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Probing the alpha-sarcin region of Escherichia coli 23S ribosomal RNA with short complementary DNA oligomers

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Probing the Alpha-Sarcin Region of Escherichia coli 23S Ribosomal RNA with Short, Complementary DNA Oligomers

by

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Probing the Alpha-Sarcin Region of Escherichia coli 23S Ribosomal RNA with Short, Complementary DNA Oligomers (62 pp.)

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The structure and function of nucleotides 2653-2667 of E. coli 23S ribosomal RNA (rRNA) was investigated with short, complementary DNA oligomers.

Availability of the α-sarcin region to DNA oligomers was assessed in nitrocellulose filter binding assays, sucrose gradient binding assays, and with ribonuclease H. The results indicate the site is not accessible to DNA probes in the 50S subunit or 70S ribosomes. Further hybridization assays revealed that probes could not bind to 50S subunits or 70S ribosomes in the presence of polyuridylic acid, phenylalanine transfer RNA, or both.

The cause of 50S ribosomal subunit collapse reportedly triggered by hybridization of a 14-base cDNA probe at the α-sarcin region of 23S rRNA was also investigated by physical measurement of probe-subunit complexes in varying buffer conditions. The results reported here show that this probe was unable to hybridize to its target site in the intact 50S subunit (see Table III, p. 44) and the physical characteristics of 50S subunits remained unchanged in its presence. Subunit collapse was induced in buffer containing 20mM Tris-HCl (pH 7.5), 600 mM NH_4Cl, 1 mM $MgCl_2$, 1 mM DTT, and 0.1 mM EDTA in the absence of probe, producing 39S and 45S subparticles of the 50S subunit. The probe bound specifically to its target site in the collapsed particle, but did not promote further unfolding. The results demonstrate that a DNA probe complementary to the α-sarcin region cannot cause the 50S subunit to unfold or cause 23S rRNA to degrade. The previously reported collapse was most probably the result of the ionic conditions used.

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ABBREVIATIONS

aa-tRNA	=	aminoacyl-tRNA
ATP	=	adenosine 5'-triphosphate
cDNA	=	complementary DNA
Ci	=	Curie(s)
cpm	=	counts per minute
ddH_2O	=	double-distilled H_2O
DNA	=	deoxyribonucleic acid
DTT	=	dithiothreitol
EDTA	=	ethylenediaminetetraacetic acid
EF-Tu	=	elongation factor Tu
g	=	force of gravity
HPLC	=	high-performance liquid chromatography
GTP	=	guanosine 5'-triphosphate
mM	=	millimolar
pmol	=	picomole(s)
poly (U)	=	poly (uridylic acid)
QLS	=	quasi-elastic light scattering
RNA	=	ribonucleic acid
rRNA	=	ribosomal RNA
tRNA	=	transfer RNA
Tris	=	tris-(hydroxyamino)methane

CHAPTER I

INTRODUCTION

The Escherichia coli Ribosome

In all organisms, protein biosynthesis is performed, with the aid of several ligands, by ribonucleoprotein complexes called ribosomes. Because of their obvious biological importance, ribosomes have been studied intensely since they were discovered some thirty years ago.

The ribosome of the bacterium Escherichia coli is by far the most extensively characterized. The E. coli ribosome has a molar mass of approximately 2.7 X 10^6 daltons, is approximately 35% protein and 65% RNA, and has a sedimentation coefficient of 70S (Hill et al., 1969). Each 70S ribosome consists of one 30S subunit (0.9 X 10^6 daltons) and one 50S subunit (1.8 X 10^6 daltons) (Hill et al., 1969). The 30S subunit contains one 16S RNA molecule and 21 proteins, ranging in size from 8500 daltons to 61,000 daltons (Wittmann-Liebold, 1986); the 50S subunit contains one 5S RNA molecule, one 23S RNA molecule, and 32 proteins from 5400 daltons to 24,500 daltons (Wittmann-Liebold, 1986).

Three-dimensional models of the ribosome and its subunits derive mostly from electron microscopy studies (Lake, 1976; Lake, 1982; Vasiliev et al., 1983; Kastner et al., 1981; Stöffler & Stöffler-Meilicke, 1984). While there are some minor differences among the models, all share the

same approximate shape and structure (Figure 1). The 30S subunit appears as a semisymmetrical, elongated particle. The upper one-third of the subunit, the head, is divided from the lower two-thirds, the base, by a neck-like indentation. A region of the subunit, called the platform, extends from the base of the subunit and forms a cleft between it and the head. The model for the 50S subunit is asymmetrical, consisting of a central protuberance and two protrusions (the "L7/L12 stalk" and the "L1 shoulder") approximately 50° 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. 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 250 Å.

While each ribosomal component is well-characterized and there is a general concensus concerning the overall shape of the particle and its subunits, the arrangement of the individual components within the ribosome has yet to be determined precisely. Techniques currently employed include immunoelectron microscopy (Stöffler-Meilicke et al., 1983; Stöffler-Meilicke et al., 1981; Lake & Kahan, 1975), protein-RNA crosslinking (Traut et al., 1980; Wower et al., 1981), and protein-protein crosslinking (Sköld, 1982;

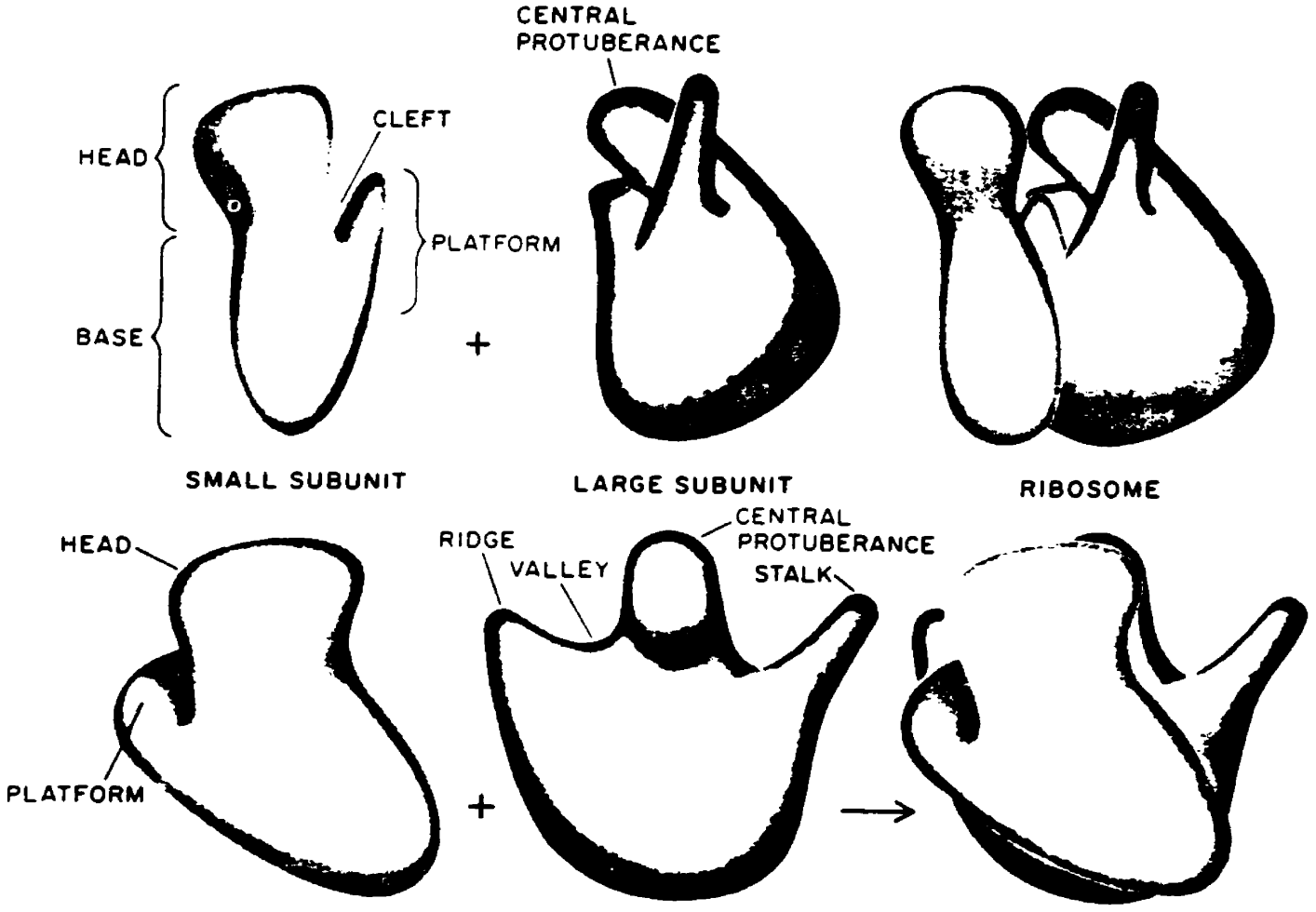


Figure 1. Three-dimensional models of the E. coli ribosome. Adapted from Oakes et al. (1986a).

Acharya et al., 1973). Promising results have also been obtained from small angle neutron scattering (Moore et al., 1986). A model for the spatial arrangement of all twenty-one 30S subunit proteins has been proposed from neutron scattering data (Capel et al., 1988; Capel et al., 1987). Neutron scattering data for the 50S subunit are less complete, but work is in progress. To date, the distances between thirteen 50S subunit proteins have been determined (Nierhaus et al., 1983; Nowotny et al., 1986).

Ribosomal RNA Structure

The primary structures of all three E. coli ribosomal RNAs have been determined completely: 5S RNA contains 120 nucleotides (Brownlee et al., 1968); 16S RNA contains 1542 nucleotides, including nine methylated bases (Carbon et al., 1979); 23S RNA contains 2904 nucleotides, including ten methylated bases, three pseudouridine residues, and one ribothymidine residue (Branlant et al., 1981).

Secondary structure interactions of the large ribosomal RNAs have been elucidated in ribonuclease protection studies (Stiegler et al., 1981; Noller et al., 1981; Branlant et al., 1981), or through use of single-strand specific chemical modifying agents (Noller & Woese, 1981; Noller et al., 1981) or chemical crosslinkers (Glotz & Brimacombe, 1980). Three secondary structure models each have been proposed for 16S RNA (Stiegler et al., 1981; Noller & Woese, 1981; Zwieb et al., 1981) and 23S RNA (Noller et al., 1981;

Glotz & Brimacombe, 1980; Branlant et al., 1981) on the basis of experimental results and phylogenetic sequence analysis. Except for minor structural differences, all models are in substantial agreement. Large single-stranded loop regions and 45 helices define three structural domains of 16S RNA. Long-range base-paired interactions of 23S RNA organize the molecule into six major structural domains containing over 100 helices (Figure 2).

While the primary and secondary structures of the ribosomal RNAs have been characterized with some certainty, much less is known about the three-dimensional organization of the RNAs within their respective subunits. Studies of protein-free rRNA have provided conflicting results. Under certain ionic conditions, the RNA molecules organize into compact structures approximating the shape of ribosomal subunits (Vasiliev et al., 1978; Dunn & Wong, 1979), while under other conditions, rRNA by itself cannot attain a high degree of tertiary structure (Boublik et al., 1982; Tam et al., 1981a; Tam et al., 1981b). Association of certain ribosomal proteins with rRNA leads to extensive folding of the RNA molecules (Boublik et al., 1982; Vasilev et al., 1977). Although it may be possible for the free RNA to form tertiary interactions in solution, the evidence indicates that rRNA in the free state is less ordered than rRNA in the subunit, and tertiary structure most probably depends upon association of specific structural proteins (Boublik et al., 1982).

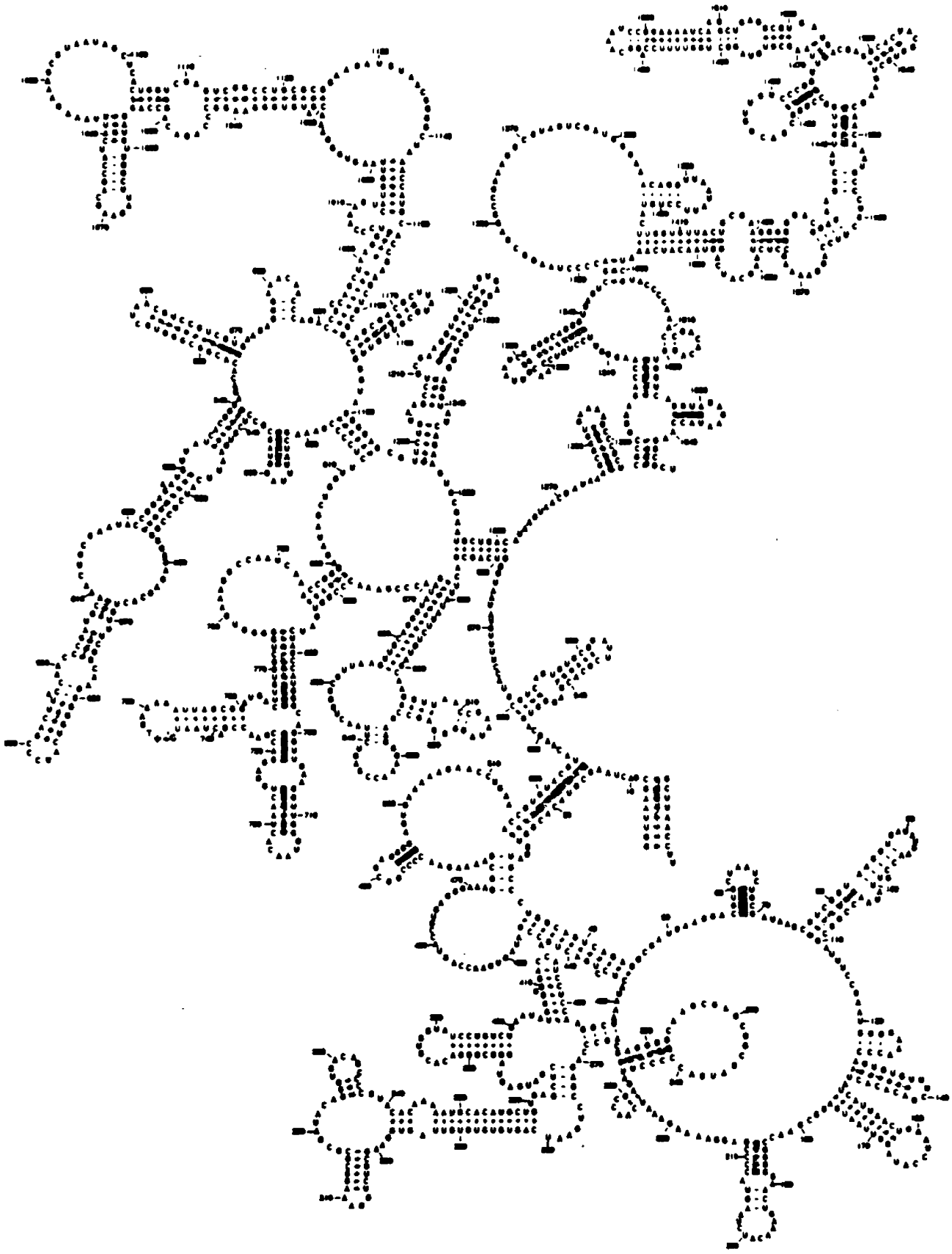


Figure 2. Secondary structure map of E. coli 23S rRNA. The α-sarcin region is outlined in yellow. Adapted from Noller (1984).

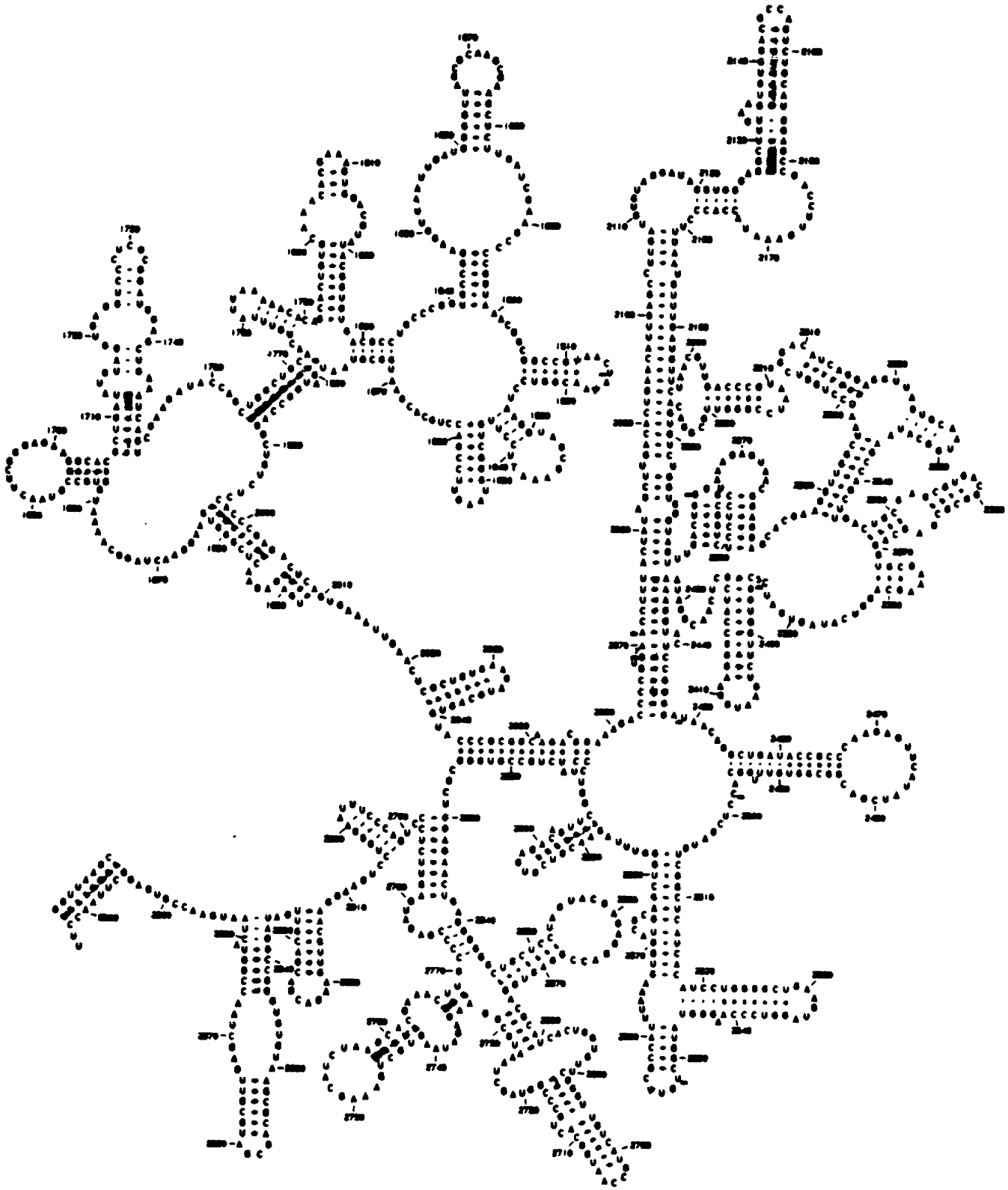


Figure 2. (continued)

Specific long-range interactions of rRNA within the subunit have been determined from intra-RNA crosslinks in rRNA induced by irradiation of subunits by ultraviolet light (Stiege et al., 1982) or by chemical crosslinkers (Stiege et al., 1982; Expert-Bezancon & Wollenzien, 1985). Ribosomal RNA regions exposed to the surface of subunits have also been localized by hybridization electron microscopy (Oakes et al., 1986a, 1986b) and by localization of N_6,N_6-dimethyl-adenosine residues by electron microscopy of ribosome-antibody complexes (Politz & Glitz, 1977). Protein-RNA and noncovalent RNA-RNA interactions have also been characterac-terized from ribonucleoprotein fragments of subunits prepared by limited nuclease digestion (Spitnik-Elson et al., 1982; Spitnik-Elson et al., 1985). The resulting fragments were characterized for protein content and rRNA sequence.

Indirect techniques such as immunoelectron microscopy (Stöffler-Meilicke et al., 1983; Stöffler-Meilicke et al., 1981; Lake & Kahan, 1975), protein-RNA crosslinking (Traut et al., 1980; Wower et al., 1981), protein-protein crosslin-king (Sköld, 1982; Acharya et al., 1973) and small-angle neutron scattering (Moore et al., 1986) have provided information on the three-dimensional arrangement of proteins and RNA within the subunit. Preliminary tertiary structure models for the rRNAs based on secondary structure, intra-molecular RNA crosslinking results, protein-RNA interac-

tions, and the locations of proteins within the subunits have been proposed (Expert-Bezancon & Wollenzien, 1985; Noller & Lake, 1984), but are far from being complete.

Ribosomal RNA Functions

While the RNAs of the E. coli ribosome were originally thought to be structural components of the particle, evidence has accumulated in recent years that they are functional as well (Woese, 1980; Noller, 1984; Cundliffe, 1986). Perhaps the earliest known and most extensively studied rRNA function is messenger RNA binding. Basepairing between the 3' terminus of 16S RNA and a conserved sequence of bacterial mRNAs is necessary for initiation of protein synthesis (Shine & Dalgarno, 1974; Backendorf et al., 1980; Steitz & Jakes, 1975). Other functions attributed to ribosomal RNA include subunit association through base-pairing interactions between specific sequences of 23S RNA and 16S RNA (Herr & Noller, 1979; Herr et al., 1979; Tapprich & Hill, 1986), transfer RNA binding (Schwartz & Ofengand, 1978; Ofengand et al., 1979; Zimmermann et al., 1979; Taylor et al., 1981; Prince et al., 1982), and initiation factor binding (Wickstrom, 1983; Wickstrom et al., 1986).

Studies of antibiotics have provided evidence for other possible functions of ribosomal RNA. Several antibiotics which bind to specific, conserved 16S RNA sequences inhibit tRNA binding to the 30S subunit (Moazed & Noller, 1987).

Thiostrepton, a modified peptide antibiotic, is able to inhibit ribosome-associated GTPase activities when bound to the region surrounding A_{1067} in 23S RNA (Cundliffe, 1986). The protein elongation inhibitors erythromycin and chloramphenicol compete for the same binding site in the peptidyl transferase center of 23S RNA (Sigmund et al., 1984; Skinner et al., 1983). If the assumption that antibiotic binding to a specific ribosomal component inhibits the function of that component is valid, then each of the RNA regions affected by antibiotic binding is a probable functional site of the ribosome.

Important features of functionally active rRNA sequences become apparent upon analysis: ribosomal RNA sequences in these regions are highly conserved phylogenetically (Noller, 1984; Cundliffe, 1986; Woese, 1980), structural features of active sequences are conserved (Moazed & Noller, 1987; Noller, 1984), and (3) they are often exposed to the surface of the subunit (Noller, 1984).

DNA hybridization has emerged recently as a promising method for probing rRNA structure and function. Small, complementary DNA probes can be advantageous over other techniques such as nuclease digestion or chemical modification because such probes can be targeted to specific RNA sequences, and presumably do not disrupt structural interactions within the ribosome. Complementary oligonucleotides have been used successfully to probe RNA secondary structure

(Hogan et al., 1984; Lewis & Doty, 1977) and to locate or identify rRNA sequences exposed on the surfaces of ribosomal subunits (Oakes et al., 1986a; Marconi & Hill, 1988). DNA probes have also helped to identify rRNA sequences involved in specific ribosomal functions, such as mRNA binding (Van Duin et al., 1984; Backendorf et al., 1980; Taniguchi & Weissmann, 1978), subunit-subunit association (Tapprich & Hill, 1986), and tRNA binding (Schmitt et al., 1980).

The Alpha-Sarcin Region

One ribosomal RNA sequence of particular interest is the α-sarcin region of Escherichia coli 23S RNA. This region spans nucleotides 2653-2667 (see Figure 2) and contains the cleavage site for the cytotoxin α-sarcin (Endo & Wool, 1982; Endo et al., 1983; Schindler & Davies, 1977). Alpha-sarcin is a 17 kd protein produced by the mold Asperigillus giganteus that possesses both single- and double-stranded ribonuclease activities specific for the 3' side of purines (Endo et al., 1983). Although it causes extensive degradation of naked RNA, α-sarcin hydrolyzes a single phosphodiester bond in a highly conserved sequence contained within 23S rRNA when incubated with 50S subunits or 70S ribosomes from E. coli (Endo et al., 1983). The clip occurs between bases G_{2661} and A_{2662} (Figure 3) and produces the 242-base "alpha fragment" from the 3' terminus of 23S rRNA (Endo & Wool, 1982; Endo et al., 1983; Schindler & Davies, 1977).

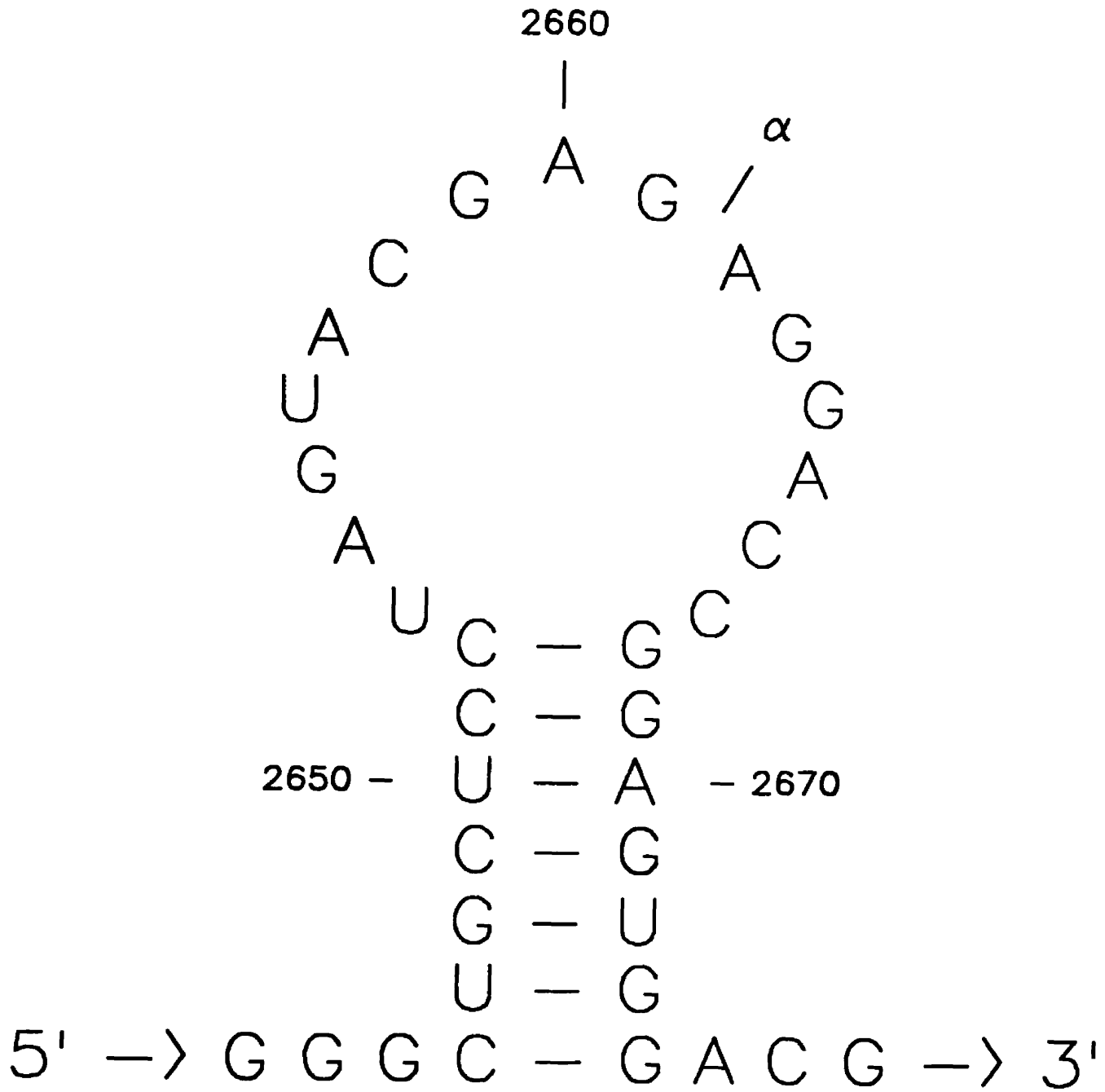


Figure 3. Detailed view of the α-sarcin region of E. coli 23S rRNA. The cleavage site is denoted by "α".

The action of α-sarcin is highly specific; no cleavage of 16S rRNA is observed when 30S ribosomal subunits are incubated with the enzyme, nor is the alpha fragment observed when protein-free ribosomal RNA is used as the substrate (Endo et al., 1983).

The nucleolytic specificity of α-sarcin is thought to be a function of the highly conserved RNA sequence at the cleavage site and of ribosome structure surrounding the region (Endo et al., 1983). Comparison of the α-sarcin region in E. coli with α-sarcin sensitive sites in rat liver 28S rRNA and yeast 26S rRNA shows there is greater than 93% sequence homology among them (Endo & Wool, 1982). In all three cases, the region is single-stranded.

Alpha-sarcin is a potent inhibitor of protein synthesis. Cleavage at the α-sarcin site inactivates the ribosome specifically by inhibiting EF-Tu-catalyzed aminoacyl-tRNA (aa-tRNA) binding at the ribosomal A site during peptide chain elongation (see Figure 4), but does not affect uncatalyzed aa-tRNA binding (Fernandez-Puentes & Vazquez, 1977; Hobden & Cundliffe, 1978). Aminoacyl-tRNA appears to protect 28S rRNA in rat liver polysomes from α-sarcin cleavage, but polysomes become sensitized to the toxin after treatment with puromycin (Chan et al., 1983). A study of the nucleic acid binding properties of eukaryotic EF-Tu revealed the factor has two RNA binding sites, one having a high affinity for tRNA and the other having a high affinity for

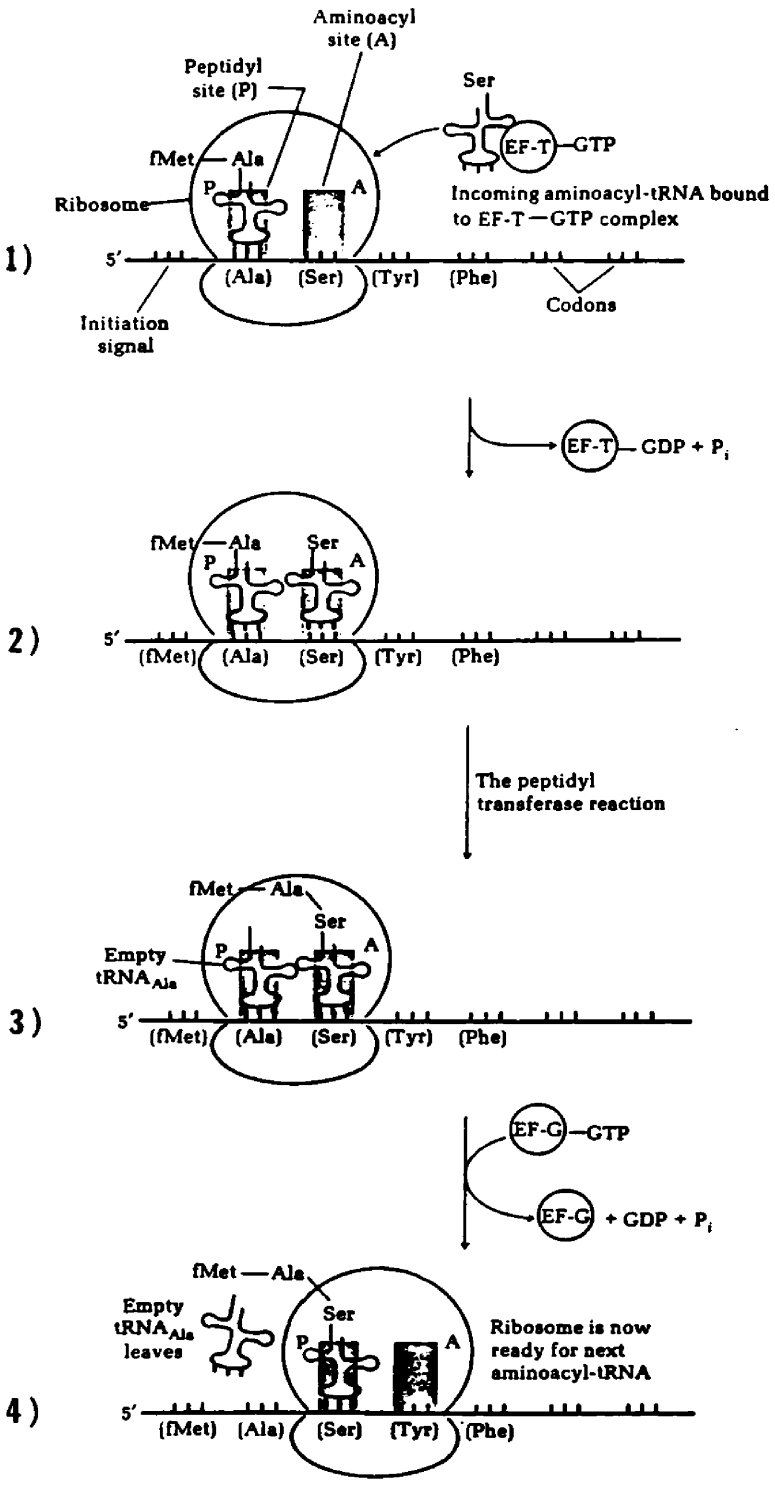


Figure 4. The E. coli protein elongation cycle. (1) At the beginning of each cycle, a peptidyl-tRNA is bound to the ribosomal P site. (2) An aminoacyl-tRNA binds to the A site in a complex with EF-Tu and GTP. (3) Upon binding, the aa-tRNA accepts the nascent peptide chain. (4) The P-site tRNA is ejected and the A-site tRNA takes its place. [Adapted from Lehninger (1975)].

poly (G) and poly (U) (Slobin, 1983). A G- and U-rich sequence in rat liver ribosomes starting 19 nucleotides downstream from the α-sarcin cleavage site has been proposed as a possible EF-Tu binding site. A similar sequence is found in yeast 26S and E. coli 23S rRNAs. This circumstantial evidence suggests that the rRNA and possibly the proteins in the site affected by the enzyme may form a domain involved in elongation factor-mediated A site binding.

Henderson and Lake (1985) recently attempted to map the location of the α-sarcin site on the surface of the 50S subunit using hybridization electron microscopy. Those authors observed what was described as an "unexpected ribosomal collapse" triggered by the hybridization of a 14-base DNA probe complementary to nucleotides 2654-2667 of the α-sarcin region. During collapse, the 50S subunit reportedly released 5S rRNA and a distinct set of five ribosomal proteins (L1, L5, L6, L7/L12) and yielded a 43S subparticle called the alpha particle. In addition to the reported collapse, three 3' end fragments of 23S rRNA of roughly 350, 250, and 170 nucleotides were produced. The observed effects were amplified by increasing the incubation temperature from 37°C to 51°C. These data would suggest that the α-sarcin region is involved in possible tertiary or quaternary interactions responsible for maintaining the structural integrity of the large ribosomal subunit.

This contradicts the results of Walker et al. (1983), who demonstrated that 5.8S rRNA and the alpha fragment remain associated with α-sarcin treated rat liver ribosomes, and the only rRNA fragments produced corresponded in size to the alpha fragment and a 5' terminal fragment of 28S rRNA lacking nucleotides released with the alpha fragment.

Proposed Problem

The goals of this project were to assess any physical changes which may occur in the 50S ribosomal subunit as a result of hybridization of cDNA oligomers at the α-sarcin region, and to elucidate the function of the region, if possible. As noted above, Henderson and Lake (1985) reported collapse of the 50S subunit when a 14-base probe was bound to the site. The validity of those results is questionable because experimental conditions used in that study have been shown to be unfavorable for maintaining ribosome structure (Hill et al., 1970; Gesteland, 1966; Weller et al., 1968; Miall & Walker, 1969; Weller & Horowitz, 1964; Cammack & Wade, 1965; T. Wood, unpublished results). While the buffer conditions were probably the contributing factor in subunit collapse, the possibility that a cDNA oligomer can contribute to structural disruption of the subunit cannot be ruled out. The cause of ribosomal collapse, whether it be buffer conditions or cDNA hybridization, remained uncertain. The structural problem, therefore, required study before the question of function could be addressed.

The first phase of the study would utilize physical techniques to characterize the effects of buffers and of a chemically synthesized copy of the 14-base oligomer described by Henderson and Lake (1985) on 50S subunit structure. The probe would be hybridized to the α-sarcin region in the experimental conditions described by Henderson and Lake and also under conditions similar to those described in other DNA-rRNA probing studies (Tapprich & Hill, 1986; Backendorf et al., 1981; Marconi & Hill, 1988). The physical properties of the resulting probe-subunit complexes would be to those of each other and to those of control subunits.

If hybridization of the 14-base probe contributed to subunit collapse in a buffer-independent manner, then hexamers complementary to overlapping subsets of the α-sarcin region would be synthesized, hybridized to the rRNA, and tested individually for structural effects on the 50S subunit. In this manner, the specific nucleotides of the α-sarcin region involved in structural interactions within the 50S subunit could be identified.

If the probe did not affect the structure of the 50S subunit, then the ribosomal collapse phenomenon could be dismissed as an experimental artifact. If this were the case, the proposed hexameric oligomers would be used as functional probes. The possible role of the α-sarcin region as a functional domain involved in aa-tRNA binding would be tested in competition experiments between probes and tRNA, EF-Tu, EF-Tu·GTP and the ternary complex aa-tRNA·EF-Tu·GTP.

CHAPTER II

MATERIALS AND METHODS

Preparation of Ribosomes and Ribosomal Subunits

Ribosomes were isolated from E. coli RNase I deficient strain MRE 600 by the method of Hill et al. (1969). Typically, 100 g of frozen cells were thawed in 100 ml of buffer A (10 mM Tris-HCl, pH 7.4; 200 mM KCl; 10 mM $MgCl_2$) in a Waring laboratory blender and pelleted by centrifugation at 10,000 x g in a Beckman JA-17 rotor for 5 minutes. The pellets were mixed into a slurry with 0.25-0.30 mm glass beads in 15-20 ml of buffer A. The cells were disrupted by grinding the slurry in a Gifford-Wood minimill for 50 min. To minimize ribonuclease digestion and heat-induced degradation of ribosomes in the cell lysate, the cracking mixture was kept cold by immersing the minimill bucket in a salted icewater bath maintained at -10°C. Following grinding, the glass beads were settled from the slurry for 10 min and the supernatant was decanted into a beaker surrounded by ice. The beads were washed with small portions (about 50 ml) of ice-cold buffer A four to five times, with each wash being added to the cell lysate until the total volume was about 250 ml. Intact cells, glass beads, and cellular debris were pelleted from the lysate mixture by centrifugation at 25,800 x g for 1 hour (low-speed spin) in a Sorvall SS34 rotor and

discarded. This and all subsequent centrifugation steps were performed at 4°C. The supernatant was centrifuged in either a Beckman Ti60 rotor (250,000 x g, 3hr) or a Beckman Ti70 rotor (370,000 x g, 2.5 hr) to pellet crude ribosomes (high-speed spin). The pellet from the high-speed spin was resuspended at least 3 hours in 100 ml of buffer A at 4°C and subjected to another low-speed and another high-speed spin. The ribosome pellet from the second high-speed spin was resuspended in 25 ml of buffer B (10 mM Tris-HCl, pH 7.4; 100 mM KCl; 1.5 mM $MgCl_2$) for subunit isolation or TC70S buffer (20 mM Tris-HCl, pH 7.4; 100 mM KCl; 6 mM $MgCl_2$) for tight-couple 70S ribosome isolation. Yield of crude ribosomes was typically 1.0-1.2 grams per 100 grams of cells.

The 50S subunits were isolated by zonal centrifugation as outlined by Tam and Hill (1981), with minor modification. The crude ribosome preparation was loaded onto a 10-34% (wt/vol) sucrose gradient in buffer B in a Beckman Ti15 zonal rotor equipped with a B-29 core and centrifuged 14.5 hours at 31,000 rpm. Following centrifugation, the gradient was displaced from the rotor by pumping distilled water into the B-29 core and collected in 10 ml fractions with a Gilson fraction collector. Absorbance of the effluent was measured at 280 nm using a Chromatronix Model 220 continuous-flow absorbance detector. Subunits were pelleted from pooled zonal fractions by centrifugation for at least 9 hrs at

370,000 x g in a Beckman Ti70 rotor. To obtain unwashed 50S subunits, pellets were resuspended in 10 ml buffer B and dialyzed 24 hr against 1 L buffer B to remove residual sucrose. To obtain salt-washed 50S subunits, the pellets were resuspended in 20-25 ml of a high-salt buffer containing 50 mM Tris-HCl (pH 7.4), 500 mM NH_4Cl, 10 mM $MgCl_2$, and divided into six equal aliquots. Each aliquot was layered onto a 23 ml sucrose cushion (20% sucrose in high-salt buffer) and centrifuged at 370,000 x g for 18 hours. Subunit pellets were resuspended in 10 ml of buffer B and dialyzed against buffer B as described. All subunit samples were stored at -70°C in small aliquots.

Tight-couple 70S ribosomes (TC70S) were purified by zonal centrifugation using a 10-34% (wt/vol) sucrose gradient in TC70S buffer in a Beckman Ti14 rotor. The crude preparation was centrifuged 5 hours at 47,000 rpm and 5 ml fractions were collected, dialyzed against TC70S buffer, and stored as described. For small-scale preparations, 500 μg of crude ribosomes in approximately 100 μl of TC70S buffer were layered onto 13 ml 10-30% (wt/vol) linear sucrose gradients and centrifuged in a Beckman SW41 rotor for 4.5 hours at 37,000 rpm. Fractions (2 ml each) were collected from the bottom of each gradient and measured for absorbance at 260 nm in a Beckman DU-8 spectrophotometer. Fractions containing TC70S ribosomes were pooled and centrifuged in a Beckman TLA 100.2 rotor for 30 min at 100,000 rpm to pellet the ribo-

somes. The pellets were washed twice with TC70S buffer and finally resuspended in 100-200 μl of TC70S buffer. Samples were stored at -70°C in small aliqouts.

Preparation of Alpha Particles

Alpha particles were prepared by dialyzing 50S ribosomal subunits against 100 volumes of alpha buffer (20 mM Tris-HCl, pH 7.5; 600 mM NH_4Cl; 1 mM $MgCl_2$; 1 mM DTT; 0.1 mM EDTA) for 24 hours at 4°C with three buffer changes. Samples were analyzed immediately following dialysis.

Preparation of DNA Probes

DNA probes were synthesized on a Biosearch Model 8600 automated DNA synthesizer using β-cyanoethylphosphoramidite chemistry. The oligomers were removed from the solid support by incubating the synthesis column in concentrated NH_4Cl for 2 hours at room temperature. Benzoyl blocking groups were removed from probes by further incubation in a sealed ampule with fresh, concentrated NH_4Cl for 5 hours at 55°C. The 5'-dimethoxytritylated (5'-DMT) probes were evaporated to dryness, resuspended in 100 μl ddH_20, and purified by gradient reverse-phase HPLC (RP-HPLC) on a 25 cm Column Engineering 10 μm ODS column. The gradient was 100% Buffer A (10 mM TEA-OAc, pH 7.3) to 70.8 % Buffer B (50% Buffer A: 50% Baker HPLC grade acetonitrile) in 20 min at 1 ml/min. Purified 5'-DMT probes were evaporated to dryness, resuspended in 1 ml of 80% acetic acid, and incubated for one hour at room temperature to detritylate. Detritylated probe was

evaporated to dryness, resuspended in 1 ml ddH_2O, and extracted six times with 1-ml portions of diethyl ether. The sample was evaporated to dryness, resuspended in 100 μl ddH_2O, and purified by gradient RP-HPLC as described. Purified oligomers were evaporated to dryness, dissolved in 1 ml of ddH_2O and stored at -70°C.

Purified DNA probes were 5'-end labeled using T4 polynucleotide kinase (Bethesda Research Laboratories) and [gamma-^{32}P]ATP (New England Nuclear) as described by Chaconas and Van de Sande (1980) with the omission of the dephosphorylation step. The labeling reactions contained 35 pmol probe, 35 pmol [gamma-^{32}P]ATP (3000 Ci/mmol), and 4 units of kinase in 50 μl of buffer containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM DTT, and 1 mM spermidine. Reactions were incubated 30-45 minutes at 37°C and stopped by the addition of 5 μl 200 mM EDTA. The mixture was extracted once with an equal volume of TNE-equilibrated phenol (TNE = 10 mM Tris-HCl, pH 8.8; 100 mM NaCl; 5 mM EDTA) and the aqueous phase was removed and saved. The remaining organic phase was back-extracted four times with equal volumes of TNE, with the aqueous phase of each extraction being added to those from the previous extractions. The extracted probes were loaded onto NACS PREPAC nucleic acid purification columns (Bethesda Research Laboratories) in TNE buffer, washed with low-salt buffer, and eluted from the column with high-salt buffer as recommended by the manufacturer. Typical

probe recovery was about 70% at a specific activity of 3.5 x 10^6 cpm/pmol.

The purity of probe preparations was monitored by gel electrophoresis. DNA oligomer (25 μg) was evaporated to dryness, resuspended in 15 μl of gel loading dye (7M urea, 5% glycerol, 0.025% bromphenol blue, 0.025% xylene cyanol) and loaded onto a 20% polyacrylamide gel (12.5 cm X 13.5 cm X 1.5 mm) containing 89 mM Tris-borate (pH 8.3) and 1mM EDTA (TBE). The sample was electrophoresed at 40 mA for 50 min at 4°C in TBE running buffer. Bands were visualized by staining the gel in a solution containing 0.2% methylene blue, 200 mM acetic acid, and 200 mM sodium acetate.

Radiolabeled probe was also monitored electrophoretically for purity. Immediately following labeling, two probe samples containing approximately 300,000 cpm were mixed with 5 μl gel loading dye and analyzed. The first was loaded onto a 20% polyacryl ıide gel and electrophoresed at 40 mA for 45 min at 4°C. The current was stopped and the second sample was loaded into an adjacent well on the gel. Electrophoresis was continued for an additional five minutes. Labeled DNA was visualized by autoradiography. A sheet of Kodak XAR-5 film was placed over the gel for 15 minutes and then developed five minutes in Kodak D-19 developer. Undegraded probe appears as a single band near the bottom of the 50-minute lane and near the top of the five-minute lane. Unreacted [gamma-^{32}P]ATP appears as a

broad band at the bottom of the five-minute lane if it is present in the sample.

Preparation of $tRNA^{phe}$

Phenylalanine transfer RNA ($tRNA^{phe}$) was 5'-end labeled with [gamma-^{32}P]ATP using the coupled phosphatase-kinase reaction described by Cobianchi and Wilson (1987). In this reaction sequence, 10 pmol RNA and 0.1 unit calf intestine alkaline phosphatase (United States Biochemical) were incubated 30 minutes at 37°C in 10 µl of buffer containing 50 mM Tris-HCl (pH 9.0), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 1 mM spermidine. After incubation, 5 µl of 10X kinase buffer (500 mM Glycine-KOH, pH 9.2; 50 mM $MgCl_2$; 100 mM DTT), 5 µl of 50 mM potassium phosphate (pH 9.2), 50 pmol [gamma-^{32}P]ATP, and 4 units T4 polynucleotide kinase were added to the reaction mixture and the final volume was brought to 50 µl with water. The final reaction mixture was incubated 30 minutes at 37°C and the reaction was stopped by the addition of 5 µl 200 mM EDTA. Radiolabeled RNA was purified with a NENSORB 20 nucleic acid purification cartridge (New England Nuclear) according to the manufacturer's instructions. Eluted nucleic acid was evaporated to dryness and dissolved in 400 µl of ddH_2O. All samples were stored in water at -70°C.

Labeled RNA was analyzed for purity and for degradation electrophoretically. Small samples containing approximately 50,000 cpm were mixed in 5 µl gel loading dye, loaded onto a 10cm x 8cm x 0.8mm 5% polyacrylamide gel containing TBE and

7M urea, and electrophoresed in TBE for 20-40 min at 12.5 mA. The RNA was visualized by autoradiography as described above.

Transfer RNA Binding

Transfer RNA binding was assayed in a procedure adapted from Gnirke and Nierhaus (1986). Reaction mixtures contained 25 pmol 50S subunits or 70S ribosomes and 25-200 pmol radiolabeled, deacylated $tRNA^{phe}$ in 50 μl of tRNA binding buffer (10 mM Tris-HCl, pH 7.4; 150 mM KCl; 15 mM $MgCl_2$; 1 mM DTT). For poly (U)-directed tRNA binding, 10 μg poly (U) was included in the 50 μl reaction. Samples were incubated 2-4 hr at 4°C, diluted to 1 ml with ice-cold tRNA binding buffer, and filtered through Millipore 0.45 μm nitrocellulose filters. Unbound tRNA was washed through the filter with two 1 ml aliquots of ice-cold tRNA binding buffer. Radioactivity retained by the filter was determined by liquid scintillation counting.

Probe Hybridization Assays

Probe hybridization assays were performed by incubating a series of reaction mixtures containing 25 pmol 50S subunits or TC70S ribosomes and increasing amounts of 5'-^{32}P end-labeled probe in 50 μl of buffer containing 10 mM Tris-HCl (pH 7.4), 60-200 mM KCl, and 5-15 mM $MgCl_2$. The incubation period varied from 2-24 hrs at 4°C or 10°C, or for 30 min at 37°C or 51°C, depending on the experiment being performed. Blanks containing equivalent amounts of radio-

labeled probe and no subunits were used as controls in these assays. Following incubation, the reaction mixtures were spotted on Millipore HAWP 0.45 μm nitrocellulose filters and washed twice with 1-ml aliquots of binding buffer. The radioactivity retained by the filters was determined by liquid scintillation. The amount of probe bound to subunits was determined by subtracting the radioactivity retained on the blank filters from that retained on the corresponding reaction filters containing subunits.

Probe hybridization was also assayed using sucrose gradient centrifugation. The reactions in these experiments contained 2 A_{260} units (approximately 140 μg) of ribosomes or 50S subunits and radiolabeled probe in 50 μl of buffer. The reaction mixtures were layered onto linear sucrose gradients and centrifuged at 4°C. Different gradient and centrifugation conditions were used depending upon the ribosome species present in the hybridization reaction: for 50S subunits, a 4 ml 5-20% gradient was centrifuged 1.75 hours in a Beckman SW60 rotor at 54,000 rpm; for TC70S ribosomes, a 13 ml 5-20% gradient was centrifuged 4.5 hours in a Beckman SW41 rotor at 37,000 rpm; for alpha particles, a 13 ml 15-30% gradient was centrifuged 12 hours in a Beckman SW41 rotor at 37,000 rpm. In all cases, 20 fractions were taken starting from the bottom of each tube. Each fraction was assayed for subunit migration by measuring absorbance at 260 nm and for probe migration by liquid scintillation.

RNase H Assays

To determine the exact site(s) of cDNA probe base-pairing, the probe-subunit complex was incubated with RNase H in conditions similar to those outlined by Donis-Keller (1979) and the digestion products analyzed electrophoretically. Reactions containing 25 μg ribosomal subunits, 2.5 μg cDNA probe and 3-5 units of RNase H (P-L Biochemicals) in 20 μl of 40 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 60 mM KCl and 1 mM DTT were incubated at 4°C for 24 hr.

Ribosomal RNA digestion products were analyzed by gel electrophoresis. The RNA was purified by extracting subunit samples three times with buffer-equilibrated phenol, precipitating with 2.5 volumes 95% ethanol at -20°C for 1 hr and pelleting by centrifugation for 1 hr in a Sorvall HB-4 rotor at 10,000 rpm. RNA pellets were evaporated to dryness and resuspended in 15 μl of gel loading dye and electrophoresed in a 5% polyacrylamide gel (12.5 cm X 13.5 cm X 1.5 mm) containing 7M urea and TBE at 12.5 mA for 4 hr at room temperature. RNA products were stained in the gel with methylene blue.

Alpha-sarcin was used to generate the RNA fragment (the alpha fragment) (Endo et al., 1983) used as the marker in this assay. In this reaction, 25 μg 50S subunits and 0.25 μg α-sarcin were incubated in 20 μl of buffer A (50 mM Tris-HCl, pH 7.6; 50 mM KCl) for 15 min at 37°C.

Physical Methods

Sedimentation velocity experiments were performed in a Spinco Model E analytical ultracentrifuge equipped with schlieren optics. Dual-cell experiments were carried out in all cases to allow direct comparison of samples; typically, control samples were injected into a standard sample cell and test samples were injected into a 1° wedge cell. Both cells were centrifuged simultaneously in a Beckman ANH rotor at 52,000 rpm and 4°C. Photographs were taken at 4-minute intervals beginning 2 min after the rotor had reached 52,000 rpm. Photographs were analyzed visually for differences in schlieren patterns and sedimentation constants were calculated from the distances traveled by each boundary.

Diffusion coefficients of subunit samples were obtained using quasi-elastic light scattering (QLS) (Bloomfield, 1981). Samples for QLS experiments contained 100 μg 50S subunits in 200 μl of buffer in a 75 x 10 mm glass test tube and an additional 5.2 μg of probe, where indicated. Prior to scattering experiments, all samples were centrifuged overnight at 5000 rpm, 4°C in a Sorvall HB4 rotor to sediment dust particles.

The correlation function was obtained using a Malvern 4300 spectrometer system and a Langley-Ford 1096 autocorrelator. A Lexel 4 watt argon ion laser was used for the incident light. The correlation functions were analyzed by the method of Blair et al. (1981) to obtain diffusion coefficients.

CHAPTER III

RESULTS

Buffer Dependence of Alpha Particle Formation

As noted, previous studies (Hill et al., 1970; Gesteland, 1966; Weller et al., 1968; Miall & Walker, 1969; Weller & Horowitz, 1964; Cammack & Wade, 1965; T. Wood, unpublished results) have shown that the buffer conditions used by Henderson and Lake (20 mM Tris-HCl, pH 7.5; 600 mM NH_4Cl; 1 mM $MgCl_2$; 1 mM DTT; 0.1 mM EDTA, denoted here as alpha buffer) may have deleterious effects on subunit structure. The following experiments were initiated to compare the physical characteristics of 50S subunits in alpha buffer to those of 50S subunits in buffer B. The same physical characteristics of 50S subunits were also measured in the presence of the 14-base cDNA probe 23S(2653-2667).

Sedimentation Velocity

To test the effects of alpha buffer on 50S subunit stability, the sedimentation behavior of 50S subunits in alpha buffer was compared to that of 50S subunits in buffer B. Following subunit isolation, a 25 mg/ml stock solution of 50S subunits in buffer B was divided into two aliquots. One aliquot was dialyzed against alpha buffer for 24 hrs at 4°C, while the other was dialyzed against buffer B at 4°C for the same period. Each sample was diluted with its respective

buffer to a final concentration of 5 mg/ml. An 800 μl portion of the buffer B solution was injected into a standard 14 mm sample cell and the same volume of the alpha buffer solution was injected into a 14 mm 1° wedge cell. Both cells were centrifuged in the same rotor simultaneously at 52,000 rpm to allow direct comparison. Photographs of the schlieren patterns appear in Figure 5A. The 50S subunits migrated as a single boundary in buffer B, while subunits in alpha buffer migrated as two distinct boundaries. Also, both particles in alpha buffer sedimented more slowly than the particle in buffer B. The $S_{20,w}$ for each particle was calculated to be 52.4S, 43.7S, and 37.5S, respectively.

To test if elevated temperature contributed to 50S subunit collapse, both sample cells were removed from the rotor, inverted several times to resuspend the subunits, incubated at 51°C for 40 min, cooled to 4°C, and centrifuged as before. The boundary representing 50S subunits in buffer B was much smaller and broader after heating, while the boundaries in alpha buffer disappeared completely (Fig. 5B).

The effect of probe 23S(2654-2667) on subunit sedimentation is shown in Figure 6. The lower profile from the first experiment (Fig. 6A) derives from a 2.5 mg sample of 50S subunits sedimenting in 500 μl of buffer B. The upper profile derives from a 2.5 mg sample of 50S subunits preincubated in buffer B and sedimented with an eight-fold excess (mol/mol) of probe for 4 hrs at 4°C. The schlieren

(A) (B)

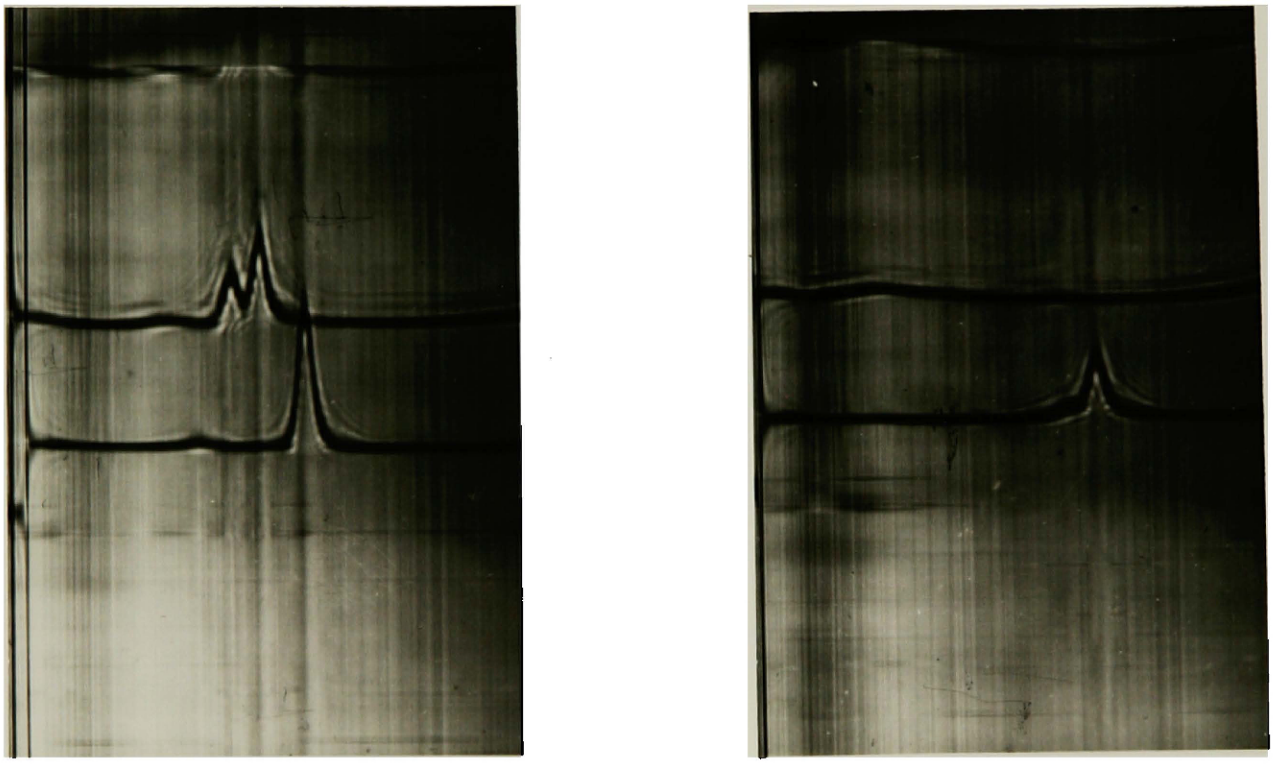
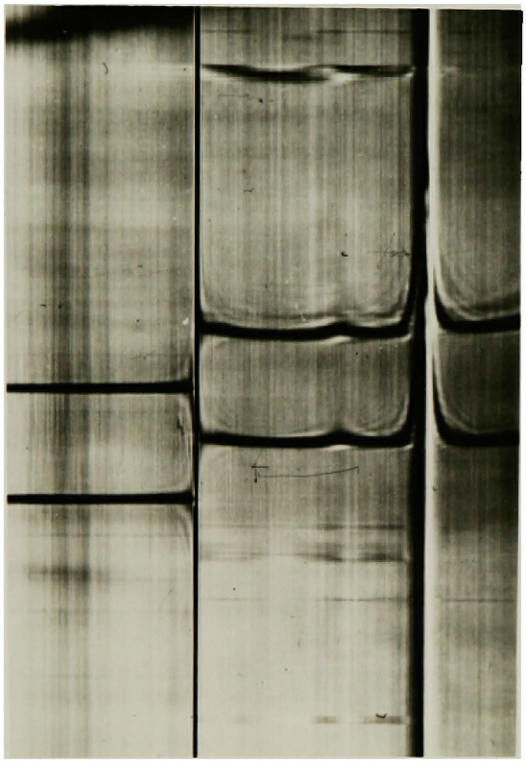


Figure 5. Schlieren patterns of 50S ribosomal subunit sedimentation in alpha buffer (upper) and buffer B (lower). (A) Following subunit isolation, a 25 mg/ml stock solution of 50S subunits in buffer B was divided into two aliquots. One aliquot was dialyzed against alpha buffer for 24 hrs at 4°C, while the other was dialyzed against buffer B at 4°C for the same period. Each sample was diluted with its respective buffer to a final concentration of 5 mg/ml. 800 μl of each sample were analyzed in a dual-cell sedimentation velocity experiment. Sample cells were centrifuged in a Beckman ANH rotor at 52,000 rpm. (B) Sample cells were removed from the rotor, inverted several times to resuspend the subunits, incubated at 51°C for 40 min, cooled to 4°C, and centrifuged as for (A). Sedimentation is from left to right. Photographs were taken 14 min after reaching 52,000 rpm using 70° phase plate angle. Temperature was 6.0°C.

(A)



(B)

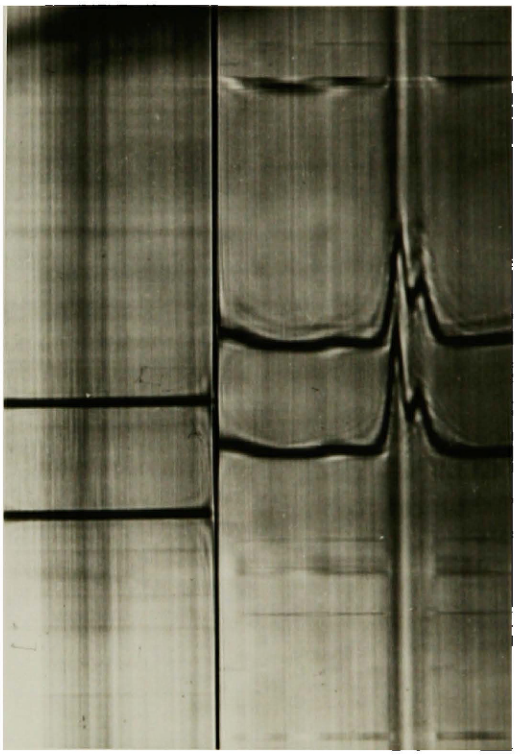


Figure 6. Schlieren patterns of 50S ribosomal subunit sedimentation in the absence of probe 23S(2654-2667) and after preincubation with an 8-fold excess (mol/mol) of probe. Each sample contained 2.5 mg 50S subunits in 500 μl of buffer and was incubated 4 hr at 4°C. (A) 2.5 mg 50S subunits in 500 μl buffer B (lower) and 2.5 mg 50S subunits plus eight-fold molar excess of probe in 500 μl buffer B (upper). (B) 2.5 mg 50S subunits in 500 μl alpha buffer (lower) and 2.5 mg 50S subunits plus eight-fold molar excess of probe in 500 μl alpha buffer (upper). Centrifugation conditions were as described in Figure 4. Sedimentation is from left to right. Photographs were taken 10 min after reaching 52,000 rpm using 75° phase plate angle. Temperature was 6.8°C.

patterns of both samples were identical. Both samples in the second experiment (Fig. 6B) were identical to those in the first experiment, except alpha buffer was substituted for buffer B. The lower schlieren pattern shows the alpha particle again formed in alpha buffer without probe. The upper pattern shows the sedimentation of subunits in the presence of an eight-fold excess (mol/mol) of probe. The sedimentation behavior of the subunit sample containing probe was essentially the same as that of the subunit sample without probe.

Quasi-elastic Light Scattering

Quasi-elastic light scattering was utilized to measure the diffusion coefficients of subunits under experimental conditions similar to those of the sedimentation velocity experiments (see Table I). Each sample contained 100 μg 50S subunits in 200 μl of the indicated buffer. Consistent with the sedimentation data, there was a significant difference in the diffusion coefficient of 50S subunits in binding buffer (1.94×10^{-7} cm^2 sec^{-1}) and the diffusion coefficient of 50S subunits in alpha buffer (1.67×10^{-7} cm^2 sec^{-1}). In binding buffer, a 20-fold molar excess of DNA probe had no significant effect on the 50S subunit diffusion coefficient, where $D = 1.96 \times 10^{-7}$ cm^2 sec^{-1}. Unexpectedly, the diffusion coefficient of subunits coincubated with probe in alpha buffer (1.81×10^{-7} cm^2 sec^{-1}) was slightly larger than the diffusion coefficient of subunits in alpha buffer alone,

TABLE I.
50S Subunit Diffusion Coefficients

Buffer	Probe	$D_{20,w}$ (cm^2 sec^{-1})
Binding Buffer	No	1.94×10^{-7}
	Yes	1.96×10^{-7}
Alpha Buffer	No	1.67×10^{-7}
	Yes	1.81×10^{-7}

Diffusion coefficients of subunit samples were obtained using quasi-elastic light scattering (QLS) (Bloomfield, 1981). Samples for QLS experiments contained 100 μg 50S subunits in 200 μl of buffer in a 75 x 10 mm glass test tube and an additional 5.2 μg of probe, where indicated. Prior to scattering experiments, all samples were centrifuged overnight at 5000 rpm, 4°C in a Sorvall HB4 rotor to sediment dust particles.

implying the presence of a smaller particle.

Ribosomal RNA Analysis

In addition to the ribosomal collapse described by Henderson and Lake, cDNA hybridization at the α-sarcin site was reported to be the direct cause of 23S rRNA degradation, producing three 3' end fragments of approximately 350, 250, and 170 nucleotides. Since alpha particle formation occurred in the absence of probe, the possibility that 23S rRNA degradation could occur in the absence of probe was tested. Subunits were incubated under conditions described by Henderson and Lake in either binding buffer or alpha buffer. The RNA was then isolated by phenol extraction and analyzed for degradation by polyacrylamide gel electrophoresis. Figure 7 clearly shows neither the probe nor increased temperatures caused the RNA fragmentation described by Henderson and Lake. In the parallel experiment, 50S subunits were incubated in alpha buffer under the same conditions. The RNA isolated from those subunits showed no sign of degradation, even after incubation at 51°C in the presence of the probe.

Probe Binding to 50S Subunits and Alpha Particles

Probe 23S(2654-2667)

Probe hybridization was quantified by nitrocellulose filter binding assays as previously described by Backendorf et al. (1981) and by Tapprich and Hill (1986). Hybridization of the 14-base DNA probe 23S(2654-2667) to 50S subunits in

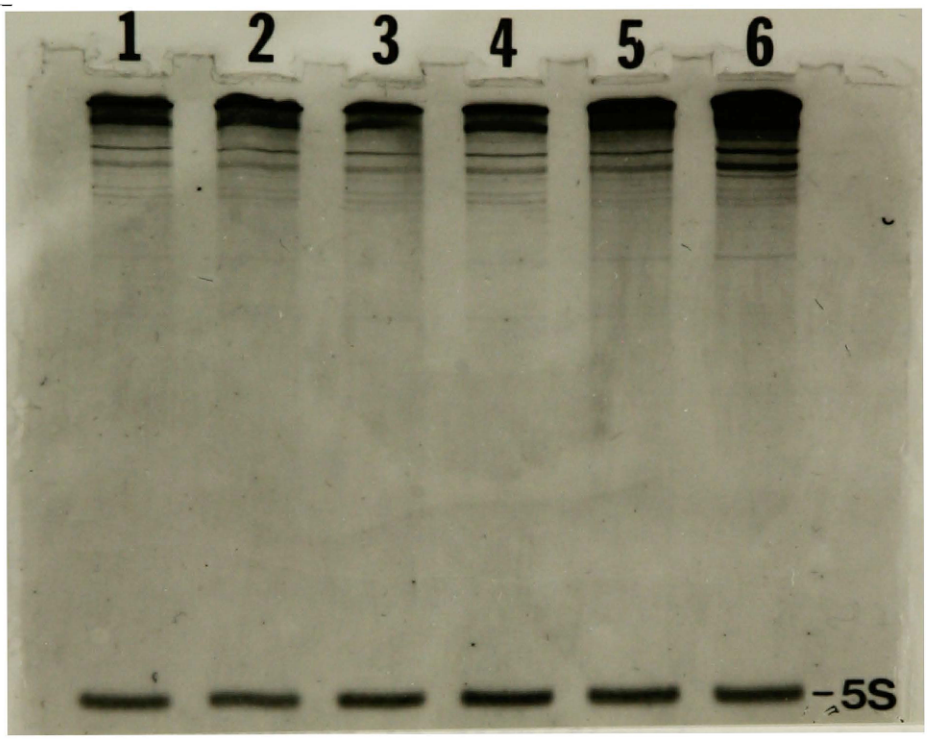


Figure 7. Electrophoretic analysis of ribosomal RNA from 50S subunits incubated in binding buffer at various temperatures in the presence or absence of probe 23S(2654-2667). Each reaction contained 25 pmol 50S subunits in 50 μl of buffer containing 10 mM Tris-HCl, 100 mM KCl, and 5 mM $MgCl_2$. Subunits were coincubated with 350 pmol probe where indicated. The RNA was purified by phenol extraction and electrophoresed in a 12.5 cm X 13.5 cm X 1.5 mm 5% polyacrylamide gel containing 89 mM Tris-borate (pH 8.3) and 1 mM EDTA for 4 hrs at 12.5 mA. Lane 1: incubated 30 min, 4°C; Lane 2: incubated 30 min, 37°C; Lane 3: incubated 30 min, 51°C; Lane 4: plus probe, 30 min, 4°C; Lane 5: plus probe, 30 min, 37°C; Lane 6: plus probe, 30 min, 51°C.

alpha buffer is shown graphically in Figure 8, demonstrating 30% of subunits in the reaction mixture complexed with probe as the amount of probe in the binding reaction was increased.

Probe binding was also assayed by sucrose gradient centrifugation. Hybridization is qualitatively demonstrated by comigration of radiolabeled probe with subunits in sucrose density gradients. Migration of probe 23S(2654-2667) and subunits in a 10-30% sucrose gradient containing alpha buffer is shown in Figure 9. The two peaks from left to right correspond to 50S subunits and to the alpha particle, respectively. In all trials, the labeled probe comigrated with the alpha particles, and not with the 50S subunits.

Hybridization of the 14-base probe to the intact 50S subunit was not detected in significant amounts by nitrocellulose filter binding assays under any of several experimental conditions. Initially, the conditions of Tapprich and Hill (1986) were used. Unwashed 50S subunits and probe were incubated in 50 μl of buffer containing 10 mM Tris (pH 7.4), 60 mM KCl, and 10 mM $MgCl_2$ for 4 hours at 4°C. When hybridization failed to occur under these conditions, salt-washed 50S subunits were assayed for their ability to bind the probe, again producing negative results. Varied salt concentrations in 10 mM Tris buffer were tested to determine if the initial buffer conditions were unfavorable for cDNA binding. Potassium chloride concentrations were varied from

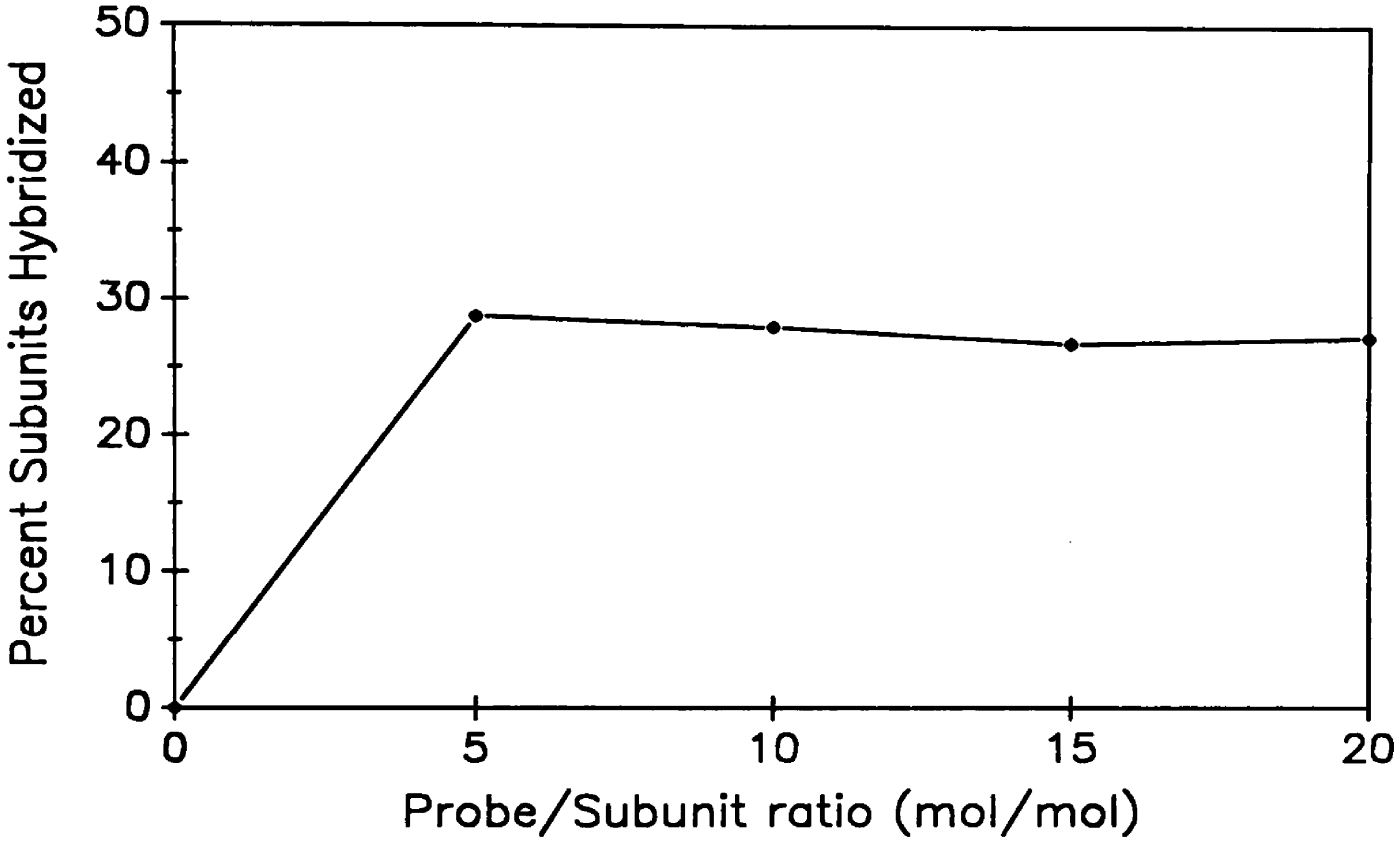


Figure 8. Filter binding assay of DNA probe 23S(2654-2667) and 50S subunits in alpha buffer. Probe hybridization assays were performed by incubating a series of reactions containing 25 pmol 50S subunits and increasing amounts of 5'-^{32}P end-labeled probe in 50 μl of buffer containing 20 mM Tris-HCl (pH 7.5), 600 mM NH_4Cl, 1 mM $MgCl_2$, 1 mM DTT, and 0.1 mM EDTA. Reactions were incubated 12 hrs at 4°C, spotted on Millipore HAWP 0.45 μm nitrocellulose filters, and washed twice with 1-ml aliquots of ice-cold buffer. Blanks containing equivalent amounts of radiolabeled probe and no subunits were used as controls. The radioactivity retained by the filters was determined by liquid scintillation. The amount of probe bound to subunits was determined by subtracting the radioactivity retained on the blank filters from that retained on the corresponding reaction filters containing subunits.

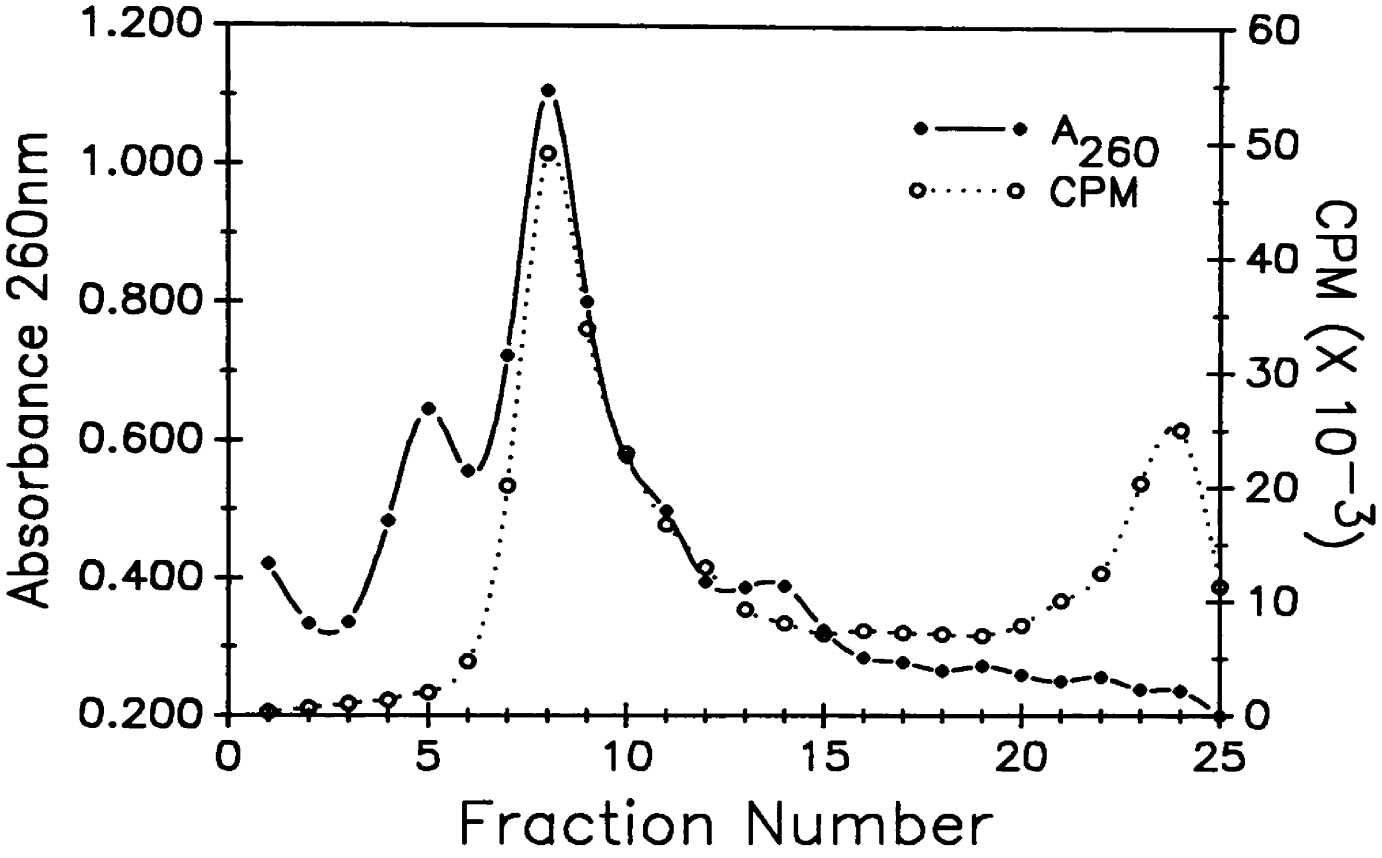


Figure 9. Gradient binding assay of DNA probe 23S(2654-2667) and 50S subunits in alpha buffer. Reactions containing 2 A_{260} units (approximately 140 μg) of 50S subunits and radiolabeled probe (300,000 cpm) in 50 μl of alpha buffer (20 mM Tris-HCl, pH 7.4; 600 mM NH_4Cl; 1 mM $MgCl_2$; 1 mM DTT; 0.1 mM EDTA) were incubated 4 hr at 4°C. Reaction mixtures were layered onto 11 ml 15-30% sucrose gradients and centrifuged in a Beckman SW41 rotor 12 hr at 37,000 rpm, 4°C. Fractions were taken from the bottom of each tube. Each fraction was assayed for subunit migration by measuring absorbance at 260 nm and for probe migration by liquid scintillation. Sedimentation is from right to left.

50 to 200 mM in 50 mM increments and magnesium chloride concentrations were varied from 5 to 15 mM in 5 mM increments. None of the twelve possible monovalent/divalent cation ratios influenced probe binding.

At this point, a buffer containing 10 mM Tris-HCl (pH 7.4), 100 mM KCl and 5 mM $MgCl_2$ (hereafter denoted as binding buffer) was chosen for subsequent hybridization assays on the basis of its ability to promote reproducible binding of a probe previously shown to hybridize to the C_{1400} region of 16S RNA (Tassanakajohn, 1987). Probe hybridization as a function of reaction time and temperature was also assayed. Binding was not observed after incubation at 0°C for as long as 16 hours, at 4°C for as long as 36 hours, at 10°C for 12 hours, or at 37°C for as long as 4 hours. Probe binding as high as 33% was measured after after incubation for 30 min at 51°C, but these results were discounted due to the subunit unfolding observed at this temperature (Figure 5B).

Gradient binding assays confirmed the inability of 50S subunits to bind the alpha-sarcin probe: only an insignificant amount of radioactivity (< 1% of that observed in Figure 9) cosedimented with intact 50S subunits in sucrose density gradients.

Other Probes

The accessibility of subsets of the alpha sarcin region to hexameric DNA probes was also tested in nitrocellulose

filter binding assays (see Table II). These assays were performed with salt-washed 50S subunits to eliminate the possibility that residual factors or tRNA would interfere with probe hybridization. Four probes were designed for complementarity to overlapping sequences within the α-sarcin region. If any portion of the region was accessible to probes, it could be identified easily. Probes 23S(2653-2658) and 23S(2656-2661), which together cover the 3' half of the single-stranded loop (see Figure 3), were unable to bind to the subunit. Probes 23S(2659-2664) and 23S(2662-2667) bound to the subunit at very low levels of 7.5% and 6.2%, respectively.

Probe Binding to 70S Ribosomes

Probe binding was also assayed for probe 23S(2654-2667) and tight-couple 70S ribosomes to test the possibility that subunit association could induce hybridization. The results of those experiments, summarized in Table III, clearly show the large probe was able to bind at low levels to the 70S ribosome, while there was no significant change in hybridization of the smaller probes. As with 50S subunits, the probes complementary to the 5' half of the single-stranded loop were able to bind weakly to the ribosome, while the two hexamers complementary to the 3' side were not.

Probe Binding in the Presence of Poly (U) and $tRNA^{phe}$

As a final hybridization experiment, the ability of

TABLE II.
Probe Binding to 50S Subunits and 70S Ribosomes

Probe	Complementary Sequence (5'-3')	Percent Binding sw50S	TC70S
23S(2654-2667)	UAGUACGAGAGGACC	0	14.5%
23S(2653-2658)	UAGUAC	0	0
23S(2656-2661)	UACGAG	0	0
23S(2659-2664)	GAGAGG	7.5%	7.0%
23S(2662-2667)	AGGACC	6.2%	10.8%

Experimental conditions were as described in Materials and Methods.

probe 23S(2654-2667) to bind 50S subunits in the presence of poly (U), $tRNA^{phe}$, or both was assayed. The assay was designed to test for accessibility of the α-sarcin region to a DNA probe with tr· sfer RNA in place at the ribosomal P site. Conditions were chosen which have been shown previously to promote poly (U)-directed P site binding of deacylated tRNA (Lill et al., 1984; Rheinberger et al., 1981; Gnirke & Nierhaus, 1986; Rheinberger & Nierhaus, 1980; Lill et al., 1986) and thus simulate a ribosomal translation initiation complex. As shown in Table III, the probe bound to the 50S subunit when deacylated $tRNA^{phe}$ was bound, but not in the presence of poly (U). P-site bound tRNA had no effect on probe binding to the 70S ribosome. Unexpectedly, poly (U) inhibited probe binding to the ribosome, both in the presence and absence of tRNA.

RNase H Assays

RNase H was used to demonstrate that the probe was binding specifically to its target site. If the probe hybridized specifically at the α-sarcin region, RNase H would cleave a 240-base 3' fragment from 23S RNA. The cleavage products from an RNase H digest of probe-alpha particle complex is shown in Figure 10. The additional fragment in Lane 3 shows that 23S RNA is cleaved by RNase H when the alpha particle is coincubated with probe, demonstrating that the probe binds to 23S rRNA. The new fragment was also the same size as the 240-base fragment generated by

TABLE III.
Probe 23S(2654-2667) Binding in the Presence of Poly (U) and $tRNA^{phe}$

	poly(U)	$tRNA^{phe}$	Percent Binding
30S subunits	-	-	13.6
50S Subunits	-	-	0
	+	-	0
	-	+	5.6
	+	+	0
70S Ribosomes	-	-	14.5
	+	-	0
	-	+	12.2
	+	+	0

Reaction mixtures contained 25 pmol 50S subunits or 70S ribosomes, increasing amounts of radiolabeled probe, and either 10 μg poly(U), 50 pmol $tRNA^{phe}$, or 10 μg poly(U) and 50 pmol $tRNA^{phe}$ in 50 μl of tRNA binding buffer (10 mM Tris-HCl, pH 7.4; 150 mM KCl; 15 mM $MgCl_2$; 1 mM DTT). Samples were incubated 6 hr at 4°C , diluted to 1 ml with ice-cold tRNA binding buffer, and filtered through Millipore 0.45 μm nitrocellulose filters. Radioactivity retained by the filter was determined by liquid scintillation counting.

an α-sarcin digest of 50S subunits (Lane 4), suggesting specific binding at the target site.

RNase H assays were also performed using intact 50S subunits incubated with each of the five probes, and using 70S ribosomes or 30S subunits incubated with probe 23S(2654-2667). No RNA fragments were produced in any of these reactions.

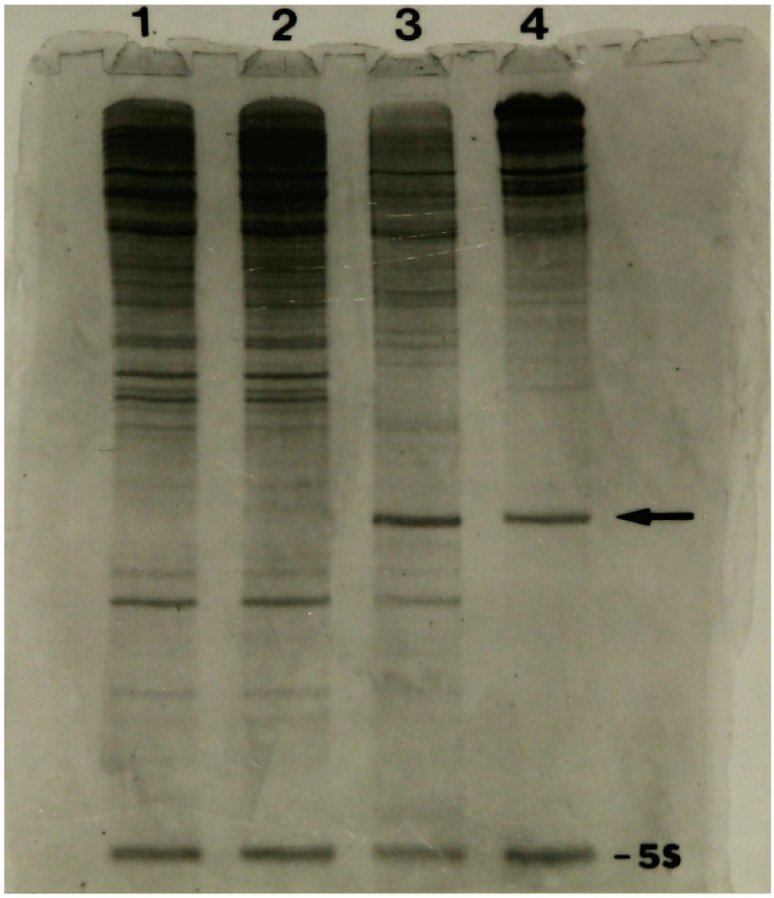


Figure 10. RNase H digestion of probe-alpha particle complex. Reactions containing 25 μg ribosomal subunits, 2.5 μg cDNA probe and 3-5 units of RNase H (P-L Biochemicals) in 20 μl of 40 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 60 mM KCl and 1 mM DTT were incubated at 4°C for 24 hr. RNA was purified by extracting subunit samples three times with buffer-equilibrated phenol, precipitating with 2.5 volumes 95% ethanol, pelleting by centrifugation. Samples were electrophoresed in a 5% polyacrylamide gel (12.5 cm X 13.5 cm X 1.5 mm) containing 7M urea and TBE at 12.5 mA for 4 hr at room temperature. RNA products were stained with methylene blue. Lane 1: control alpha particles; Lane 2: alpha particle + probe; Lane 3: alpha particle + probe + RNase H; Lane 4: α-sarcin digest of 50S subunit. The arrow indicates the alpha fragment.

CHAPTER IV

DISCUSSION

The goal of this study was to probe the structure and function of the α-sarcin region (nucleotides 2653-2667) of E. coli 23S rRNA in situ with complementary oligodeoxyribonucleotides. DNA hybridization was measured in nitrocellulose filter binding assays, sucrose gradient binding assays, and with RNase H. Surprisingly, those binding assays clearly demonstrated that the 50S ribosomal subunit has little or no affinity for any of the site-specific probes tested.

The first probe, a 14-mer complementary to bases 2654 through 2667, did not bind to 50S ribosomal subunits or to salt-washed 50S subunits in any of several buffers, even though the ionic conditions of those buffers are similar to those of buffers used in previous cDNA probing studies of 30S or 50S subunits (Tapprich & Hill, 1986; Backendorf et al., 1981; Marconi & Hill, 1988). Further hybridization reactions utilizing this probe, incubated at varied temperatures and varied periods of time, failed to induce probe binding. The inability to detect more than trace probe binding in sucrose gradient binding assays or to generate RNA fragments with RNase H suggests the α-sarcin site is not accessible to a 14-base DNA probe in the intact 50S subunit.

Hexameric subsets of the region were subsequently assayed for probe binding. The results of filter binding and sucrose gradient binding assays suggested the nucleotides on the 5' terminus of the α-sarcin sequence were also unavailable for cDNA hybridization. Bases 2659 to 2667 are possibly exposed to such probes, since a low level of binding was detected for oligomers complementary to that portion of the α-sarcin sequence. It is impossible to determine if this is actually the case, since no RNase H fragment was produced with either of these probes bound.

The 70S ribosome was probed next to test the accessibility of the 15-base sequence to the same set of probes, and the results are summarized in Table II. The 14-mer 23S(2654-2667) was observed to bind an average of 14.5% of the ribosomes, while the four hexamers bound at essentially the same levels as with the 50S subunits. As with the 50S subunits, binding specificity could not be determined, since RNase H did not cleave 23S rRNA in the presence of any of the five probes.

To test the possibility of a conformational change in the α-sarcin region, a simulated initiation complex was formed *in vitro* and probed with the 14-base probe. The synthetic mRNA poly (U) and deacylated $tRNA^{phe}$ were bound nonenzymatically to tight-couple 70S ribosomes in conditions previously shown to promote P site saturation (Lill *et al.*, 1984; Rheinberger *et al.*, 1981; Gnirke & Nierhaus, 1986;

Rheinberger & Nierhaus, 1980; Lill et al., 1986; Moazed et al., 1986). As summarized in Table III, the 50S subunit was unable to bind the probe in the presence of poly (U) or poly (U) plus tRNA, and bound only a small amount of probe in the presence of $tRNA^{phe}$. Probe binding to 70S ribosomes was essentially unchanged when only the tRNA was present. Surprisingly, poly (U) had a definite inhibitory effect on probe binding. An explanation for this observation cannot be offered on the basis of these results, since it was impossible to establish binding specificity.

In summary, apparent lack of probe binding to 50S subunits, 70S ribosomes, 50S-poly (U)-tRNA complexes or 70S-poly (U)-tRNA complexes demonstrates that the α-sarcin region is not accessible to DNA oligomers. This observation is somewhat surprising when one considers the action of α-sarcin on 50S subunits or 70S ribosomes. Alpha-sarcin is able to cleave 23S rRNA at a site that is seemingly inaccessible to a DNA probe that is only one-fourth its molar mass. No data concerning the mechanism of α-sarcin cleavage are yet available, so any explanation of the observed discrepancies would be purely speculative. There are several possible explanations for the lack of probe binding, including protection of the site by a 50S subunit protein(s), protection by an unknown protein factor, or involvement of the single-stranded sequence in tertiary interactions with other single-stranded regions within the subunit. A similar lack

of evidence about the α-sarcin region precludes an unambiguous answer. To date, no ribosomal proteins have been located at the α-sarcin region, and no tertiary interactions involving the region have been identified.

Results of the hybridization experiments in this study invalidate the reasons for the ribosomal collapse as described by Henderson and Lake (1985). Reportedly, hybridization of a 14-base DNA probe complementary to the α-sarcin region of 23S rRNA triggers collapse of the 50S subunit (Henderson & Lake, 1985). However, hybridization reactions in that study were performed in a high salt, low magnesium buffer at 37°C or 51°C. These conditions are unusual when compared to buffers and incubation conditions used in other cDNA hybridization studies (Tapprich & Hill, 1986; Backendorf et al., 1981; Marconi & Hill, 1988). In this study, the α-sarcin region of 23S rRNA was probed with cDNA oligomers in differing buffers and temperatures and the resulting probe-subunit complexes were characterized by physical methods. The results show that the ribosomal collapse was not caused by hybridization of a 14-base DNA probe complementary to the α-sarcin region, but by the ionic and thermal conditions utilized in the earlier study. The result was the formation of the alpha particle, a somewhat unfolded 50S subunit.

Although the 14-base probe was unable to bind the intact 50S ribosomal subunit, the α-sarcin region was

available to the probe in the unfolded alpha particle. Gradient binding assays (Fig. 9) demonstrate that the probe can bind only to the alpha particle and RNase H assays confirm that RNase H, which degrades RNA in DNA-RNA heteroduplexes (Donis-Keller, 1979), cleaved 23S rRNA when incubated with a probe-alpha particle reaction mixture (Fig. 10). The size of the RNA fragment generated by RNase H cleavage confirmed that the probe binds to 23S rRNA since its size matched the size of the alpha fragment, suggesting that the probe bound specifically at the α-sarcin site. These results indicate that the probe apparently does not hybridize to the α-sarcin region in the intact 50S subunit, but binds specifically to its target site in the alpha particle after the subunit unfolds.

The sedimentation velocity data shown in Figure 5 demonstrate alpha buffer was directly responsible for disruption of subunit structure. This result is consistent with those from early studies of the physical characteristics of ribosomes at various magnesium ion concentrations (Gesteland, 1966; Weller et al., 1968; Miall & Walker, 1969; Weller & Horowitz, 1964; Cammack & Wade, 1965). In those studies, the stability of 50S ribosomal subunits decreased in buffers containing less than 2.5 mM Mg^{2+}, especially when the monovalent/divalent cation ratio is greater than 60.

In low-magnesium buffers, the subunit unfolded into several intermediates ranging from 16S to 45S without

suffering any rRNA degradation or loss of ribosomal material. The unfolding process generally occurred over extended periods of time, but was accelerated at elevated temperatures (Weller & Horowitz, 1964). High concentrations of monovalent cations, particularly ammonium ion, also affect the physical characteristics of the ribosome. The sedimentation coefficient of the 50S subunit has been observed to decrease to 45S in 0.5 M NH_4Cl at nominal Mg^{2+} concentrations with no apparent shape change of the subunit or a loss of subunit material (Hill et al., 1970). The only change in the subunit was an increase in the partial specific volume of the particle. A similar decrease in sedimentation coefficient seen here suggests an increase in partial specific volume as well.

The presence of the probe in these ionic conditions had no further effect on the physical characteristics of the alpha particle. The results reported above do not necessarily preclude the possibility that probe hybridization at the α-sarcin region could aid subunit unfolding in alpha buffer. This possibility was tested by QLS and sedimentation velocity experiments. The schlieren patterns in Figure 6 demonstrate that the sedimentation behavior of 50S subunits in binding buffer did not change in the presence of probe and no further subunit degradation was observed when the probe was incubated with alpha particles in alpha buffer. Similarly, the diffusion coefficient of subunits in binding

buffer was unaffected by the presence of probe. Unexpectedly, the diffusion coefficients of subunits in alpha buffer increased slightly when probe was added, the opposite effect one would expect if the probe actually triggers ribosomal collapse. An explanation for this observation cannot be provided on the basis of these data.

Because the probe reportedly triggered 23S rRNA degradation (Henderson & Lake, 1985), the RNA from the alpha particles was analyzed and compared to RNA from 50S subunits. The RNA samples in Figure 7 clearly show neither the DNA probe nor elevated temperature could disrupt 23S RNA primary structure. The 23S RNA from 50S subunits incubated with probe in alpha buffer at elevated temperatures was also unaffected.

In light of the results presented here, the ionic conditions of alpha buffer and elevated temperature were probably the cause of the reported subunit collapse. The results of this study indicate that upon heating in alpha buffer the 50S subunits unfold and undergo degradation as well.

The importance of the α-sarcin region for ribosome function cannot be overstated. Unfortunately, the results of this study do not provide any insight into the function of the α-sarcin region. Ribosomes are able to translate the N-terminal portion of the bacteriophage R17 coat protein gene after unspecific digestion of 23S rRNA with pancreatic

ribonuclease (Kuechler et al., 1972), but hydrolysis of a single phosphodiester bond at G_{2661} of 23S rRNA completely inhibits protein synthesis by preventing enzymatic A site binding during the elongation cycle (Fernandez-Puentes & Vazquez, 1977; Hobden & Cundliffe, 1978). Recently, Hausner et al. (1987) demonstrated α-sarcin specifically blocked the binding of both elongation factors EF-Tu and EF-G to the ribosome without affecting any elongation factor-independent functions, i.e. tRNA binding at both A and P sites, peptidyl transferase, and spontaneous translocation. These data support the premise that the α-sarcin region binds EF-Tu, but Hausner et al. failed to eliminate secondary effects of 23S RNA cleavage as the cause of the observed inhibition.

It was hoped that competition experiments between EF-Tu and an oligonucleotide bound specifically at the α-sarcin site would help to answer this question unambiguously. This study was begun with the premise that any ribosomal RNA sequence which functions as a binding site for a ribosomal ligand would be exposed on the surface of the subunit, and therefore be accessible to a DNA probe. As this study has demonstrated, an RNA sequence accessible to the protein α-sarcin is not accessible to a DNA probe, and therefore the premise may not necessarily be true. Assuming the α-sarcin region is indeed the binding site for EF-Tu, the protein may find its recognition site in the same way α-sarcin finds it.

If one assumes the α-sarcin region binds EF-Tu, and the

binding site must be exposed to EF-Tu before it binds, then a conformational change must occur to expose the region. The most likely stage for this occur is during elongation, particularly immediately following initiation complex formation or immediately following translocation (see Figure 4). The purpose of the binding assays in the presence of poly (U) and tRNA was to test this possibility. The results of this study neither prove nor disprove the possibility. The ribosome complex probed here is undoubtedly different from the corresponding physiological system, and must be considered only as a "first approximation". First, poly(U) and deacylated tRNA were bound nonenzymatically to the ribosome. In the physiological situation, these ligands are directed to their respective binding sites by initiation factors. Second, deacylated tRNA was chosen for this experiment because of its high affinity for the ribosomal P site (Lill et al., 1986; Gnirke & Nierhaus, 1986), but one should remember that the P site is always occupied by an aminoacyl-tRNA or a peptidyl-tRNA during translation. Third, poly (U), while able to serve as a suitable mRNA for in vitro protein biosynthesis, is not a natural messenger. The interactions between poly (U) and the ribosome may not necessarily be the same as the interactions between natural mRNAs and the ribosome. Finally, protein translation factors were missing from these experiments. Several translation functions can occur in the absence of these factors in

vitro, it is doubtful that translation occurs without them in vivo. For these reasons, the ribosomal species studied here should be considered somewhat artificial, and the results possible artifacts of the experimental protocols used in this study.

Methods have been developed for the enzymatic formation of initiation complexes (Steitz, 1981) and for purification of post-translocational ribosomes (Baranov et al., 1979). Probing studies of these complexes are absolutely necessary before comprehensive conclusions can be made about the availability and function of the α-sarcin region during translation.

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