosomes, and the localization on 23S RNA of sites crosslinked to proteins L4,L6,L21,L23,L27 and L29. Nucl.Acids Res. **9**:4285-4302.

111. **Zamir, A., R. Miskin, Z. Vogel, and D. Elson.** 1974. The Inactivation and Reactivation of E. coli Ribosomes. Methods in Enzymology **30**:406-426.

112. **Zimmermann, R.A.** 1980. Interactions Among Protein and RNA Components of the Ribosome, p.135-170. In G. Chambliss, G.R. CRAVEN, J. Davies, and K. Davis (eds.), Ribosomes: Structure, Function and Genetics. University Park Press, Baltimore, MD.

113. **Zimmermann, R.A., S.M. Gates, I. Schwartz, and J. Ofengand.** 1979. Covalent Cross-linking of Transfer Ribonucleic

Acid to the Ribosomal P Site. Site of Reaction in 16S Ribonucleic Acid. Biochemistry **18**:4333-4339.