

AN ABSTRACT OF THE THESIS OF

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Title: Accessibility of Highly Conserved RNA Sequences in
Escherichia coli 70S Ribosomes and Implications for
Developing Antibacterial Agents

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Escherichia coli 16S-like ribosomal RNAs from eubacterial, archaebacterial and eukaryotic organisms have several universally conserved sequence zones which are proposed to be single-stranded. A clear function for all of these regions has not yet been defined. To evaluate the roles of these sequence zones in protein synthesis, knowledge of their disposition in small ribosomal subunits and in intact ribosomes is essential. Here I report the results of experiments which were undertaken to quantitate the extent to which these sequences are single stranded and exposed in 30S subunits and 70S ribosomes from Escherichia coli D-10 cells. The approach was based upon the protection from nuclease S1 digestion of short synthetic DNA oligomers hybridized to target sequences. In

Escherichia coli 16S rRNA nucleotides 520-530, 1396-1404, 1493-1504 and 1533-1542 were all found to be single stranded and stoichiometrically unrestricted for hybridization to complementary short (9-11 nucleotide) DNA oligomers. The disposition of the sequences in 30S subunits and 70S ribosomes varied with each position.

To test the hypothesis that blockage of the normally open conserved sequences were essential for inhibition of protein synthesis, a set of novel synthetic short DNA analogs (3-6 nucleotides) and normal DNA (10 and 11 nucleotides) complementary to the regions 520-530 and between nucleotides 1533-1542 in 16S rRNA were tested for their ability to inhibit protein synthesis. The inhibitory activity of the DNAs were assessed in vitro and in vivo. Oligonucleotide analogs centered about position 1536-1539 inhibited protein synthesis in vitro and also inhibited colony formation by permeable and nonpermeable E. coli cells.

Accessibility of Highly Conserved RNA Sequences of
Escherichia coli 70S Ribosomes and Implications
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DEDICATION

I would like to dedicate this thesis to my wonderful parents for their sacrifice and generosity in giving me the opportunity to advance to higher studies. This work is further dedicated to my uncle Mohammad Abul Hassan, for giving me a love of learning in my first knowledge of the alphabet.

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TABLE OF CONTENTS

	Page
1. INTRODUCTION.....	1
2. LITERATURE REVIEW.....	5
2.1 MANUSCRIPT I: Nuclease S1 Mapping of 16S rRNA in Ribosomes.....	5
2.1.1 The Ribosome.....	5
2.1.2 Morphology of Ribosomes.....	10
2.1.3 Ribosome Structure and Function.....	11
2.1.4 Protein synthesis.....	14
Initiation.....	15
Elongation.....	17
Termination.....	17
2.1.5 The 30S subunit and the 16S rRNA of E. coli.....	20
2.1.6 Secondary structure of the E. coli 16S rRNA.....	23
2.1.7 The 'Domain' organization of small subunit rRNAs.....	25
2.1.8 Function of rRNA.....	28
2.1.9 New Approaches.....	29
2.2 MANUSCRIPT II: Inhibition of Translation and Antibacterial Activity by New Short DNA Analogs.....	32
2.3 REFERENCES.....	37
3. Manuscript I: Nuclease S1 Mapping of 16S Ribosomal RNA in Ribosomes.....	46
3.1 ABSTRACT.....	47
3.2 INTRODUCTION.....	48
3.3 MATERIALS AND METHODS.....	51
3.3.1 Preparation of 70S ribosomes, 30S subunits and 16S rRNA.....	51
3.3.2 Synthesis and labeling of DNA oligomer probes.....	51
3.3.3 3' end labeling of 16S rRNA.....	52
3.3.4 RNA-DNA hybridization and RNase H cleavage.....	53

3.3.5	Hybridization of DNA probes to 16S rRNA, 30S subunits, 70S ribosomes and digestion with nuclease S1.....	53
3.4	RESULTS.....	55
3.4.1	Screening for potential hybrids.....	55
3.4.2	DNA binding to 16S rRNA.....	55
3.4.3	RNase H cleavage pattern.....	68
3.4.4	Protection of DNA oligonucleotide probes in the presence of 16S rRNA, 30S subunits and 70S ribosomes.....	71
3.5	DISCUSSION.....	78
3.6	REFERENCES.....	83
4.	Manuscript II: Inhibition of Translation and Antibacterial Activity by new Short DNA Analogs.....	88
4.1	ABSTRACT.....	89
4.2	INTRODUCTION.....	90
4.3	MATERIALS AND METHODS.....	94
4.3.1	The Neu-genes.....	94
4.3.2	DNA oligomers with normal phosphodiester bonds.....	94
4.3.3	Cells.....	99
4.3.4	Other chemicals and biochemicals.....	99
4.3.5	In vitro translation assay.....	99
4.3.6	Test for colony formation.....	101
4.4	RESULTS.....	103
4.4.1	In vitro translation.....	103
4.4.2	In vivo evaluation using permeable cells.....	107
4.4.3	In vivo evaluation using nonpermeable cells.....	116
4.5	DISCUSSION.....	120
4.6	REFERENCES.....	125
5.	BIBLIOGRAPHY.....	127

LIST OF FIGURES

		Page
Figure 2-1	A sketch of the prokaryotic ribosome....	7
Figure 2-2	Composition of the prokaryotic ribosome.....	9
Figure 2-3	A sketch of the 30S subunit, 50S subunit and 70S ribosome as determined by electron microscopy.....	12
Figure 2-4	Initiation of protein synthesis.....	16
Figure 2-5	Elongation of protein synthesis.....	18
Figure 2-6	Peptide bond formation and translocation.....	19
Figure 2-7	Termination of protein synthesis.....	21
Figure 2-8	Secondary structure model of <u>E. coli</u> 16S rRNA.....	26
Figure 3-1	Nuclease S1 activity on free DNA probe 520.....	59
Figure 3-2	Saturation hybridization of DNA 520 with 16S rRNA.....	61
Figure 3-3	Saturation curve of DNA 520 and 1396 binding to 16S rRNA.....	63
	a) Saturation curve of DNA 520.....	64
	b) Scatchard plot for DNA 520.....	65
	c) Saturation curve of DNA 1396.....	66
	d) Scatchard plot for DNA 1396.....	67
Figure 3-4	Nuclease S1 digestion of control DNA 1475.....	69
Figure 3-5	RNase H cleavage of 16S rRNA in the presence of DNA 520.....	72
Figure 3-6	Protection of region-specific DNA probes from nuclease S1 digestion in the presence of ribosomal components....	74
Figure 4-1	Internucleoside linkage with methylphosphonate bonding.....	95

Figure 4-2	Methyl carbamate internucleoside linkage of neu-genes.....	96
Figure 4-3	Neu-gene cGcGcAcG and PEG ₁₀₀₀ bonded to the 5' end.....	97
Figure 4-4	Effect of neu-genes on <u>in vitro</u> translation.....	104
Figure 4-5	Alignment of some neu-genes with the antiSD sequence of 16S rRNA and their effect on <u>in vitro</u> translation.....	106
Figure 4-6	Effect of neu-genes on colony formation by the permeable <u>E. coli</u> ML308-225 cells.....	114

LIST OF TABLES

	Page
Table 3-1 Synthetic DNAs.....	56
Table 3-2 Potential hybrids between DNA 520 and 16S rRNA.....	57
Table 3-3 Binding stoichiometry of DNAs complementary to 16S rRNA.....	77
Table 4-1 The neu genes with their code designation and sequences.....	98
Table 4-2 DNA oligomers with normal phosphodiester bonds.....	100

LIST OF PLATE SETS

	Page
Plate set 4-1 Effect of DNA 520 and 1533 on colony formation by the permeable E. coli ML308-225 cells.....	108
Plate set 4-2 Effect of neu-genes on colony formation by the permeable E. coli ML308-225 cells.....	111
Plate set 4-3 Inhibition of colony formation by E. coli D-10 cells in presence of neu-genes.....	117

Accessibility of Highly Conserved RNA Sequences of
Escherichia coli 70S Ribosomes and Implications
for Developing Antibacterial Agents

1. INTRODUCTION

Protein synthesis in all cellular systems is carried out within large macromolecular complexes called ribosomes. They catalyze the formation of polypeptide chains from activated amino acid residues. The polymerization of amino acids into polypeptides is directed by an mRNA, but dependent upon over 100 macromolecules half of which are ribosomal.

Ribosomes consist of ribonucleic acids (RNA) and proteins organized in a three dimensional structure through very specific RNA-RNA and RNA-protein interactions. Ribosomal RNAs appears to be directly involved in the process of protein synthesis (Brimacombe and Stiege, 1985; Dahlberg, 1989; Noller, 1984). They do more than provide a scaffolding upon which proteins are properly arranged for carrying out functions. Sequence comparison of rRNAs from different species has revealed that there are regions of rRNAs which are universally conserved in both prokaryotic and eukaryotic organisms. For example, the sequence regions 517-533, 1394-1408 and 1492-1506 of Escherichia coli (E. coli) 16S rRNA are essentially identical in bacteria and human cells. The zone 1533-1542 is highly

conserved in all prokaryotic organisms (Gutell et al., 1985), but not in eukaryotes. Of the highly conserved portions of 16S rRNA the region 1394-1408 is believed to be involved in binding transfer RNA (tRNA) (Prince et al., 1982; Ofengand et al., 1986). tRNA carries the charged amino acids into the ribosome during protein synthesis (translation). In 16S rRNA the extreme 3' end region 1533-1542 is believed to interact with mRNA during initiation and elongation phases of protein synthesis (Weiss et al., 1988). However, at this point a complete assessment of the function of all of these conserved sequences has not been completed. Information regarding the spatial disposition of the universal zones in ribosomes is minimal. No single method so far has been used to probe the disposition of these sequence zones in ribosomes or their subunits.

Manuscript I describes the results of experiments designed to provide quantitative data on the accessibility of any single stranded sequence in ribosomes in their native state. The method used was based upon hybridization of short synthetic DNA oligomers complementary to the regions of interest and the subsequent protection of the hybridized DNA from nuclease S1 digestion. I found that the regions 520-530, 1396-1404, 1493-1504 and 1533-1542 are single stranded in E. coli 16S rRNA and nearly so in 30S subunits. However in 70S ribosomes, sequences 520-530 and 1396-1404 are open in about one half of the ribosome

population. The sequences 1493-1504 and 1533-1542 are accessible in only about 30% of the 70S ribosomes.

Knowing the state of the conserved sequences raised the possibility of using DNA probes as antibiotics. For example, the 3' end region sequence CCUCC (position 1535-1539) is highly conserved in prokaryotic 16S rRNAs, but not in eukaryotes. It is generally accepted that the 3' end sequence CCUCC of 16S rRNA interacts with a complementary sequence in prokaryotic mRNAs during initiation and elongation phases of protein synthesis. Therefore, blocking this region with a complementary DNA should interfere with mRNA binding and inhibit protein synthesis.

DNA oligomers containing normal phosphodiester bonds are unsuitable as antibacterial agents because they do not easily pass through bacterial cell membranes due to their ionic character. They are also readily degraded by bacterial nucleases. Several workers have tested DNA analogs of various forms, but none have been able to create a satisfactory antibiotic based upon the use of DNA probes. Antivirals, Inc. of Corvallis, Oregon has constructed DNA analogs of three to six nucleotides in which the normal phosphodiester bonds are replaced by methyl carbamate internucleotide linkages. These analogs are stereoregular in spatial organization and uncharged. These were named neutral genes or 'neu-genes' by Antivirals, Inc. and have characteristics which makes them potential candidates for

antibiotics.

As described in manuscript II, several of the neu-genes complementary to the ACCUCC sequence of E. coli 16S rRNA were tested for their capacity to inhibit in vitro protein synthesis and colony formation by E. coli cells. Neu-genes cGcGcAcG, cAcGcG effectively inhibited in vitro protein synthesis and colony formation by a permeable E. coli strain ML308-225. When polyethylene glycol (MW 1000) was attached to the 5' end to enhance solubility, they did not inhibit colony formation by the permeable strain, but inhibited colony formation by the nonpermeable E. coli D-10 cells.

2. LITERATURE REVIEW

2.1. MANUSCRIPT I: Nuclease S1 Mapping of 16S rRNA in Ribosomes

2.1.1 The Ribosome

Ribosomes are complex, subcellular particles composed of RNA and proteins. They are found in the cytoplasm of all cells (Petermann and Hamilton, 1957; Schachman et al., 1952; Tissieres and Watson, 1958). It is within the ribosome that peptide bonds are formed and the primary structural assembly of proteins takes place (Gierer, 1963; Littlefield et al., 1955; McQuillen et al., 1959; Yonath and Wittmann, 1989). There is an emerging perception of the ribosome as an 'enzyme', directed by mRNAs, that catalyzes peptide bond formation using aminoacyl-tRNAs as substrates (Capel et al., 1988).

Ribosomes are composed of two unequal subunits in all organisms. In both prokaryotic and eukaryotic cells they are found in the cytoplasm or in association with organelle membranes such as the endoplasmic reticulum. In eukaryotic cells, ribosomes are also found in mitochondria and chloroplasts. These ribosomes frequently manifest a lower sedimentation rate and are less complex than those found outside of the organelle (reviewed in Spirin, 1986). Although associated with a eukaryotic cell they have a

strong resemblance to prokaryotic ribosomes. Organelles make proteins which function exclusively within the organelle (reviewed in Brimacombe et al., 1978; Nomura, 1973; Wittmann, 1983). Ribosomes from E. coli are representative of prokaryotic ribosomes. Eukaryotic ribosomes are organized similarly but have more and larger molecular components (Wool, 1986). Figure 2-1 depicts a sketch of a prokaryotic ribosome.

Ribosomes play a unique role in the growth and metabolism of a cell because protein synthesis is a prerequisite for sustaining other cellular metabolic activities. The Cellular ribosome concentration rises in direct proportion to the rate of growth (Gausling, 1977). In rapidly growing E. coli, a major portion of cellular activity is directed at ribosome biosynthesis, to a point at which ribosomes constitute about 50% of the cell mass (reviewed in Nomura et al., 1984).

As indicated above, ribosomes are particles composed of RNA and protein. An individual ribosome is assembled from a specific set of ribosomal proteins (r proteins) and ribosomal RNA (rRNA). The individual r protein interacts with specific regions of rRNA and other ribosomal proteins during the assembly process (Cowgill and Schaup, 1979; Gregory et al., 1988; Schaup et al., 1971; Stern et al., 1988). In vitro reconstitution experiments have shown that ribosomes are capable of self assembly through specific protein-RNA and protein-protein interactions (Schaup et

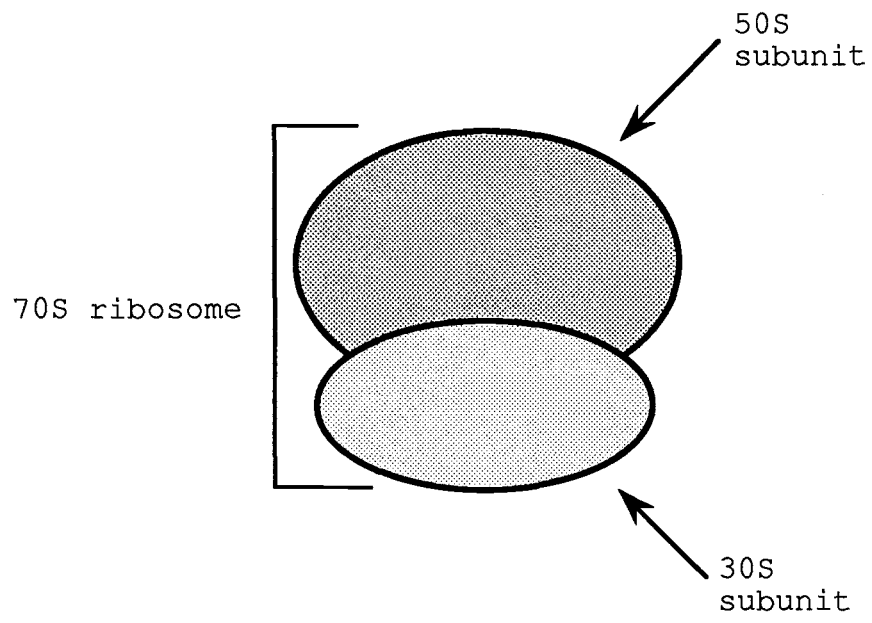


Figure 2-1 A sketch of the prokaryotic ribosome.

al., 1970; Traub and Nomura, 1968). During biosynthesis, rRNAs and r-proteins are synthesized in the stoichiometric amounts required for the assembly of the ribosome (Lindahl and Zengel, 1986; Nomura et al., 1984). The synthesis rate of rRNA usually determines the synthesis rate of ribosomal proteins. Ribosomal RNA synthesis is in turn feedback regulated by other components associated with protein synthesis, such as Initiation Factor IF-2 in E. coli (Cole et al., 1987).

The composition of the prokaryotic ribosomes is shown in Figure 2-2. It has a molecular weight of about 2.7×10^6 daltons and sediment at 70S. The 70S particle can be dissociated into a 30S particle of molecular weight 0.9×10^6 daltons, and a 50S particle of molecular weight 1.8×10^6 daltons (Gesteland, 1966; Tissieres and Watson, 1958; Wittmann-Liebold, 1986). The eukaryotic ribosome has molecular weight of 4.0 million daltons and typically has a sedimentation coefficient near 80S. The two subunits have sedimentation coefficients of about 40S and 60S. With some exceptions, the basic principles and mechanics of protein synthesis are common in both prokaryotes and in eukaryotes.

The small subunit (30S) of E. coli ribosomes contains one RNA which has a sedimentation coefficient of 16S (hence it is called 16S rRNA) and 21 different proteins (numbered S1 to S21 where the acronym denotes the small subunit) (Brimacombe et al., 1976; Kaltschmidt and Wittmann, 1970;

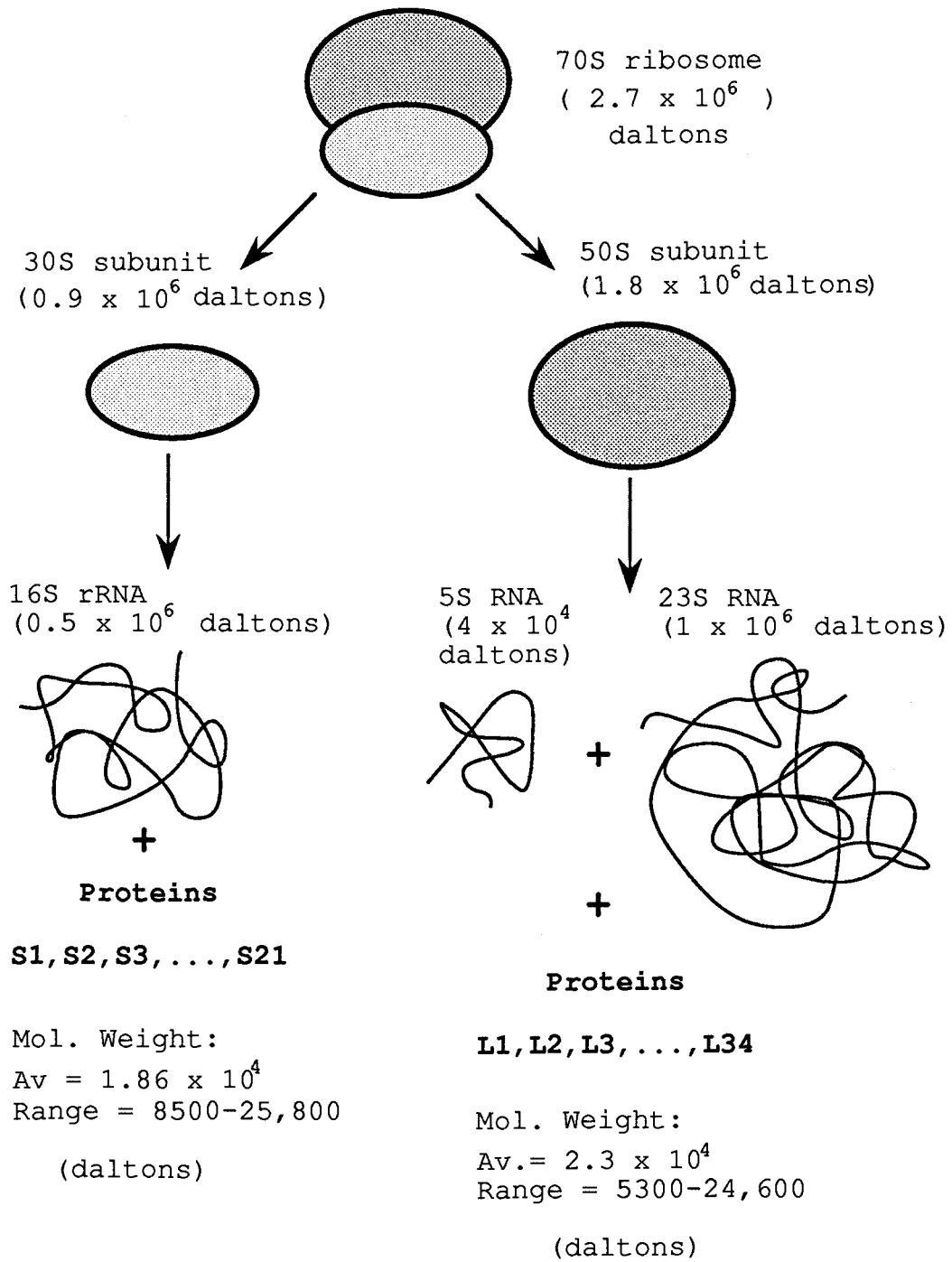


Figure 2-2 Composition of the prokaryotic ribosome.

Wittmann, 1982). The proteins are of varying molecular weights. The E. coli 50S subunit contains 34 different proteins (numbered L1 to L34, where L denotes the large subunit) and two rRNAs namely the 23S and 5S rRNAs (Kaltschmidt and Wittmann, 1970; Wittmann, 1982). One protein occurs both in the small subunit (S20) and in the large subunit (L26) when the intact ribosome dissociates into two subunits. The large subunit contains another protein which is present in four copies per large subunit but gives rise to two spots (L7/L12) in gel electrophoretic analysis, because its amino terminal end is partly acetylated.

The eukaryotic small subunit contains one rRNA called 18S rRNA and the large subunit contains three rRNAs, namely the 23S, 5.8S and 5S RNAs. The protein composition of eukaryotic ribosomes is presently not very well known; in general they contain more proteins of higher molecular weight than the bacterial ribosomes.

2.1.2 Morphology of Ribosomes

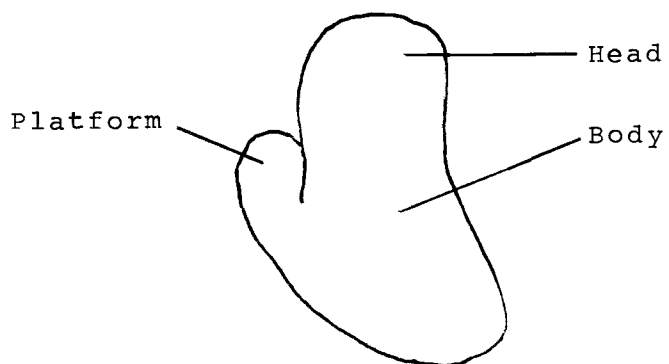
Advances have been made in elucidating the physical characteristics of ribosomal subunits. Both low and high resolution electron microscopy have been used to yield a consensus structure of the particles (Lake, 1976; Lake, 1985; Moore, 1988). More recently crystals of ribosomes have been obtained and analyzed by X-ray crystallographic

methods (Yonath, 1984; Yonath and Wittmann, 1989). Ribosomes from different organisms and cells have very similar appearance (Boublik and Hellmann, 1978; Lake et al., 1982). The individual subunits appear to be both irregular and asymmetric in shape. Figure 2-3 shows a sketch E. coli ribosomal subunits as they appear when analyzed by low resolution electron microscopy (Lake, 1985). The small subunit appears to be organized into a 'head', a 'body' and a 'platform' (side bulge). The large subunit appears to have three peripheral protuberances. The one in the center is called the 'head' or the central protuberance. The lateral fingerlike protuberance is called the L7/L12 'stalk' and the protuberance located on the other side of the central protuberance is called the 'ridge' or the side lobe. The large subunit has similar shape in prokaryotic and eukaryotic ribosomes (Boublik and Hellmann, 1987; Lake, 1985).

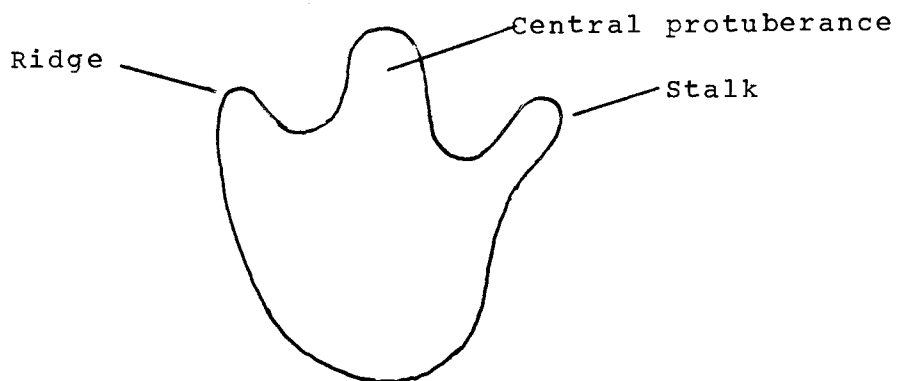
A major effort will be required to map with certainty the features of a fully active ribosome engaged in protein synthesis. It is worth noting that the morphology of ribosomes as determined by the X-ray crystallographic methods deviates somewhat from that based upon electron microscopy (Yonath and Wittmann, 1989).

2.1.3 Ribosome Structure and Function

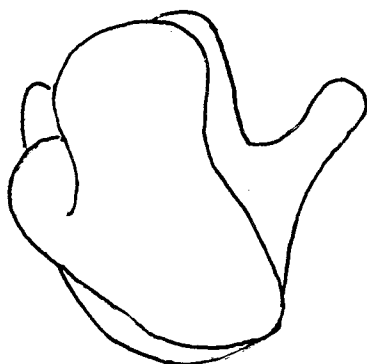
The three dimensional structure of the ribosome is



30S subunit



50S subunit



70 S ribosome

Figure 2-3 A sketch of the 30S subunit, 50S subunit and the 70S ribosome as determined by electron microscopy (from Lake, J.A., 1985).

emerging but not yet fully elucidated (Moore, 1988). The present structure-function models have not yielded sufficient data to deduce a consistent depiction of ribosome-mediated phases of protein synthesis. To grasp how the information encoded by mRNA is translated into a protein with the correct amino acid sequence, it will be necessary to probe the events which take place coordinately within ribosomes. Ribosomal components active at various phases of protein synthesis must be identified and their properties correlated with regard to their structure in ribosomes. Because early attention was focused on prokaryotic ribosomal proteins much information about their physico-chemical properties is in the literature (Engelman et al., 1975; Traut et al., 1980).

More recently information concerning the primary and secondary structure of rRNAs has become available (Brosius et al., 1978; Brosius et al., 1980; Glotz and Brimacombe, 1980; Noller and Woese, 1981; Stiegler et al., 1981a, 1981b; Woese et al., 1980; Zimmermann, 1980; Zwieb et al., 1981). Lamentably, even armed with these data it has not been possible to seize upon the detailed dynamics of protein synthesis.

Evidence that the ribosomal RNAs are important in the function of ribosomes was recently provided by Jacob et al. (1987), and Hui and de Boer (1987). Their experiments demonstrated that ribosomes with mutations at the 3' terminus of 16S rRNA translated mRNA far less efficiently

than normal ribosomes. Earlier studies involving the interactions of the Shine and Dalgarno (SD) sequence of mRNA (Shine and Dalgarno, 1974; Steitz and Jakes, 1975), tRNAs (Denman et al., 1989; Prince et al., 1982) with 16S rRNA implicated rRNA as a functional component of ribosomes. There is also a notion that ribosomes undergo structural transitions during various phases of protein biosynthesis (Noller, 1984).

The translating ribosome population is complexed with initiation, elongation and release factors as well as mRNA, tRNAs charged with amino acids, ATP, GTP etc. The intact 70S particle must dissociate into 30S and 50S subunits before initiation of translation can commence. In prokaryotic cells, ribosomes start translating mRNAs even before the completion of mRNA biosynthesis (Gold et al., 1981). This is in sharp contrast to what happens in eukaryotes where in the nucleus mRNAs are processed to remove introns (Krainer et al., 1984), packaged with proteins, and moved to the cytoplasm prior to the initiation of protein synthesis.

2.1.4 Protein synthesis

In general, protein synthesis occurs in three steps: initiation, elongation, and termination or release of the polypeptide chain. Figures 2-4, 2-5, 2-6, and 2-7 summarizes the different stages of translation in

prokaryotic systems.

Initiation

Prior to initiation the ribosome dissociates into the large and small subunits (Figure 2-4), held apart by initiation factor 3, or IF-3 (Gold et al., 1981; Guthrie and Nomura, 1968; Subramanian and Davis, 1971). The mRNA binds to the free 30S subunit in the presence of initiation factor 1 (IF-1), initiation factor 2 (IF-2), and GTP. The charged initiator tRNA (fmet-tRNA_{fmet}) binds to the AUG (or less frequently at the GUG or UUG) start codon of mRNA (Clark and Marcker, 1966; Files et al., 1974; Khorana et al., 1966; Sundararajan and Thach, 1966). The binding of mRNA is enhanced by the base-pairing interaction between the Shine and Dalgarno (SD) sequence of mRNA and the anti Shine and Dalgarno (antiSD) sequence at the 3' end of 16S rRNA (Shine and Dalgarno, 1974; Steitz, 1980). Binding of 50S subunit to the 30S initiation complex is associated with the displacement of IF-3 (reviewed in Grunberg-Manago, 1980; Weissbach, 1980). The peptidyl (P) and the aminoacyl (A) sites are generated upon formation of the 70S initiation complex. The initiator tRNA (fmet-tRNA_{fmet}) binds to the P site and the incoming aminoacyl-tRNA binds to the A site.

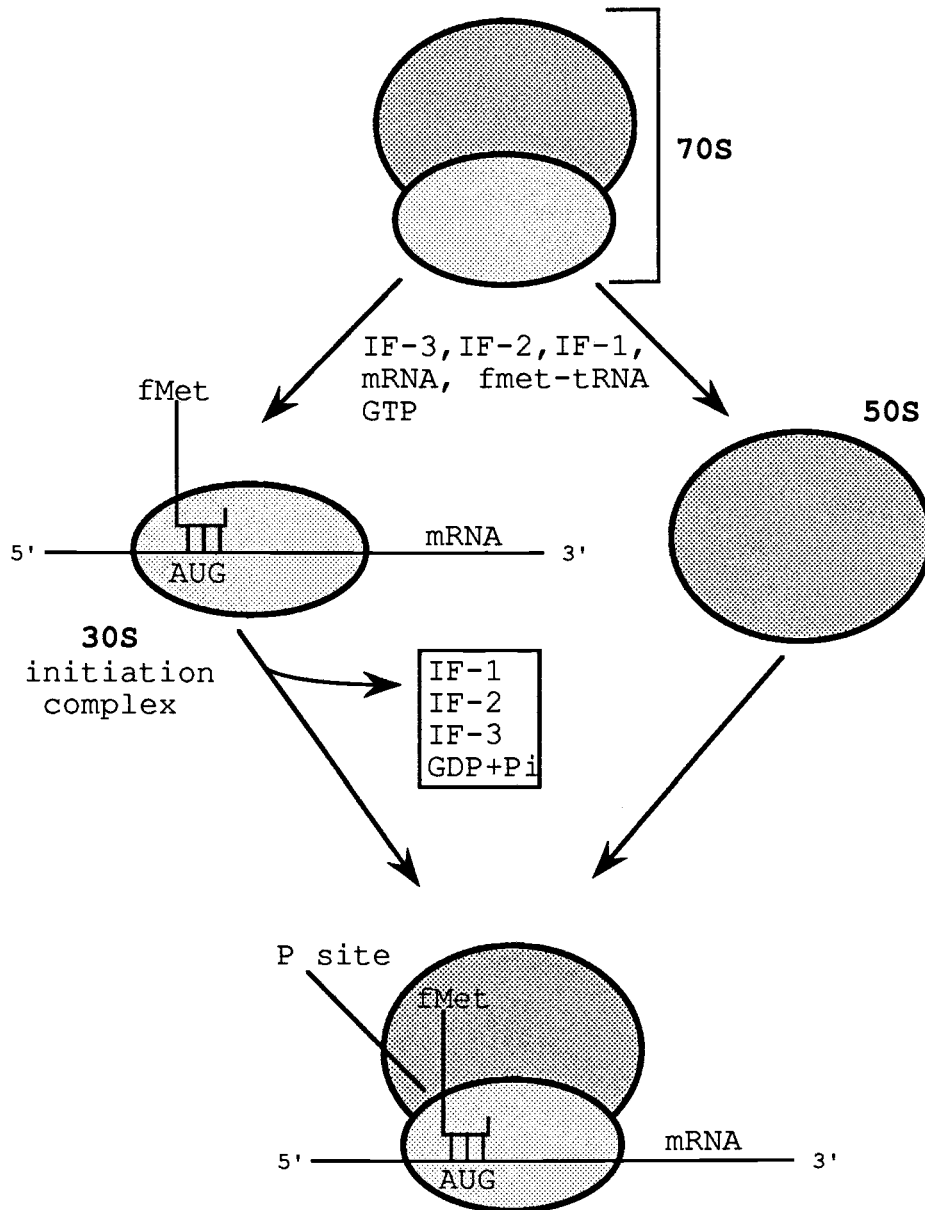


Figure 2-4 Initiation of protein synthesis.

Elongation

The elongation step involves peptide bond formation (transpeptidation) between the initiator amino acid bound to the tRNA (and subsequently the peptidyl-tRNA) at the P site and the incoming amino acid attached to the amino acyl-tRNA which binds to the A site (Figure 2-5 and 2-6). This event is followed by translocation of the newly formed peptidyl-tRNA from the A site to the P site. The elongation reactions are catalyzed by EF-Tu (Allende et al., 1974; Lucas-Lenard and Lipmann, 1966) and EF-Ts in bacteria. GTP is required in this reaction (Nishizuka and Lipmann, 1966). Translocation of peptidyl-tRNA from A site to P site is catalyzed by EF-G.

There are several binding sites on the ribosome for the incoming aminoacyl-tRNA. These binding sites can bind any aminoacyl-tRNA, but the specificity of the sequence of tRNAs or the selection of tRNAs is entirely determined by the codon (usually the triplet of nucleotides in the coding region of mRNA) arrangement of the mRNA. A specific codon on ribosome-bound mRNA recognizes the complementary nucleotide triplet (known as the anticodon) present in the cognate tRNA (Crick, 1966; Goodman et al., 1968).

Termination

After the initiation of translation the ribosome reads

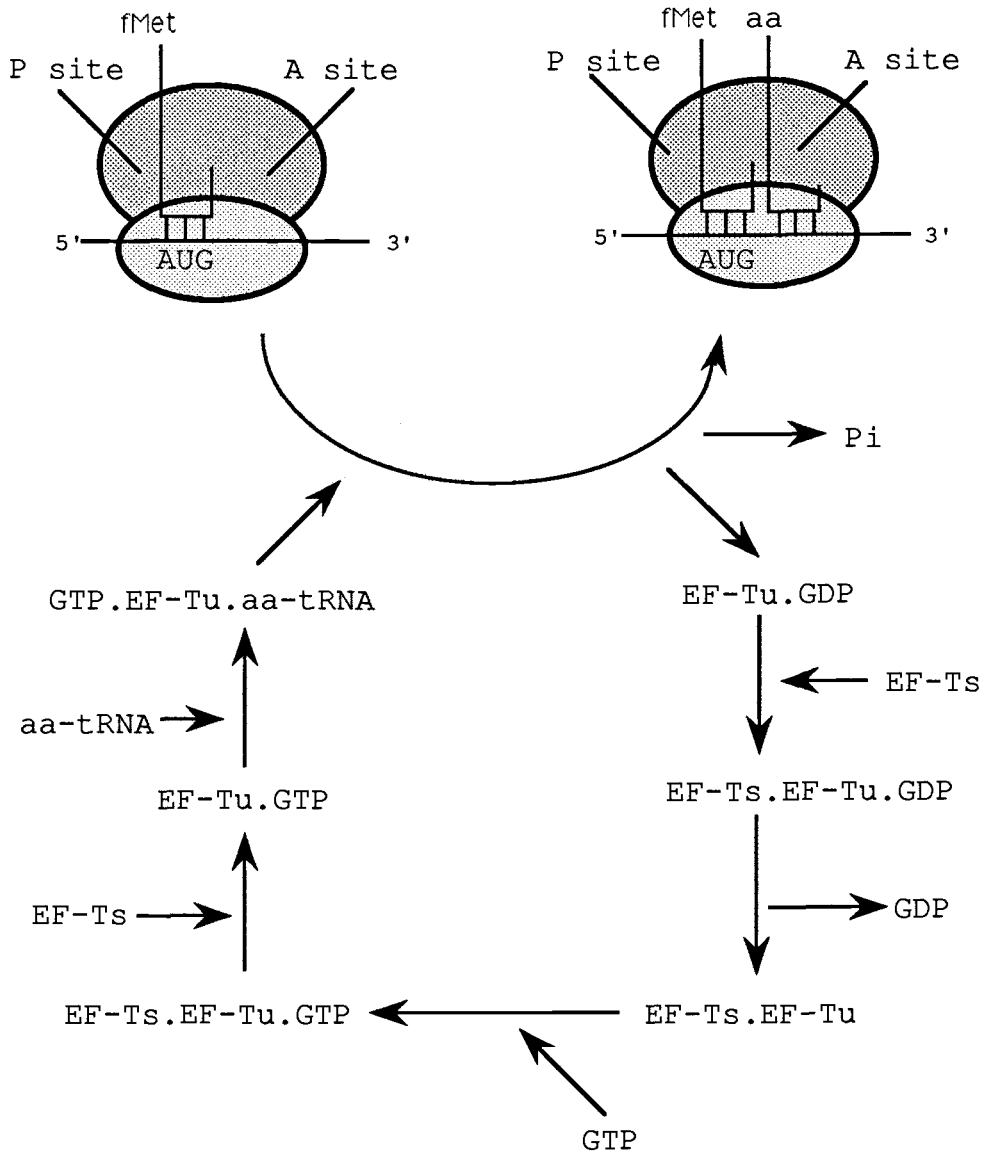


Figure 2-5 Elongation of protein synthesis

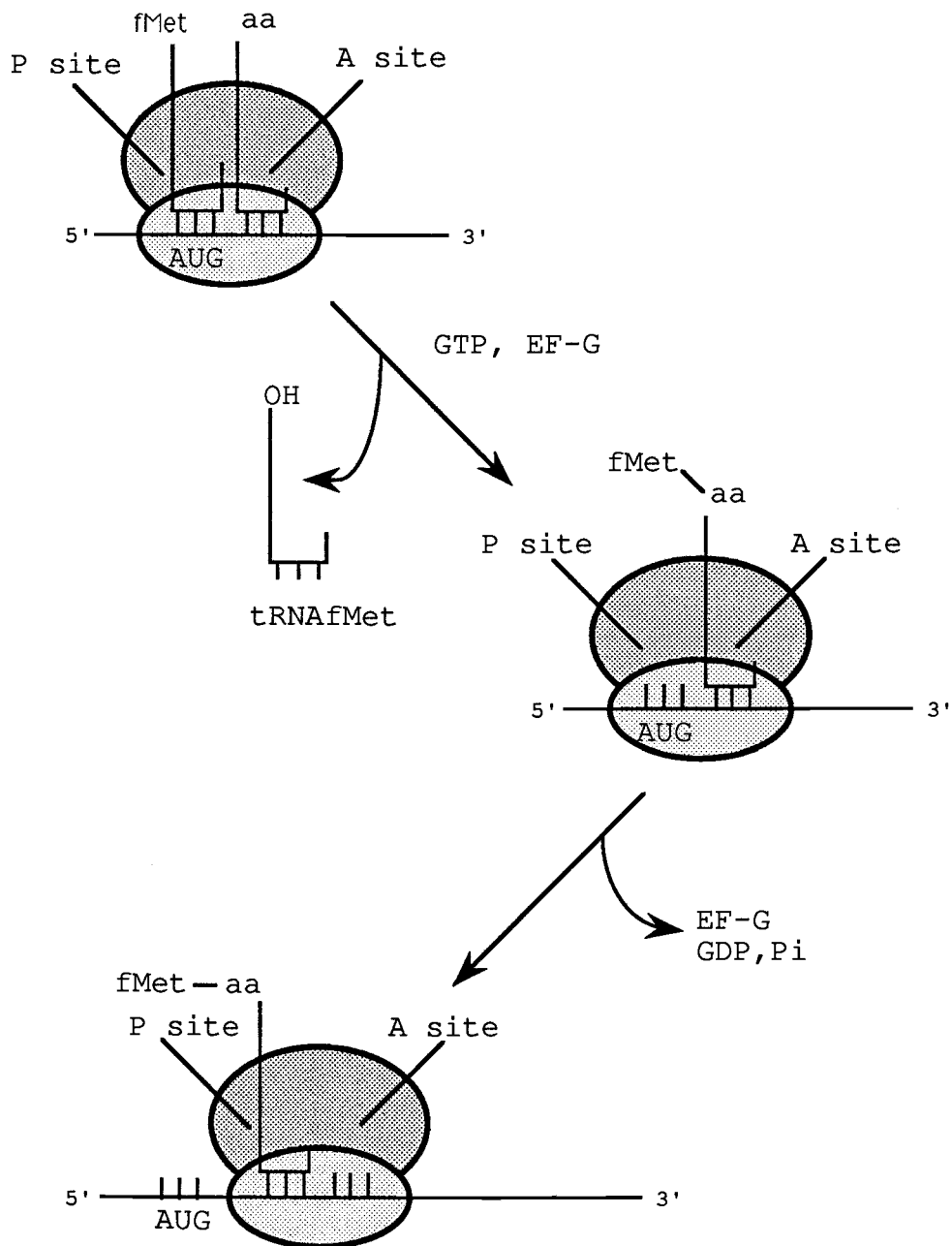


Figure 2-6 Peptide bond formation and translocation

the mRNA codon by codon (or triplet by triplet) as it continues to elongate the polypeptide chain. The ribosome stops when it reaches the termination codons UAA, UAG or UGA (Figure 2-7). These three codons do not call for any cognate tRNA; and hence, they stop translation (Brenner et al., 1965, 1967; Garen, 1968). When the ribosomal A site reaches the termination codon it is recognized by soluble proteins called the termination or release factors (RF). The release factor binds to the ribosome and hydrolyzes the ester bond between the polypeptide and the tRNA of the peptidyl tRNA which is bound to the P site. In E. coli the protein responsible for termination of translation is known as RF-2 (Caskey and Campbell, 1979).

2.1.5 The 30S subunit and the 16S rRNA of E. coli

The 30S subunit of E. coli ribosomes is composed of one molecule of 16S rRNA which is 1542 nucleotides in length and associated with 21 different proteins which range in molecular weight from 8500 to 61,200 daltons (Held et al., 1974; Traub and Nomura, 1966, 1968). Nuclease susceptibility data indicates that extended regions of rRNAs in the ribosomes are exposed to the environment. The rRNA of the ribosomal subunits is self-folded into a compact structure of unique shape that is held together by noncovalent interactions with the r-proteins (Serdyuk et al., 1983; Stern et al., 1989).

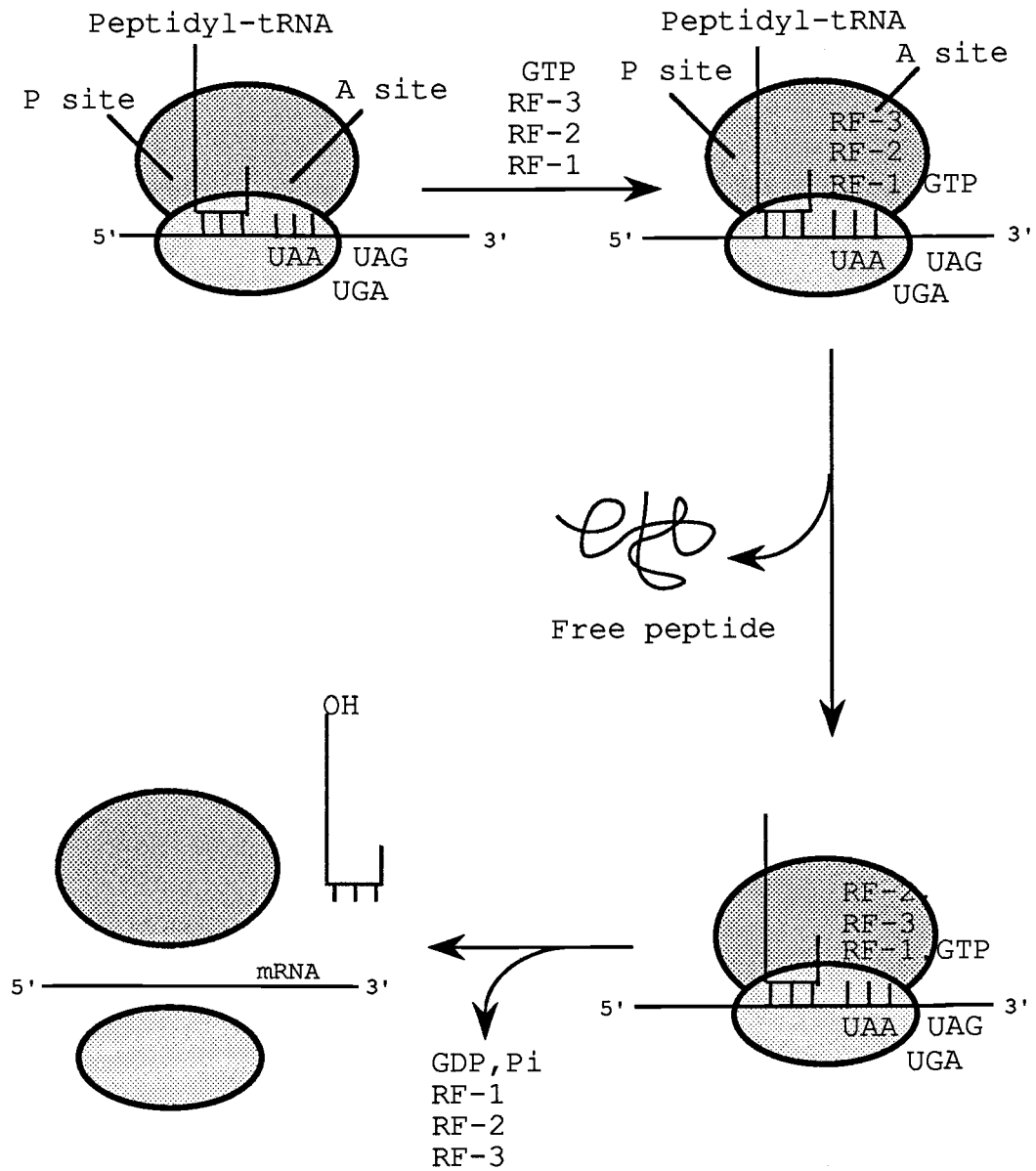


Figure 2-7 Termination step of protein synthesis.

Recently three different laboratories have proposed three dimensional models for E. coli 16S rRNA (Brimacombe et al., 1988; Expert-Benzancon and Wollenzien, 1985; Stern et al., 1988). These models incorporate data accumulated from diverse experimental approaches. All of these representations are premised upon previously proposed universal secondary structural icons of 16S rRNA (Glitz and Brimacombe, 1980; Noller and Woese, 1981; Stiegler et al., 1981a, 1981b; Woese et al., 1980; Zwieb et al., 1981). Small subunit rRNA secondary structure has been inferred from phylogenetic comparisons of hundreds of small subunit rRNAs, chemical and nucleolytic probing (reviewed in Gutell et al., 1985; Maly and Brimacombe, 1983; Noller, 1984; Woese et al., 1980; Woese et al., 1983). The three dimensional structural paradigms are based upon data discussed above, and intra-RNA crosslinking (Atmadja et al., 1986; Stiege et al., 1986; Zwieb and Brimacombe, 1980), immunoelectron microscopic localization of specific nucleotides on the subunit (Gornicki et al., 1984; Mochalova et al., 1982; Politz and Glitz, 1977), and three dimensional coordinates of the centers of mass of the 30S ribosomal proteins (Moore et al., 1986).

A low resolution model of the arrangement of 16S rRNA in 30S subunits was constructed by Brimacombe et al. (1988) based on the above mentioned data and RNA-protein crosslink sites of 13 r proteins recently determined (Gruer et al., 1987; Osswald et al., 1987). According to the proposed

model, the 16S rRNA has the dimension of 220A x 140A x 90A.

2.1.6 Secondary structure of the *E. coli* 16S rRNA

A large number of small subunit rRNA sequences has been determined for rRNAs from a wide phylogenetic range of organisms. Woese and his collaborators have compared phylogenetic differences among the small subunit rRNA sequences from a wide variety of organisms and organelles (Woese and Fox, 1977; Fox et al., 1980). For example: (a) 16S rRNAs from *E. coli* (Brosius et al., 1978; Carbon et al., 1978) and *Proteus vulgaris* (Carbon et al., 1980), (b) 16S rRNA from *Zea mays* chloroplast (Schwarz and Kossel, 1980), (c) 18S rRNA from *Saccharomyces cerevisiae* (Rubstov et al., 1980) and *Xenopus laevis* (Salim and Maden, 1981), (d) 12S rRNAs from human placental (Eperon et al., 1980) and mouse mitochondria (Van Etten et al., 1980), and (e) 15S rRNA from yeast (*Saccharomyces cerevisiae*) mitochondria.

A seminal secondary structure model for *E. coli* 16S rRNA was first deduced by Woese et al., 1980 using data from comparative nucleotide sequence analysis and chemical data. Another model proposed by Glotz and Brimacombe (1980) incorporated results from structural mapping experiments using enzymatic digestions and identification of interacting sequences by two-dimensional gel electrophoresis. A third model proposed by Stiegler et

al. (1981) was based on the following:

- (1) electrophoretic pattern of ribonucleoprotein (RNP) particles generated by digestion of RNA-protein complexes by either RNase T1 or RNase A (Ehresmann et al., 1980)
- (2) search for RNA-RNA interaction sites by RNase A digestion of RNA-protein complexes
- (3) electrophoretic analysis of RNA fragments generated by RNase T1 digestion of 30S subunits, partially unfolded 30S subunits and 16S rRNA (Ehresmann et al., 1975)
- (4) Cobra venom ribonuclease hydrolysis of 30S subunit and 70S ribosomes under progressively stronger conditions and analysis of the digestion products by polyacrylamide gel electrophoresis.
- (5) Sequence analysis of uniformly labeled RNA fragments digested by either RNase T1 or RNase A (Sanger et al., 1965) and localization of enzymatic cleavage points.

Data from all these experimental approaches and sequence comparison were employed in using a computer algorithm to search for base-paired regions (Zuker and Stiegler, 1981). The outcome was a triad of models showing that small subunit rRNAs could be folded into a secondary structure that shared a common base-pairing scheme (Glotz and Brimacombe, 1980; Stiegler et al., 1981; Woese et al., 1980) as presented in Figure 2-5.

Sequence comparison of small subunit rRNAs has shown that a high degree of conservation of primary structure

exist even between evolutionary distant species (Gutell et al., 1985; Stiegler et al., 1981a; 1981b). In addition to this, a large number of secondary structural elements proposed for E. coli 16S rRNA appear to be conserved in other species, and there are about 40 invariant nucleotides conserved at equivalent positions in a common structural motif. Most of these invariant nucleotides in current representations of 16S rRNA are not base paired and are clustered in sequences corresponding to positions 517-533, 1391-1408, 1491-1506 in E. coli 16S rRNA. The nucleotide sequence at position 1531-1542 is highly conserved in prokaryotes, but with the exception of *Giardia lamblia* (Sogin et al., 1989), not conserved in eukaryotes (Gutell et al., 1985). The position of highly conserved single stranded nucleotide sequence regions of E. coli 16S rRNA is depicted in Figure 2-8.

These invariant nucleotides may be involved in tertiary interactions with nonribosomal components such as tRNAs (Denman et al., 1989; Prince et al., 1982), mRNA (Hui et al., 1987; Jacob et al., 1987), and protein factors, etc. Alternatively they may mediate ribosomal function associated with protein synthesis, ribosome assembly, or control of ribosomal functions which remain unidentified.

2.1.7 The 'Domain' organization of small subunit rRNAs

In all of the icons of 16S rRNA, the RNA within the

Figure 2-8 Secondary structure model of E. coli 16S rRNA
(from Stern et al., 1988).

The solid lines indicate the universal, and the hatched line indicate prokaryotic conserved single stranded zones. The dashed lines shows the domains of 16S rRNA.

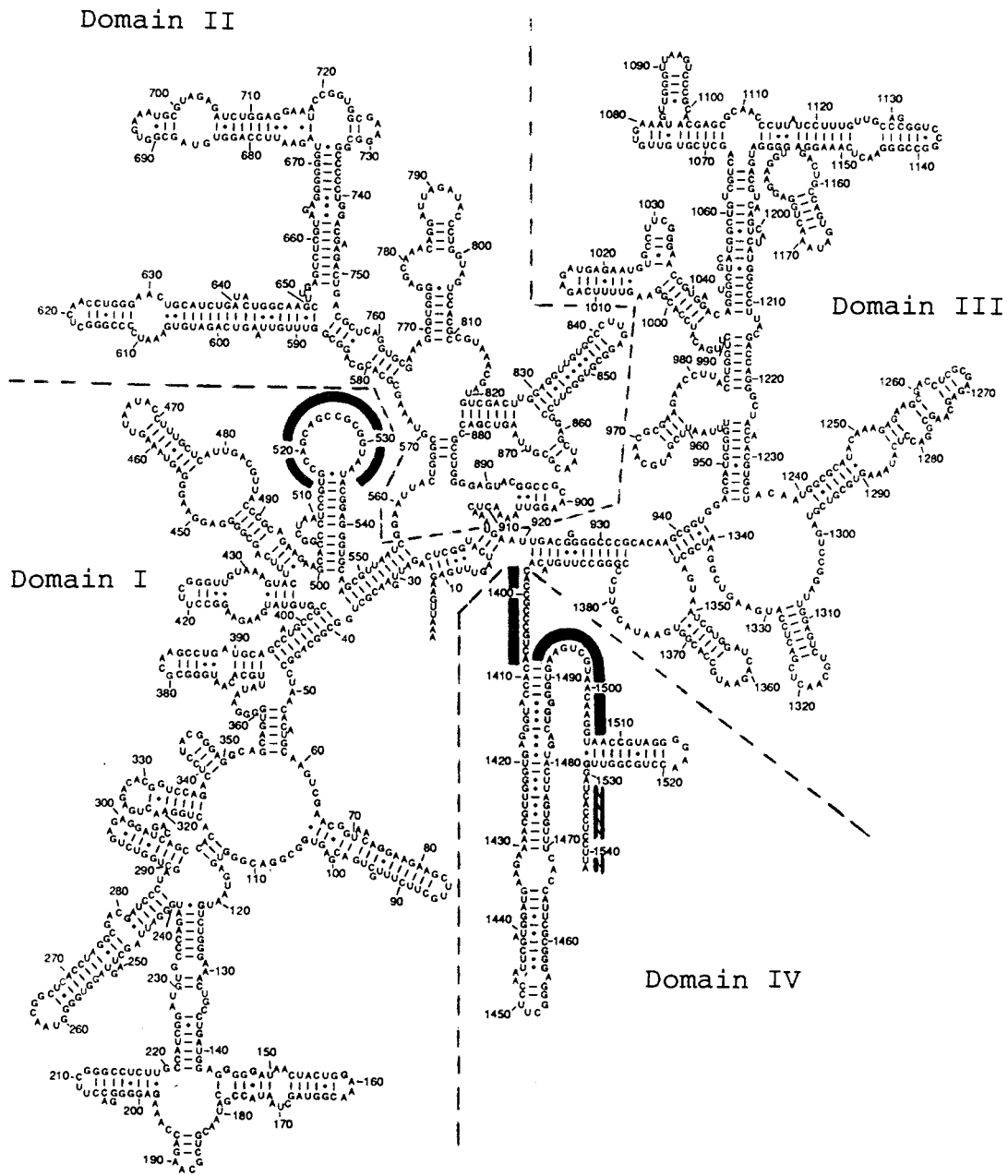


Figure 2-8

subunit appears to be organized into 4 distinct domains generated by secondary structure (Figure 2-8) (reviewed in Noller, 1984). Each domain was defined in terms of its (i) intrinsic susceptibility to enzymatic digestion and chemical modification (ii) its relation with either an individual r-protein or a group of proteins (iii) its ability to generate well defined and stable RNA or RNA-protein particles after mild enzymatic digestion.

The domains were named the 5'-domain (nts 1-556), the central domain (nts 557-916), the 3'-major domain (nts 917-1391) and the 3'-minor domain (nts 1392-1542). The organization of these domains is believed to be enhanced by the interactions of specific r-proteins with the respective rRNA fragment. Partial nuclease digestion experiments revealed that these domains form autonomous ribonucleoprotein (RNP) structures within the assembled 30S subunit (Powers et al., 1988; Yoki and Brimacombe, 1975).

2.1.8 Function of rRNA

The secondary structure of rRNAs have been well conserved in all species examined to date (reviewed in Noller, 1984; Brimacombe and Steige, 1985) which implies that RNAs are likely to participate in ribosomal function. Small subunit rRNA participation in ribosomal function was first demonstrated by Noller and Chaires (1972). They reported that 16S rRNA is a component of tRNA binding sites

within the ribosome. More recent studies by Moazed and Noller (1986, 1987) have further characterized tRNA and antibiotic binding sites on 16S rRNA. Further support for the involvement of rRNA in ribosomal function came from the finding that mRNA binds directly to 16S (Hui and de Boer, 1987; Jacob et al., 1987; Shine and Dalgarno, 1974).

It would not be surprising to learn that rRNA possess functions which we customarily associate with enzymes, because recently it has been shown that RNAs can even function as enzymes (Guerrier-Takada et al., 1983; Sharp and Eisenberg, 1987; Uhlenbeck, 1987; Zaug and Cech, 1986). The interactions of tRNA (Barta et al., 1984; Ofengand et al., 1986) and mRNA (Steize and Jakes, 1975), and their participation in the decoding sites is well established. Moreover, conformational switches and RNA-RNA interactions may play an important role during the translocation process (Brimacombe et al., 1986; Noller, 1984). Several antibiotics also bind to rRNAs (Cundliffe, 1986).

2.1.9 New Approaches

It is clear that rRNAs are directly involved in ribosome mediated protein synthesis. Although several three dimensional structures have been proposed, they are based solely on secondary structure models of the E. coli 16S rRNA. The experimental evidence for those models is based on chemical modification, RNA-protein and RNA-RNA

interaction data and neutron scattering data. These techniques have provided valuable information about the helical segments of 16S rRNA and how the rRNA is folded in the 30S subunit to attain the overall structural conformation. The models do not furnish any information concerning the disposition of single stranded regions in the intact 30S subunits and in 70S ribosomes.

The only approach taken to probe for single stranded sequence zones employed base-specific digestion with enzymes such as RNase T1 and RNase A. Also Ross and Brimacombe (1979) have used nuclease S1 to digest single stranded regions. They then characterized the remaining resistant double stranded regions. The major drawback with all of these methods is that nuclease digestion could disrupt weak secondary and tertiary interactions within the particles, leading to an underestimate of the extent of secondary structure.

Manuscript I of this thesis is involved with the application of a method based on the RNA-DNA hybridization and subsequent protection of hybrids from nuclease S1 digestion. This method has been used here to probe the exposure and accessibility of universal single stranded sequence zones in 30S subunits and 70S ribosomes. Single stranded exposed regions of rRNAs offer potential sites for the interaction with other nonribosomal components of protein synthesis. Furthermore, universal conservation implies a special global role for these sequences in

protein synthesis. The novelty of this approach compared to other methods has been discussed in the manuscript.

The approach is unique in that the protection of the DNA oligonucleotide probe depends entirely on sequence accessibility, and successful hybridization of the probe to the sequence. Once it is hybridized, S1 nuclease will digest only the single stranded nucleotides leaving the RNA-DNA hybrid form intact. Any single strand generated during or after digestion will be immediately subjected to further digestion. Furthermore, free single stranded DNA is digested by nuclease S1 at a rate seven times faster than single stranded RNA (Vogt, 1973). Thus, only RNA-DNA hybrids initially formed will survive the nuclease S1 attack. The protected DNA-RNA hybrid can be visualized in a denaturing polyacrylamide gel if the DNA is terminally labeled with ^{32}P .

This method is rapid, simple and relies on established procedures. This method offers a unique opportunity to examine any molecular suprastructure containing DNA or RNA and their involvement during different biological functioning. Involvement of a particular nucleic acid segment can be identified in reaction intermediates. It can be further used to elucidate the participation of different rRNA single stranded zones during different phases of protein synthesis.

2.2 MANUSCRIPT II: Inhibition of Translation and Antibacterial Activity by New Short DNA Analogs

One of the most interesting features of the 16S-like ribosomal RNA secondary structure is the high conservation of helical and single stranded sequence regions. Among them the regions 322-329, 515-533, 691-699, 1047-1061, 1390-1407 and 1492-1506 are nearly universal and single stranded in 16S-like rRNAs (Woese et al., 1983). A striking difference occurs at the extreme 3' end where the sequence CCUCC (corresponding to positions 1535-1539) is highly conserved in prokaryotic 16S-like rRNAs, but not conserved in eukaryotic rRNAs (Gutell et al., 1985), with exception in *Giardia lamblia* (Sogin et al., 1989). This pyrimidine-rich sequence is known as the anti Shine and Dalgarno (antiSD) sequence.

It is now accepted that this sequence region participates and influences the initiation of translation (Gold et al., 1981; Kozak, 1983; Shine and Dalgarno, 1974; Steitz and Jakes, 1975) through base pairing with the Shine and Dalgarno (SD) sequence found about 10 nucleotides upstream from the initiation codon AUG or GUG of all prokaryotic mRNAs. Recently it has been shown that modification of the antiSD sequence at the 3' end of *Escherichia coli* 16S rRNA by nucleotide substitutions drastically alters the efficiency of translation (Jacob et

al., 1987; Hui and de Boer, 1987; Weyens et al., 1988). Modification of the antiSD sequence was also found to effect the feedback regulation of rRNA synthesis (Yamagishi et al., 1987), which in turn controls the synthesis of ribosomal proteins.

Weiss et al. (1988) have recently shown that the antiSD sequence involved in initiation also base pairs with mRNA during the elongation phase of translation. They have indicated that mRNAs contain some internal SD-like sequences and the 3' end of 16S rRNA scans the mRNA during elongation through efficient detection and continuous base pairing to retain the correct reading frame (Dahlberg, 1989; Weiss et al., 1988).

The presence of the antiSD sequence at the extreme 3' end of prokaryotic 16S-like rRNAs and its absence in eukaryotes makes them potential targets for the development of antibacterial agents. One approach is to block the antiSD sequence with short complementary DNA oligonucleotides, as they readily form hybrids through Watson-Crick type base pairing. Because their size is much smaller (3-6 nucleotides used here) than mRNA, they are readily available for binding to the antiSD site and prevent binding of mRNA. This would abolish initiation of translation and ultimately stop all cellular processes. Using oligonucleotides with regular phosphodiester internucleotide linkage is not suitable for this purpose because they are susceptible to degradation by cellular

nucleases. DNA oligomers with altered internucleotide linkages are nondegradable by nucleases.

The other requirements for an antibacterial agent are that: (1) the size of the agent must be suitable for penetration into the bacteria; (2) the properties of the agent should not prevent it crossing cell membranes; and (3) it should bind to the target sequence with sufficient specificity and inhibit a critical biological activity of the organism.

Jayaraman et al. (1981) have reported that E. coli B cells were permeable to DNA oligonucleotide analogs 3 residues in length and complementary to the antiSD region of 16S rRNA, but they were not permeable to DNA analogs 5 residues in length. The question of whether or not a 4 residue analog can penetrate the cells was not addressed. Furthermore, they have shown that 3- and 5-residue oligomers bind to the antiSD sequence of the E. coli B 16S rRNA and inhibit in vitro translation and colony formation by permeable cells. Other experiments have shown that DNA oligomer analogs complementary to specific sites of mRNA can inhibit mRNA translation to a limited extent (Hastie and Held, 1978; Paterson et al., 1977). As antibiotics mRNA-directed oligomers are of limited value, because the oligomers are displaced from the mRNA by the translating ribosome (Gupta, 1987; Liebhaber et al., 1984; Shakin and Liebhaber, 1986).

The best evidence available suggest that complementary

oligomers that block attachment sites on ribosomes are likely to be the most effective antibiotics (Liebhaber, 1984; Blake et al., 1985a; Lawson et al., 1986). The specificity and efficiency of inhibition by any oligomer analogs seems to be dependent upon the sequence, the chain length, the location and the structural organization of the binding site (Van der Krol et al., 1988). Unmodified oligomers are poorly absorbed by cells (Zamecnik et al., 1986) and they are very sensitive to intracellular nucleases (Cazenave et al., 1986); this limits their use as *in vivo* antibiotics.

Several laboratories have developed modified nucleotide analogs to overcome lability and cell penetration problems. For example, oligonucleoside methylphosphonates were used for hybridization arrest of globin synthesis (Blake et al., 1985a; 1985b) and for the control of growth of herpes simplex virus (Smith et al., 1986). The oligonucleoside methylphosphonate-RNA complex is not a substrate for RNase H. A high concentration of this oligomer is required for antibacterial or antiviral activity (Van der Krol et al., 1988). This is attributed to the substitution of an oxygen atom by a methyl group which yields four different substituents on the phosphorus atom producing diastereoisomers. Some of them bind very poorly to the target sequence. Other analogs used for hybridization arrest include the phosphorothioates (Matsukura et al., 1987), alpha-anomeric DNA oligomers (Cazenave et al.,

1987), oligomers with modified ends (Cosstick and Eckstein, 1985), oligomers linked to reactive agents such as alkylating agents (Knorre and Vlassov, 1985), and metal complexes such as EDTA-Fe(II) (Chu and Orgel, 1985). These modified nucleosides are limited by one or more of the problems discussed above. To date the most effective anti-sense oligonucleotide antibiotics appear to be the methylphosphonates of Jayaraman et al. (1981).

The research presented in this thesis was undertaken in collaboration with Antivirals Inc. to explore the antibiotic potential of DNA oligomer analogs that have the normal phosphodiester bonds replaced by methylcarbamate linkages. The chain lengths were from 3 to 6 nucleotides and the oligomers were complementary to the highly conserved ACCUCCU sequence region in prokaryotic small subunit 16S rRNAs. Various sequences and combinations of sequences were tested. The synthetic oligomers used in this work are neutral with regard to the internucleotide linkages and stereoregular in steric organization. They are called the "neu-genes" by Antivirals Inc. They are resistant to nucleases and autodegradation and their neutral properties allow them to pass through the cell membrane. Polyethylene glycol of molecular weight 1000 (PEG₁₀₀₀) was coupled to some of the test oligomers to improve their solubility.

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3. NUCLEASE S1 MAPPING OF 16S RIBOSOMAL
RNA IN RIBOSOMES

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3.1 ABSTRACT

Escherichia coli 16S rRNA and 16S-like rRNAs from other species have several universally conserved sequences which are believed to be single stranded in ribosomes. The quantitative disposition of these sequences within ribosomes is not known. Here we describe experiments designed to explore the arrangement of universal 16S rRNA sequences in 30S particles and 70S ribosomes. Unlike previous investigations, quantitative data on the arrangement of conserved portions of 16S rRNA within ribosomes was acquired. Uniquely, the experimental design also permitted investigation of cooperative interactions involving these portions of 16S rRNA. The basic strategy employed ribosomes, 30S subunits, and 16S rRNA, which were quantitatively analyzed using synthetic DNA in combination with nuclease S1. Recovery of useful data did not depend upon nuclease access to the probed site. In deproteinated E. coli 16S rRNA and 30S subunits, the regions 520-530, 1396-1404, 1493-1504, and 1533-1542 are all single stranded and unrestricted for hybridization to short synthetic DNAs. However, the accessibility of the sequences in 70S ribosomes varies with each position. There appear to be no cooperative interactions between 16S rRNA universal sequences in 30S subunits.

3.2 INTRODUCTION

Ribosomes, particles composed of proteins and RNA, play a prominent part in the synthesis of proteins. Concise roles for RNA elements in ribosomes have not been established. Highly conserved single-stranded regions of 16S rRNA and 16S-like rRNAs in 30S ribosomal subunits offer simple structural elements which may be envisioned to participate in protein synthesis through base interactions with non-ribosome components (Brimacombe and Stiege, 1985; Noller, 1984). Among the universally conserved single-stranded sequences, the regions 517-533, 1394-1408 and 1492-1506 have the longest single stranded stretches (Gutell et al., 1985; Stiegler et al., 1981; Woese et al., 1981; Zwieb and Brimacombe, 1981). The 3' terminal sequence CCUCC (position 1535-1539) is universally present in eubacteria and archaebacteria (Gutell et al., 1985; Woese et al., 1981; Zwieb and Brimacombe, 1981) with one exception (Sogin et al., 1989), but not in eukaryotes. The remarkable conservation of the single-stranded portions of 16S like rRNAs implies that they must be active in some global function in protein synthesis. For example, the conserved eubacterial 3' terminal sequence CCUCC is clearly essential for initiation of protein synthesis (Abdul-Latif and Schaup, 1988; Gold, 1981; Kozak, 1983; Shine and Dalgarno, 1974; Steitz and Jakes, 1975; Weiss et al., (1988). The zone centered about C₁₄₀₀ in 16S rRNA has

been implicated in tRNA binding (Ciesiolka et al., 1985; Denman et al., 1988; Gnirke and Nierhaus, 1986; Ofengand and Liou, 1981; Ofengand et al., 1982; Prince et al., 1982).

A knowledge of rRNA sequence department is necessary to propose and support theories about the function of conserved portions of rRNA in ribosomes and ribosomal subunits. There are current models showing the three-dimensional arrangement of E. coli 16S rRNA in 30S subunits (Brimacombe et al., 1988; Expert-Bezancon and Wollenzien, 1985; Moazed et al., 1986; Stern et al., 1988). Support for these representations comes from various techniques such as comparative sequence analysis, chemical and enzymatic footprinting, RNA-RNA and RNA-protein crosslinking, and neutron diffraction studies. However, these techniques do not readily permit determination of the number of ribosomes in a given configuration. We have used short site-specific DNA oligomers to infer the single strandedness of conserved portions of 16S rRNA in 30S subunits and 70S ribosomes from E. coli. Unlike previous work by others, the probing with the DNA should not cause artifactual alterations in the particle being investigated. The experiments were undertaken using conditions which maintain ribosome configuration. We have found that the regions centered about positions 525, 1400, 1500, and 1535 are always single stranded in 16S rRNA and nearly so in 30S subunits. In contrast, in active 70S ribosomes, sequences

centered about positions 520 and 1400 are open in roughly one-half of the ribosomes. The sequence zones centered about positions 1500 and 1538 are open only in about 30% of the ribosomes. We have also found that the presence of DNA probes complementary to other highly conserved single-stranded zones does not alter the interactions observed when the DNA probes are individually tested.

3.3 MATERIALS AND METHODS

3.3.1 Preparation of 70S ribosomes, 30S subunits and 16S rRNA. The 70S ribosomes, 30S subunits and 16S rRNAs were prepared from E. coli D-10 cells harvested in the early log phase of growth. The cells were stored frozen at -70°C . Ribosomes and ribosomal subunits were prepared by zone ultracentrifugation using previously described salt washing procedures (Hardy et al., 1969; Kurland, 1966). These procedures yield 70S particles which are associated with mRNA and tRNAs. Ribosomal RNA was prepared by zone ultracentrifugation from phenol extracts of cell lysates cleared of cell debris as described elsewhere (Schaup et al., 1970; Traub and Nomura, 1968).

3.3.2 Synthesis and labeling of DNA oligomer probes. DNA oligomer probes (Table 1) were chemically synthesized and supplied by the Central Service Laboratory of the Center for Gene Research and Biotechnology, Oregon State University. The probes were made with an Applied BioSystems Model 380A DNA Synthesizer using phosphoramidite chemistry (Beaucage and Caruthers, 1981; Matteucci and Caruthers, 1981). HPLC purified probes were 5' end labeled with γ -[^{32}P]ATP (adenosine 5'-triphosphate [γ - ^{32}P], specific activity 7000 mCi/mMol, crude; ICN Radiochemicals) and

T4 polynucleotide kinase (BRL) according to established methods (Chaconas et al., 1975; Chaconas and van de Sande, 1980; Maniatis et al., 1982). The kinased probes were purified by 20% polyacrylamide-7M urea gel electrophoresis. After autoradiography the zone containing the full length 5' end-labeled probe was cut out and the DNA was eluted from the gel essentially as previously described (Maniatis et al., 1982).

3.3.3 3' end labeling of 16S rRNA. 16s rRNA was 3' end labeled by the method described by England et al. (1980) using 5' [³²P] pCp (cytidine 3',5' bis(phosphate), [5'-³²P], NEN, specific activity 3000 Ci/mMol) and T4 RNA ligase (BRL) incubated overnight at 4°C in ligase buffer (50 mM HEPES/20 mM MgCl/3.3 mM DTT/0.015 µg µl⁻¹ BSA). The labeled RNA was purified using a 2.8% polyacrylamide 7M urea gel electrophoresis system and was recovered from the gel by two extractions with equal volumes of SSC buffer [150 mM NaCl/ 15 mM Na-Citrate (pH 7.2)] and redistilled phenol (equilibrated with SSC buffer) (Cory et al., 1972), followed by ethanol precipitation at -20°C. The suspension was then passed through a CF11 (Cellulose Powder, Whatman) column prepared in a 1 ml pipet tip plugged with siliconized glass wool. The column was washed with 5 ml of 80% ethanol and then with 5 ml of 80% methanol. This process removed acrylamide. The

pCp labeled 16S rRNA was eluted from the CF11 column with 500 μ l of sterile distilled water, lyophilized and dissolved in 20 μ l of TSM buffer (10 mM Tris-base, pH 8.0, 3 mM succinic acid/10 mM $MgCl_2 \cdot 6 H_2O$ /6 mM 2-mercaptoethanol) (Kurland, 1966) and the specific activity was determined by liquid scintillation counting.

3.3.4 RNA-DNA hybridization and RNase H cleavage.

3' end-labeled 16S rRNA was mixed with a 30 fold molar excess of unlabeled DNA probes in 10 μ l of E. coli RNase H hybridization buffer [40 mM Tris-base (pH 7.9)/4 mM $MgCl_2$ /1 mM dithiothreitol/0.03 μ g μ l⁻¹ BSA] and calf thymus RNase H hybridization buffer [50 mM Tris-base (pH 8.3)/25 mM $MgCl_2$ /0.1 M KCl/0.5 μ g μ l⁻¹ BSA], was heated at 50°C for 20 min, then cooled to 32°C and further incubated at 32°C for 30 min (Donis-Keller, 1979). After addition of RNase H (0.5 U/ μ g of 16S rRNA) the reactions were incubated at 32°C for 50 min. A 20 μ l aliquot of tracking dye [10 M urea/0.05% xylene cyanol/0.05% bromophenol blue/5 mM Tris-borate/1.0 mM EDTA (pH 8.3)] (Peattie, 1979) was added to the reactions which were then analyzed electrophoretically on 2.8% polyacrylamide-7M urea gels.

3.3.5 Hybridization of DNA probes to 16S rRNA, 30S subunits, 70S ribosomes and digestion with nuclease S1.

Hybridization of DNA probes to 16S rRNA, 30S subunits

and 70S ribosomes in nuclease S1 protection experiments was carried out with minor modifications of described procedures (Belfort et al., 1985; Berk and Sharp, 1977; Sharp, 1980). The experimental samples were incubated with a 15-fold molar excess of DNA probe in 20 μ l of S1 hybridization buffer modified by the addition of $MgCl_2$ to stabilize the configuration of the ribosomes and the RNA [40 mM PIPES/400 mM NaCl/3 mM $MgCl_2$ (pH 6.8)]. The incubations were done at 37°C for 30 min, slowly cooled to 30°C, and the incubation was continued at 30°C for 3 hours.

Nuclease S1 (E.C.3.1.4.x, Pharmacia) was diluted to a concentration of 5 U/ μ l with nuclease S1 digestion buffer prior to use and kept at 0°C [30 mM Na-acetate/280 mM NaCl/4.5 mM zinc sulfate/5% glycerol (v/v) (pH 4.6)]. Thirty μ l of S1 digestion buffer was added to each reaction, followed by incubation at 30°C for 45 min. The reaction was stopped by the addition of 25 μ l of termination mix (2.5 M ammonium acetate/50 mM EDTA] as well as 25 μ g of tRNA as carrier (Sharp et al., 1980). The mixture was then precipitated by adding 2.5 vol of ethanol and the precipitates were dissolved in 25 μ l of loading buffer [95 mM Tris-borate (pH 8.3)/1.0 mM EDTA/ 7 M urea/0.01% xylene cyanol/0.01% bromophenol blue] (Peattie) and analyzed by 20% acrylamide-7 M urea gel electrophoresis.

3.4 RESULTS

3.4.1 Screening for potential partial hybrids.

The DNA probes used in these investigations are shown in Table 3-1. An analysis was made to find all potential points within 16S rRNA where partial hybrids could be formed which could cause ambiguity in interpreting nuclease S1 experiments. DNA probe 520 forms potential hybrids of two or more contiguous base pairs with 16S rRNA (Table 3-2). When compared with current secondary structure models for 16S rRNA, most of the alignments are in double stranded or partially double stranded zones of the molecule. Stable hybrids cannot form at these sites under the conditions used in these investigations. The only alignment that would yield full nuclease S1 protection of the DNA is one in which the DNA probe is fully complementary when the zone is single stranded. Other interactions would yield digestion products very much smaller than the intact probe. This is also true for all the other DNA probes used in these investigations.

3.4.2 DNA binding to 16S rRNA. The nuclease S1 hybridization buffer employed here had a pH of 6.8 and contained magnesium. Buffers commonly used for S1 nuclease digestions contain no magnesium and the pH is usually 4.6. Therefore an assessment of the enzyme's

Table 3-1 Synthetic DNAs.^a

DNA	Position ^b	Length	Sequence ^c
520	520-530	11	TCGTCGGCGCC
1396	1396-1404	9	ACACCGCCC
1475	1475-1485	11	CTAAGTACTGA
1493	1493-1504	12	TCAGCATTGTTC
1533	1533-1542	10	GTGGAGGAAT

a: Position and sequence are referenced to E. coli 16S rRNA.

b: Position span on 16S rRNA.

c: Sequences are shown in the 3' to 5' direction.

Table 3-2 Potential hybrids between DNA 520 and 16S rRNA

Position ^a	Hybrid ^b	Position ^a	Hybrid ^b
98-108	ACGAGUGGCGG : :: : TCGTGGCGCC	350-360	GGCAGCAGUGG :::: : TCGTGGCGCC
435-445	ACUUUCAGCGG : : : TCGTGGCGCC	520-530	AGCAGCCGCGG ::::: TCGTGGCGCC
681-691	AGGUGUAGCGG :: : : TCGTGGCGCC	932-942	CGCACAAGCGG :: : : TCGTGGCGCC
935-945	ACAAGCGGUGG : : : : TCGTGGCGCC	1346-1356	AGUAAUCGUGG :: : : : TCGTGGCGCC
1516-1526	GGAACCGCGG : : : : TCGTGGCGCC		

a: Position span on 16S rRNA.

b: rRNA is shown in the upper sequence in 5' to 3' orientation and DNA 520 is represented in the lower sequence

activity under our conditions was necessary. The results of a polyacrylamide gel analysis of nuclease S1 digestions at various enzyme concentrations in the modified buffer are presented in Figure 3-1.

The autoradiograph presented in Figure 3-2 shows the effect of increasing the concentration of DNA probe 520 relative to a fixed level of 16S rRNA. After autoradiography of the gel, the protected bands were cut out and quantitated using a liquid scintillation spectrometer. The molar amount of probe DNA bound to 16S rRNA was calculated using the known specific activity of the DNA probe and plotted against molar excess to generate the saturation hybridization curve presented in Figure 3-3 a. A Scatchard plot (Scatchard, 1949; van Holde, 1985) of the same data, which shows $n \approx 1$ (where n is the number of binding sites per molecule of 16S RNA) is presented in Figure 3-3(b). Similar analyses using data from similar experiments with DNA 1396 are presented in Figures 3-3c and 3-3d. From the Scatchard plots (3-3b and 3-3c) the binding (number of moles DNA bound per mole of 16S rRNA) appears to be one-to-one as expected for specific interactions. From Figures 3a and 3c it is evident that the binding of probe 520 as well as probe 1396 to 16S rRNA is maximum in the presence of a 15- to 20-fold molar excess of probe. DNA probes 1493 and 1533 show comparable stoichiometric interactions with 16S rRNA.

Figure 3-1. Nuclease S1 activity.

5×10^{-12} moles of 5'- ^{32}P -labeled DNA 520 was digested with various amounts of nuclease S1. The lane numbers correspond to the number of units per μl of the nuclease S1 used in each reaction. Lane M shows intact DNA 520 and lane T shows a digestion using 1 $\mu/\mu\text{l}$ of nuclease S1 in the presence of 3.43×10^{-12} moles of yeast tRNA. The polyacrylamide gel was a 20% denaturing gel (26 cm x 24.8 cm x 0.08 cm) run at 25 mA for 2' 30". The digestion conditions were as described in materials and methods.

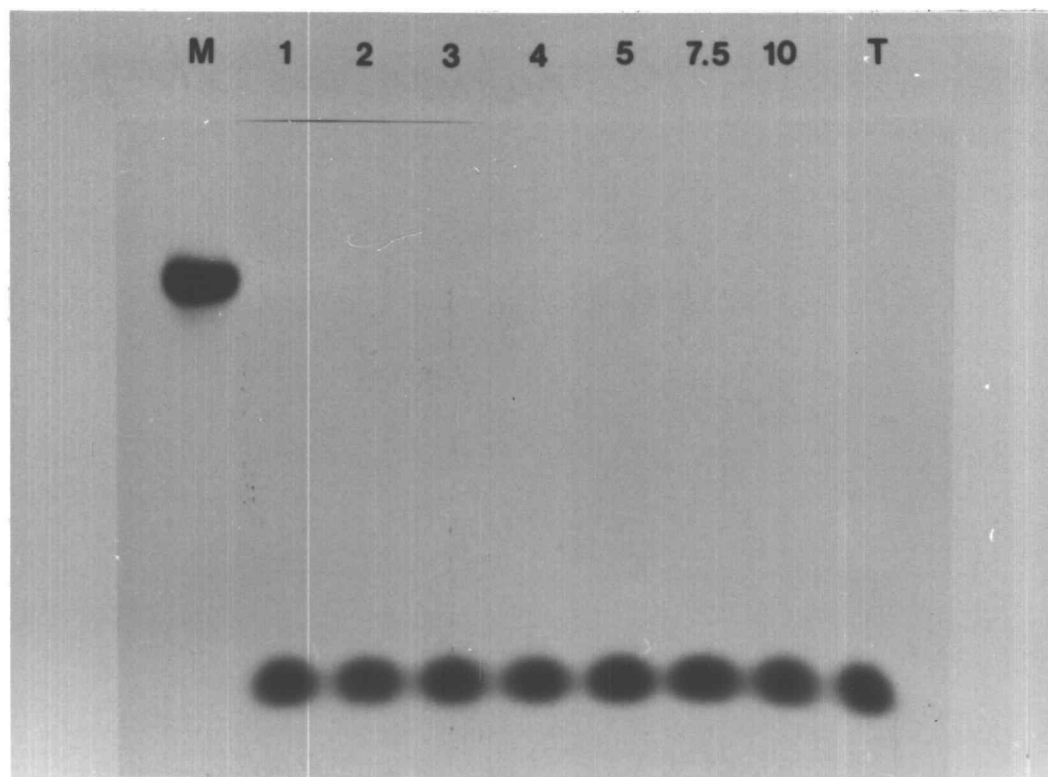


Figure 3-1

Figure 3-2. Saturation hybridization of DNA 520 with 16S rRNA.

Autoradiograph of a 20% polyacrylamide gel analysis of nuclease S1 resistant 5' [^{32}P] labeled DNA 520 hybridized to 1.67×10^{-13} moles of 16S rRNA. The lane numbers indicate the molar ratio of DNA to 16S rRNA in the reactions. M shows intact DNA 520. The reaction conditions are as described in the Materials and Methods.

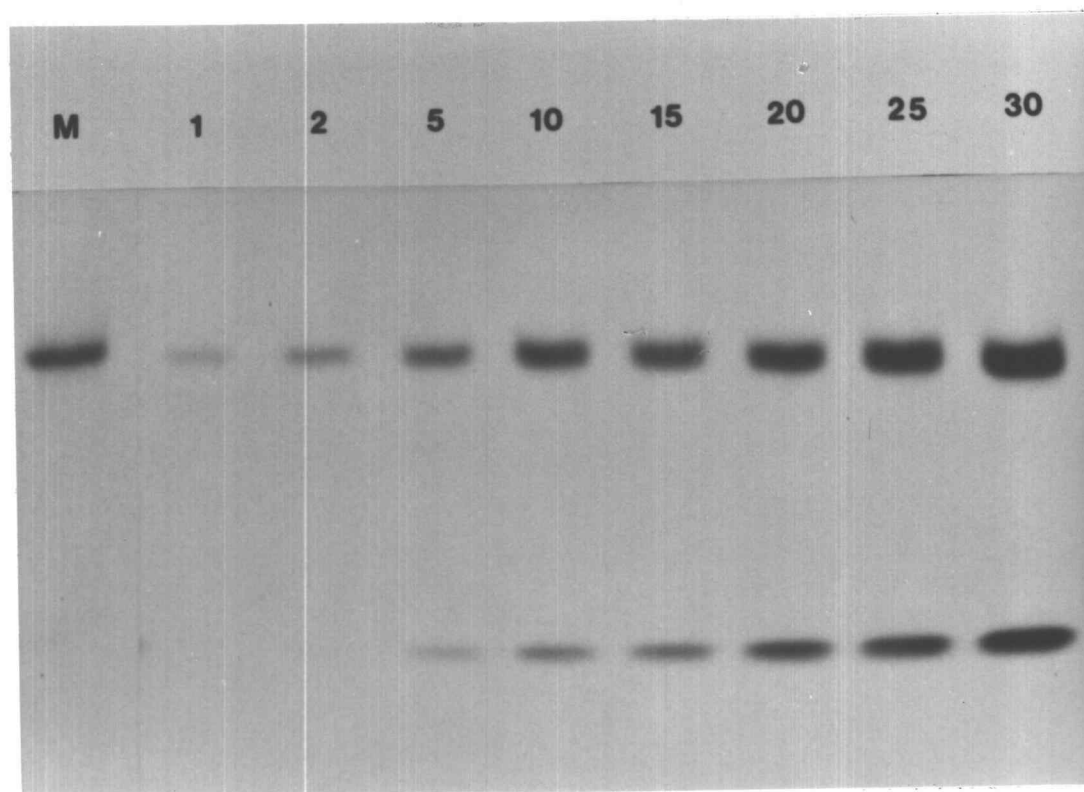


Figure 3-2

Figure 3-3. Saturation curve of DNA 520 (3-3a) and DNA 1396 (3-3c) binding to 16S rRNA.

The zones containing protected DNA similar to those shown in Fig. 3-2 were cut out of the gel and quantitated by liquid scintillation spectrometry in a Triton X-100 cocktail. The number of moles of DNA in the protected bands were calculated using the specific activity of the respective probes. The ratio of moles DNA bound per mole of 16S rRNA is plotted against molar excess of DNA to 16S rRNA. Data represents the average of three separate experiments. Data shown in (3-3a) and (3-3c) were used to generate the Scatchard plots for DNA 520 (3-3b) and DNA 1396 (3-3d). From the Scatchard plots the number of binding sites for DNA 520 and 1396 are, respectively, 0.987 and 1.10.

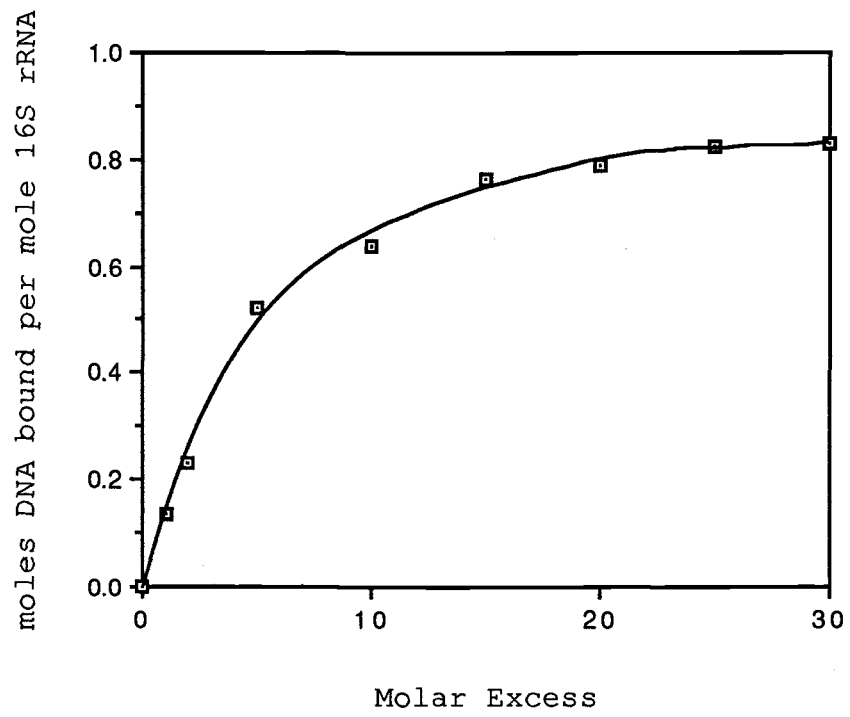


Figure 3-3a

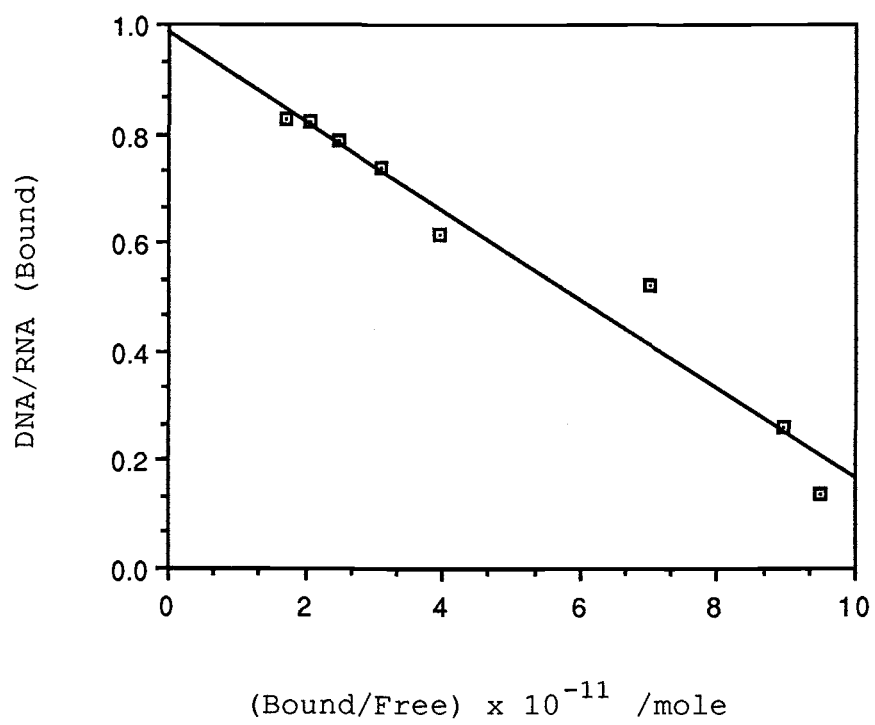


Figure 3-3b

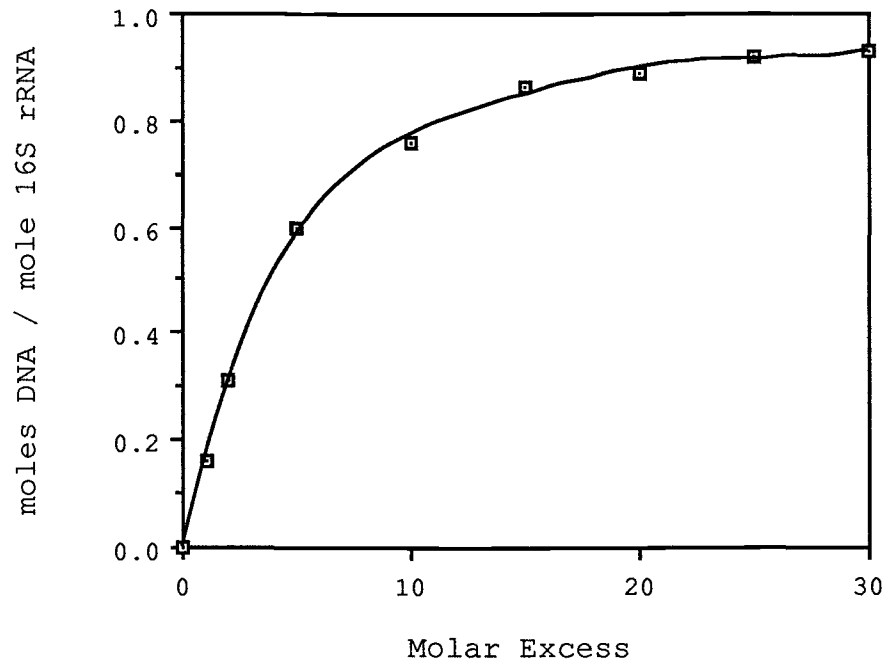


Figure 3-3c

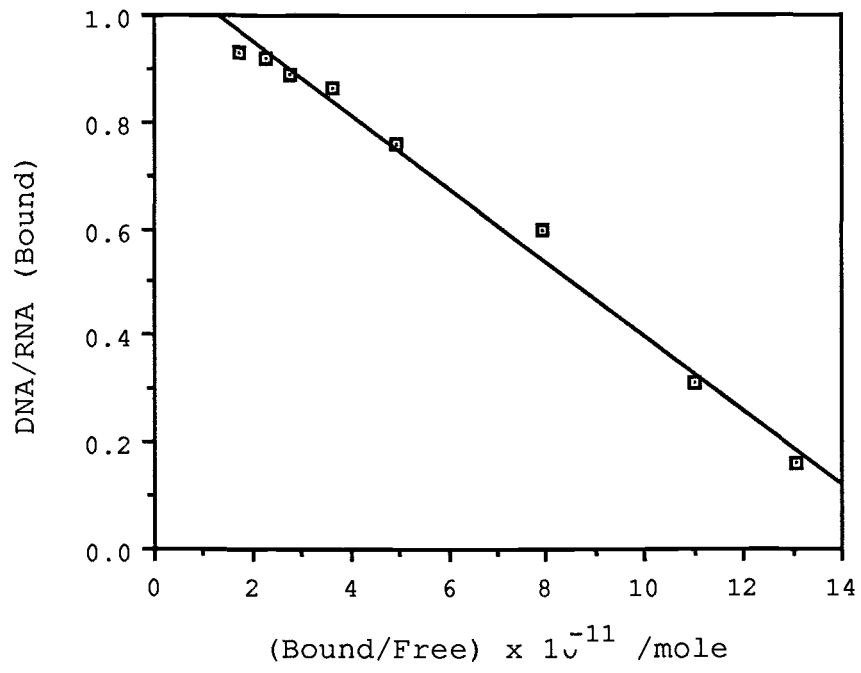


Figure 3-3d

We used probe 1475 as a control for nonspecific DNA-rRNA or DNA-ribosomal protein interactions. It was an 11 nucleotide DNA complementary to positions 1475-1485 in E. coli 16S rRNA. The complement to this probe in current secondary structural models of 16S rRNA is shown to be in a conserved double-stranded region of the molecule. Protection of the probe from nuclease S1 digestion would not be expected unless the DNA was involved in secondary artifactual interactions. There was no observable protection from nuclease S1 of probe 1475 after incubation with 16S rRNA, 30S subunits, or with 70S ribosomes (Figure 3-4). This experiment shows that hybridization of the probes to 16S rRNA is essential for protection. It also shows that secondary interactions, possibly with ribosomal proteins, are not likely to produce artifacts in these experiments.

3.4.3 RNase H cleavage pattern. To provide further assurance that the probes were interacting with the expected zones, we analyzed RNase H digestion patterns of the 16S rRNA hybridized to our probes. The conditions used were comparable to those adapted for nuclease S1 digestions. RNase H will only cleave RNA/DNA hybrids. Therefore, the size of the digestion products in these experiments is predictable only if the probe has specifically hybridized to the 16S rRNA

Figure 3-4. Nuclease S1 digestion of control DNA 1475.

Autoradiograph of a 20% polyacrylamide gel where a 30 fold molar excess of DNA 1475 was incubated in presence of 16S rRNA (lane 2), 30S subunits (lane 3) and 70S ribosomes (lane 4) followed by digestion with nuclease S1. Lane 1 shows the intact DNA 1475. Digestion conditions were as described in materials and methods.

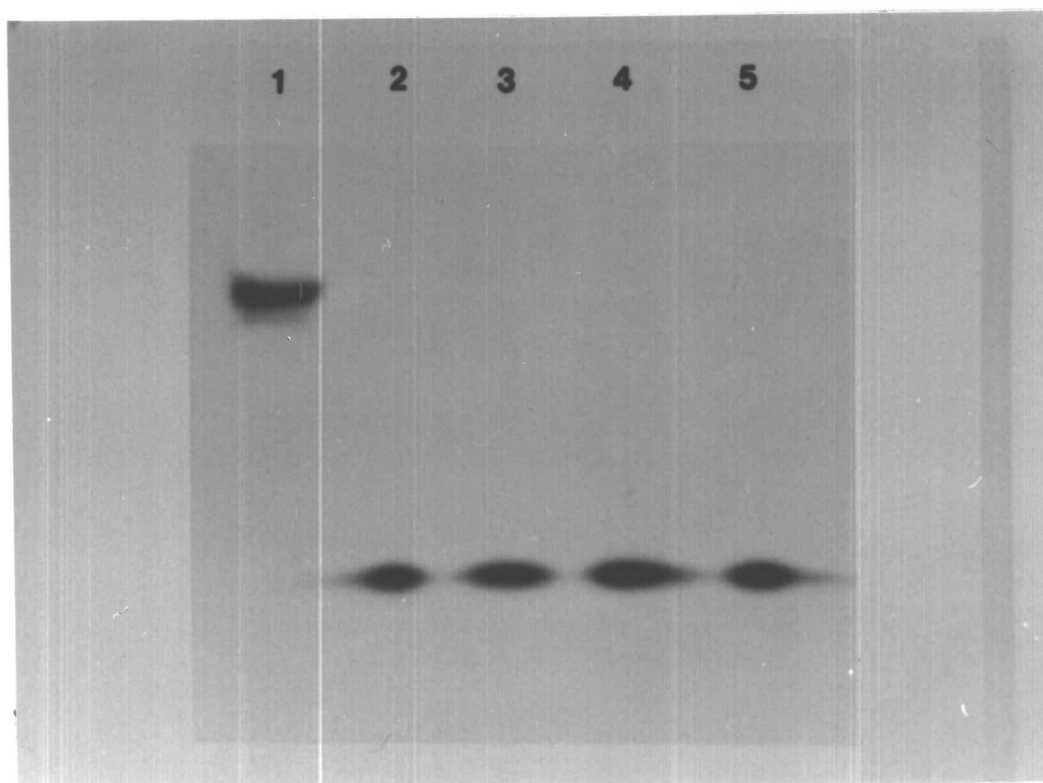


Figure 3-4

(Donis-Keller, 1979; Mankin et al., 1981; Tapprich and Hill, 1986). The results of such an experiment with probe 520 are shown in Figure 3-5. The 16S rRNA used in these experiments was 3'-end labeled. Specific cleavage in the presence of probe 520 would and did yielded a 3' end-radiolabeled fragment of about 1015 nucleotides in length (determined by using incomplete RNase T₁ digests of 3' end labeled 16S rRNA as marker in a separate experiment). Similar experiments with probes 1396, 1493 and 1533 have yielded only one RNase H digestion product and those were of the expected size (not shown).

3.4.4 Protection of DNA oligonucleotide probes in the presence of 16S rRNA, 30S subunits and 70S ribosomes. The protection of the site specific DNA probes 1396 and 1493 in the presence of 16S rRNA, 30S subunits and 70S ribosomes is shown in Figure 3-6A,B. In these experiments equimolar amounts of ribosomes or components from them were incubated with a 15 fold molar excess of individual DNA probes (5'-end labeled) and then subjected to nuclease S1 attack. After polyacrylamide gel separation and autoradiography the bands were cut out of the gel and quantitated by liquid scintillation spectrometry. The number of moles of DNA bound per mole of 16S rRNA, 30S subunits or 70S ribosomes were calculated from the specific activity of

Figure 3-5. RNase H cleavage of 16S rRNA in the presence of DNA 520.

Autoradiograph shows a separation of digestion products on a 3% denaturing polyacrylamide gel. The 16S rRNA was 3' end labeled with 5' [^{32}P]pCp and T4 RNA ligase. The DNA was present in 30 fold molar excess. The gel was 15 cm x 10 cm x 0.08 cm and was run at 6 mA for 2 hr 30 min. The specific activity of the labeled 16S rRNA was about 4×10^5 cpm/ μg . Lanes are as follows: Lane 1: intact 16S rRNA; Lane 2: 16S rRNA incubated with RNase H but in the absence of DNA 520; Lane 3: 16S rRNA with DNA 520 and RNase H from calf thymus; Lane 4: 16S rRNA with DNA 520 and RNase H from E. coli.

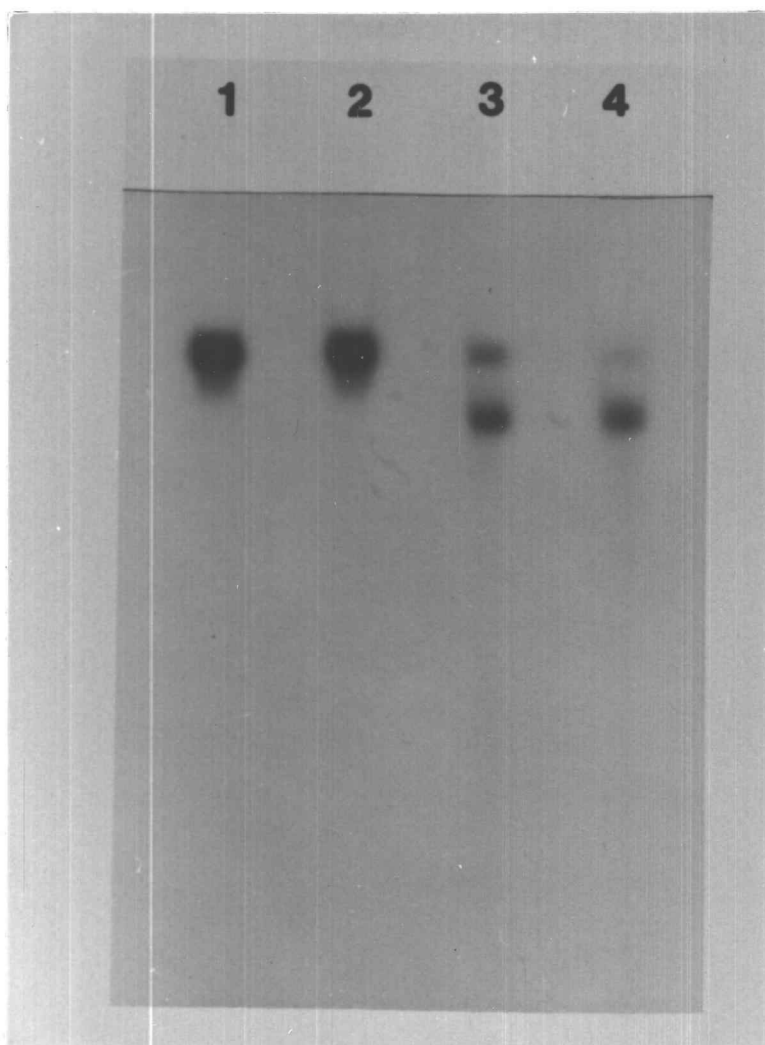


Figure 3-5

Figure 3-6. Protection of region specific DNA probes from nuclease S1 digestion in presence of ribosomal components.

Fifteen-fold molar excess of DNAs 1396 (A), 1493 (B) were hybridized to 1.67×10^{-13} moles of 16S rRNA (lanes A4, B2), 30S subunits (lanes A3, B3) and 70S ribosomes (lanes A2, B4), and then digested with nuclease S1 as described in Materials and Methods. Lanes A1 and B1 shows undigested DNA 1396 and 1493. Electrophoretic conditions are identical to those described in Figure 3-1.

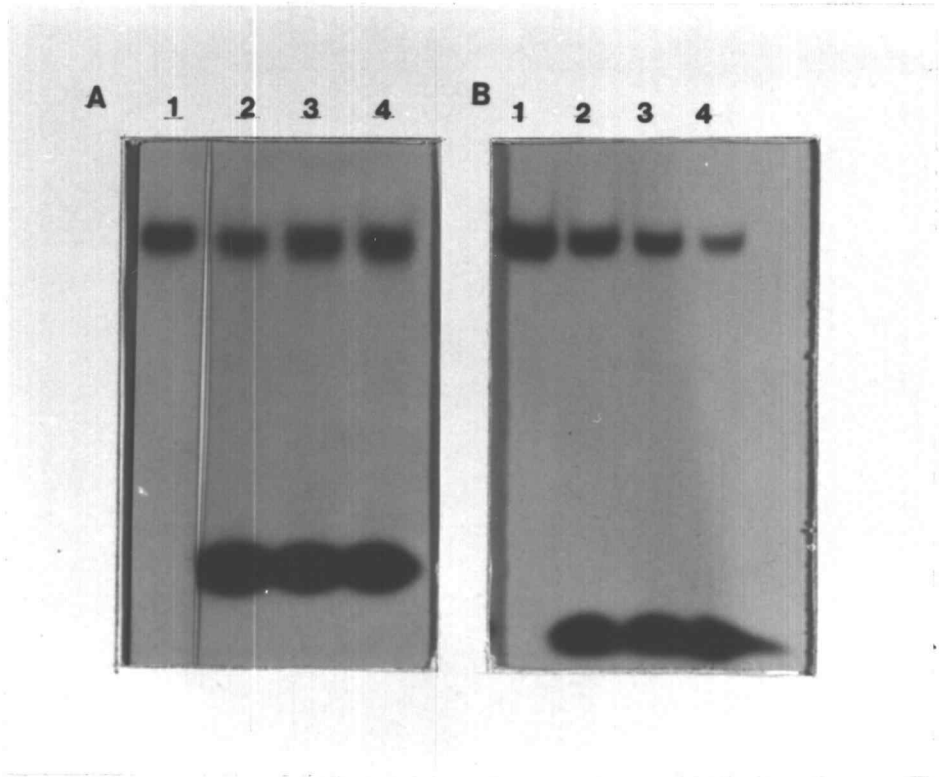


Figure 3-6

the respective probes. The binding stoichiometry was determined for all of the DNA probes by this method and the results are presented in Table 3-3. These experiments were also conducted in the presence of unlabeled mixtures of each of the other probes. Each unlabeled probe was present at 20 fold molar excess over the ribosomal component. The results of those experiments were no different from those observed when the individual probes were present. This means that there are no cooperative interactions which would reduce or increase probe binding, and that each of the probes bound to unique positions.

Table 3-3 Binding stoichiometry of DNAs
complementary to 16S rRNA.^a

DNA	DNA/16S	DNA/30S	DNA/70S
520	0.87	0.69	0.61
1396	0.99	0.90	0.57
1493	0.80	0.73	0.24
1533	1.02	0.73	0.30

a: Expressed as molar ratios.

3.5 DISCUSSION

Hill et al. (1986) have used RNase H and hybridization of synthetic DNAs to probe the structure of 16S rRNA in 30S ribosomal subunits. They quantitated DNA/RNA hybrid formation on 30S particles isolated by zone ultracentrifugation or with a nitrocellulose filter assay. Neither of these methods provide clear quantitative information nor do they confirm whether the DNA/particle interaction is complete and specific. To overcome this difficulty, they employed RNase H cleavage of the RNA/DNA hybrid in 30S particles to provide a confirmation that the target site was specifically hybridized with the DNA probe, but the cleavages were not stoichiometric. In addition, as few as four complementary bases in a DNA/RNA hybrid are sufficient for RNase H cleavage (Donis-Keller, 1979). This means that the assay really only confirms the presence of a single stranded zone which is potentially much smaller than the probes that were used. Furthermore, transient hybrid formation between portions of the DNA probe and RNA outside of the target site during the RNase H digestion could unfold the particle and artifactually make the intended target site accessible. In this regard, it is also worth noting that for the assay to be successful the site of the DNA/RNA hybrid must be accessible to RNase H. Other methods used to probe single strandedness in

ribosomes also probe only one or two bases and not entire lengths of RNA within particles (Dowthwaite et al., 1983; Ehresmann et al., 1987; Noller, 1974; Woese et al., 1980). These approaches also offer limited quantitative information and may, as with RNase H, introduce undesirable perturbations in the particles during the analysis. The experiments described here overcome these difficulties, permitting a direct quantitation and size assessment of a single-stranded zone within a 30S particle or ribosome. The novelty and virtue of this approach centers upon the elimination of artifactual generation of hybridizable sites during the analysis. This is because the hybrid is formed first, followed by nuclease S1 digestion under conditions that rapidly destroy excess probe, as well as single-stranded nonhybridized target sites in the ribosomes. Since both the target-site and probe are destroyed by nuclease S1, the post-hybridization analysis is not prone to the artifacts that other approaches embody.

Ribosomal RNAs are only functional in protein synthesis when they are complexed with ribosomal proteins. Here we have described the use of nuclease S1 to determine whether a specific portion of 16S rRNA is single stranded and accessible for hybridization in 70S ribosomes or 30S subunits. With this approach it is also possible to approximate the proportion of the

particles which are in a configuration that permits the interaction to occur.

Based upon the data presented here, it would appear that the zones encompassed by all of the probes are accessible in 30S subunits. This is consistent with observations made by others who probed for a single base or a few bases (Dowthwaite et al., 1983; Ehresman et al., 1987; Noller, 1974; Woese et al., 1980). However, in 70S ribosomes about half of the particles are open to probes 520 and 1396, while only one-third to one-fourth are open to 1493 and 1533. We prepared ribosomes from cells in a way which leaves tRNA and mRNA associated with them, but free of initiation or elongation factors. The differential access may then be due to close association between these RNAs and the universal sequences. For example, previously published investigations by others have provided strong evidence for placing tRNAs in the p site near the sequence with which probe 1396 interacts (Ciesiolka et al., 1985; Denman et al., 1988). The ribosomes which are open to the probe could be those in which peptide bond formation had taken place, but in which translocation had not yet occurred. The deacylated tRNA in the p site may then be shifted into a position which opens this part of 16S rRNA. The partial availability of the binding zone for probe 520 on 70S ribosomes may indicate that it too is associated with the basic mechanics of peptide elongation

and positioning of tRNAs.

The lower binding observed for probes 1493 and 1533 in 70S ribosomes may be caused by tRNA and or mRNA interactions. Probe 1533 covers a portion of 16S rRNA which has been shown to be important in the process of initiation through interaction with mRNA. This interaction at initiation may also sequester the sequence within the ribosome for the duration of the elongation phase in synthesis. Several three dimensional models have placed the 3' end of 16S rRNA close to the central portion of 30S subunits (Stern et al., 1988; Expert-Bezaneon and Wollenzien, 1985; Brimacombe et al., 1988). In this location it is positioned in a way which would make it unavailable once initiation of translation had taken place. Recent evidence implies that the 3' end anti SD sequence of 16S interacts with mRNA during elongation phase of translation (Weiss et al., 1988). Those 70S particles that are available for interaction with probe 1533 may be associated with ribosomes in a phase transition to the next protein coding sequence on polycistronic mRNAs.

The site complementary to DNA 1493 is believed to be involved in subunit association and thus might not be expected to be exposed in active 70S particles. However, if there is a phase transition between coding

zones on polycistronic mRNAs, as with site 1533, a fraction of the ribosomes may present accessible sites.

ACKNOWLEDGMENTS

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4. Inhibition of Translation and Antibacterial
Activity by New Short DNA Analogs

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4.1 Abstract

Protein synthesis, which takes place within ribosomes, is essential for the survival of any living organism. Ribosomes are particles composed of both proteins and RNA. Specific interaction between the 3' end CCUCC sequence of prokaryotic 16S rRNA and a partially complementary sequence preceding the initiating codon of mRNA is believed to be a prerequisite for initiation of protein synthesis. Here we report the use of short (3-6 nucleotides) synthetic DNA analogs complementary to this sequence to block protein synthesis in vitro and in vivo in Escherichia coli cells. In the DNA analogs the normal phosphodiester bonds were replaced by methyl carbamate internucleoside linkages to enhance transport across plasma membranes. Of the analogs tested, those with the sequence cAcGcG and cGcGcAcG inhibit protein synthesis, and colony formation by E. coli strains with and without an outer cell wall.

4.2 Introduction

Inhibition of cellular protein biosynthesis is an effective way of controlling bacterial propagation and infection. Streptomycin and tetracycline are examples of antibiotics that interfere with translation of mRNA through inhibition of functions associated with prokaryotic ribosomes (reviewed in Chopra and Ball, 1982; Cundliffe, 1980; Spirin, 1986). Streptomycin, an aminoglycoside antibiotic, exerts its effect by altering fidelity of translation and yielding proteins of incorrect primary structure (Anderson and Kurland, 1983). Incorporation of such proteins into the bacterial plasma membrane creates leaky membranes through which low molecular weight metabolites leak out (Anand et al., 1960). This causes cell death (Davis et al., 1986; Davis, 1987; Thompson et al., 1981).

Because ribosomes are central components of the protein synthetic apparatus, these types of antibiotics are of general biomedical utility. Sensitivity to these antibiotics is dependent upon the presence of ribosomal components, usually ribosomal proteins, but sometimes rRNAs (Moazed and Noller, 1987; De Stasio et al., 1989). The primary structures of the molecular targets of these antibiotics on the ribosome are known to vary in different strains. One major limitation of these natural antibiotics is that the primary structures of these molecules can vary

from strain to strain and bacteria can develop resistance to the antibiotics (Birge and Kurland, 1969; De Stasio et al., 1989; Ozaki et al., 1969; Spirin, 1986). This implies that there is no great pressure to conserve the target molecule for the antibiotic in the ribosome. To obtain a generally stable antibiotic--one for which resistance is not achievable through primary structural changes in the sensitive molecule--the target locus should be universally conserved in the target organism. In addition, an antibiotic must be easily transported through the cell membrane and bind to the target locus with high specificity.

In considering the development of a broad spectrum prokaryotic antibiotic the highly conserved anti Shine and Dalgarno (antiSD) sequence CCUCC sequence at position 1535-1540 in prokaryotic 16S-like ribosomal RNA presents an ideal target. This sequence is highly conserved in all prokaryotic 16S-like rRNAs but not conserved in eukaryotic 18S-like rRNAs (reviewed in Gutell et al., 1985; Woese et al., 1983; Brimacombe and Steige, 1985). It is now accepted that this part of 16S rRNAs forms a transient hybrid with a portion of mRNA, called the Shine and Dalgarno (SD) sequence, preceding the initiating AUG start codon (Shine and Dalgarno, 1974; Steitz and Jakes, 1975). Organisms which have alterations in the antiSD sequence in small subunit ribosomal RNAs have been reported to be defective in protein synthesis (Dunn et al., 1978; Hui and

de Boer, 1987; Jacob et al., 1987; Shinedling et al., 1987; Singer et al., 1981). Recently Weiss et al. (1988) have reported that during the elongation phase of translation the antiSD sequence participates in reading frame shifts in naturally occurring mRNAs.

Jayaraman et al. (1981) have investigated the antibiotic activity of nonionic oligonucleotide analogs complementary to the 3' terminal ACCUCCU sequence of E. coli 16S rRNA. The analogs were chemically synthesized and had methyl phosphonate linkages in place of normal phosphodiester bonds. These nonionic DNA oligomers manifested modest antibacterial activity only with E. coli mutants with altered permeability properties, that is they did not have an outer cell wall. Normal E. coli cells were not affected by the synthetic DNAs. However, normal cells were permeable to trinucleotides but not pentanucleotides or longer sequences (Jayaraman et al., 1981). The former would be less likely to form stable hybrids with 16S rRNA under normal 37°C growth conditions.

Here we report the testing for antibacterial activity of novel short DNA analogs complementary to portions of the antiSD sequence. These analogs differ from the uncharged diastereoisomeric and/or stereoirregular methyl phosphonate analogs reported by Miller and coworkers (1981) and Jayaraman et al. (1981) in that they are stereoregular and joined by methyl carbamate internucleoside bonds. PEG₁₀₀₀ (poly-ethylene glycol, MW 1000) was attached to the 5' end

of some of the test DNAs. This was done to enhance the solubility of the modified DNA analogs. The analogs with neutral internucleoside linkages are called neutral genes or "neu-genes". We have found that neu-genes cAcGcG and cGcGcAcG which are complementary to CCUCCU (position 1536-1439) in the 3' end of 16S rRNA inhibit in vitro protein synthesis and manifest a bactericidal effect upon permeable cells without cell walls. E. coli with a cell wall were also sensitive to cAcGcG and cGcGcAcG but only when PEG₁₀₀₀ was attached to the 5' end of the analog DNAs.

4.3 Materials and Methods

4.3.1 The Neu-genes

The synthesis of the neu-genes were carried out according to the method described by Stirchak et al. (1987). Phosphodiester bonds of the oligonucleotides have been replaced by neutral stereoregular methyl carbamate bonds (comparison of methyl phosphonate and methyl carbamate internucleoside linkages is shown in Figures 4-1 and 4-2 respectively). The neu-gene cGcGcAcG with PEG₁₀₀₀ attached at the 5' end is depicted in Figure 4-3. The sequences which were tested are listed with their code designation in Table 4-1.

The analog DNAs were solubilized in DMSO and diluted into dd-water before use. Typical working concentrations were in the range of 40-50 μM for in vitro translation assays and 160-200 μM for cell growth experiments in M9 medium.

4.3.2 DNA oligomers with normal Phosphodiester Bonds

Test oligonucleotides with normal phosphodiester bonds (DNA 520 and 1533) were supplied by the Central Service Laboratory for the Center for Gene Research and Biotechnology, Oregon State University. The DNAs were synthesized using the phosphoramidite chemistry method

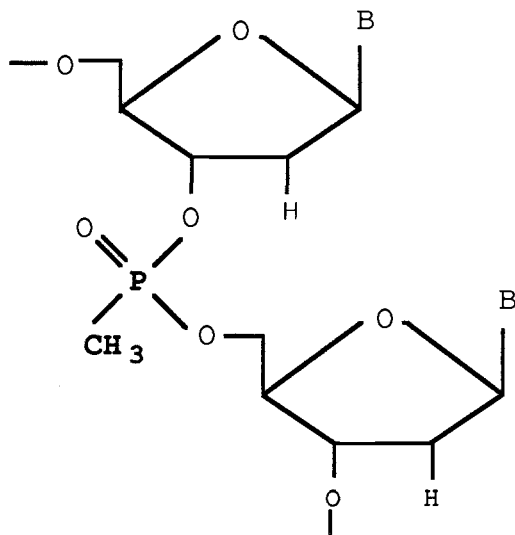


Figure 4-1 Internucleoside linkage with methyl phosphonate bonding. B indicates any of the bases A,T,G,C

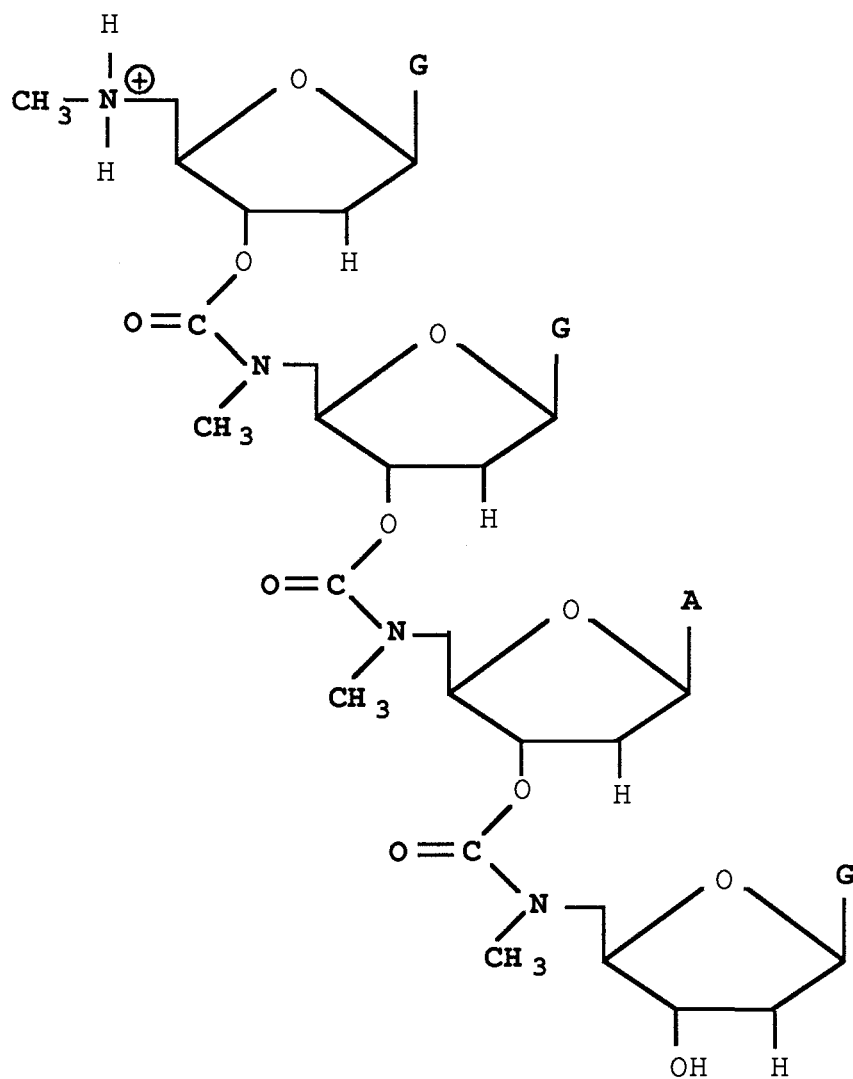


Figure 4-2 Methyl carbamate internucleoside linkage of neu-genes. cGcGcAcG sequence shown.

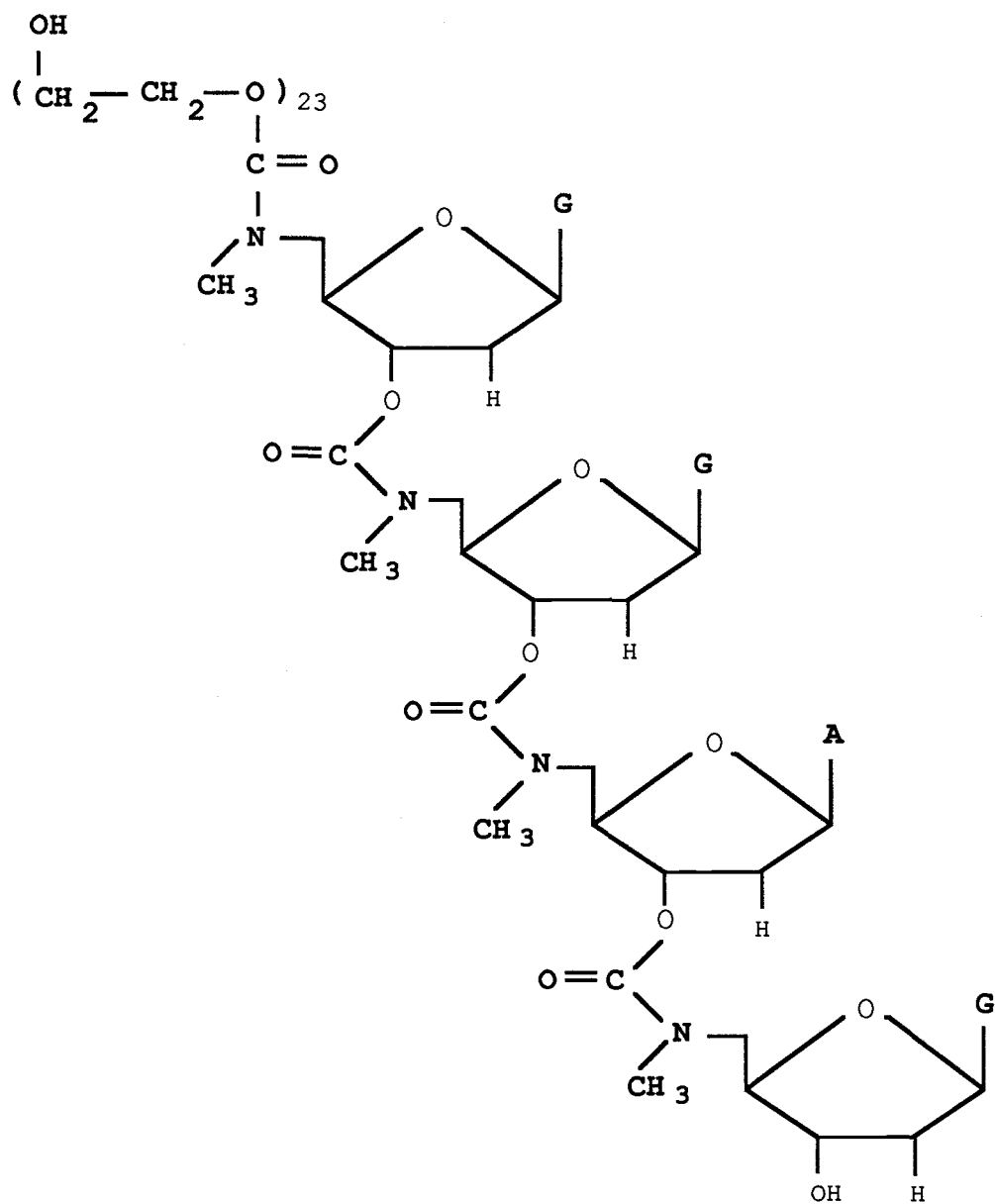


Figure 4-3 Neu-gene cGcGcAcG with PEG₁₀₀₀ bonded to the 5' end

Table 4-1 The neu-genes with their code designation and sequences.

Neu-gene	Sequence ^a
N88-14	*cGcGcAcG
N88-15	*cAcGcG
N88-16	cGcGcAcG
N88-17	cAcGcG
N88-18	*cAcAcGcG
N88-19	*cGcGcAcGcG
N88-21	cAcAcGcG
N88-22	cGcGcAcGcG
N89-24	*cAcGcGcT
N89-25	cAcGcGcT
N87-26	cAcAcAcAcAcA
N87-27	*cAcAcAcAcAcA
N89-28	*cAcGcGcbrU
N89-29	cAcGcGcbrU

a: * indicates Poly Ethylene Glycol (MW 1000) attached to the 5' end of sequence.

c indicates carbamate internucleoside bonding.

(Beaucage and Caruthers, 1981; Matteucci and Caruthers, 1981) on an Applied Biosystems model 380B DNA Synthesizer. Before using the DNAs were purified by electrophoresis in 20% polyacrylamide gel. The lengths, sequences and their positions on E. coli 16S rRNA is shown in Table 4-2.

4.3.3 Cells

E. coli ML 308-225 cells were kindly provided by Prof. Chien Ho, Carnegie-Mellon University, PA. These cells have altered permeability properties which allow DNA analogs to readily pass through their membranes and this strain was used by Jayaraman et al. (1981). E. coli D-10 cells which are RNase I- and auxotrophic for methionine were used as control cells with normal permeability properties.

4.3.4 Other chemical and Biochemicals

Bacteriophage MS2 was obtained from Boehringer Mannheim Biochemicals. Other chemicals were purchased from Sigma Chemicals and were analar or reagent grade. Bacto dextrose was a product of BDH Chemicals. ³H- Lysine (40-60 Ci/mmol) was purchased from ICN Radiochemicals.

4.3.5 In vitro Translation Assay

For in vitro translation, the initiation factors and

Table 4-2 DNA oligomers with normal phosphodiester bonds.

DNA	position ^a on 16S rRNA	Sequence (5' to 3')
520	520-530	CCGCGGCTGCT
1533	1533-1542	GTGGAGGAAT

a: E. coli 16S rRNA 5' to 3' direction.

the supernatant (S-100) fraction were prepared according to the methods described by Hershey et al. (1981). The translation reactions were carried out as previously described (van Dieijen et al., 1969; Abdul-Latif and Schaup, 1988). Test DNA (final concentration 40 μ M) was added to the translation system at the same time as MS2 mRNA to start the reaction. Reactions (100 μ l) were incubated at 37°C for 30 min. After 30 min 0.1 ml BSA (1 mg/ml) was added to each reaction before adding 1 ml of 10% Trichloroacetic acid (TCA) to stop the reaction. This was followed by heating at 75°C for 15 min after which the tube containing the mixture was transferred to an ice bath. Precipitates were collected on GF/C glass fiber filters in an Amicon filter apparatus and washed with 3 ml of cold 10% TCA. The filters were then dried and the precipitable radioactivity was determined by placing the filters in 10 ml of scintillation cocktail containing 0.55% PPO (2,5-Diphenyloxazole) and 0.05% Dimethyl POPOP (1,4-bis[2(4-methyl-5-phenyloxazolyl)]benzene), (Sigma Chemicals).

4.3.6 Test for Colony Formation

Mid-log phase E. coli D-10 and E. coli ML 308-225 cells grown in M9 medium (Maniatis et al., 1982) supplemented with 1.5% bactodextrose (BDH Chemicals) and 0.03 mg/ml L-methionine (Sigma Chemicals) were used to investigate the

effect of the modified DNA oligonucleotides on colony formation. Under sterile conditions 10 μ l of cell cultures (approximately 0.05 A₅₆₀/ml) were thoroughly mixed with 1 ml of M9 medium. From this mix 10 μ l were aliquoted to a sterile Eppendorf tube containing 400-500 μ g (approximately 160-200 μ M) of test DNA in 100 μ l of M9 medium. After the cells were incubated in a shaking water bath at 37°C for 2 hours, the samples were diluted to 300 μ l with M9 medium. A 100 μ l aliquot of the diluted growth medium was transferred and layered onto 10.0 cm plates containing 1.5% bactoagar in M9 medium. The final concentration of test DNA on the plates was in the range of 2.67-3.3 μ M. The plates were incubated at 37°C for 36-48 hours before colonies were counted. Controls included transfers of sterile media to monitor the effectiveness of procedures to maintain sterility and transfers into reactions where no test DNA was present were also done.

4.4 Results

4.4.1 In vitro translation

The effects of the various test DNAs on in vitro translation are presented in Figure 4-4. All of the analog DNAs were complementary to portions of the antiSD sequence in 16S rRNA. The attachment of PEG₁₀₀₀, for enhancement of solubility of neu-genes did not enhance their capability to inhibit in vitro protein synthesis. The sequences cAcGcG, *cAcGcG, cGcGcAcG and *cGcGcAcG have the greatest inhibitory effect on in vitro protein synthesis. A marginal effect was observed with cAcGcGcbrU and *cAcGcGcbrU.

The oligomers 520 and 1533, with normal phosphodiester bonds and complementary to positions 520-530 and 1533-1542, respectively, also inhibited in vitro protein synthesis at comparable levels (70% to 87%).

The alignment of these sequences relative to the 16S rRNA Shine and Dalgarno target zone are summarized in Figure 4-5. The stacking of two cAcGcGs to their target sequence is assumed to occur. The most effective inhibitory agent, cGcGcAcG, appears to embody elements of the other two inhibitory DNAs. DNAs which could form complements downstream or upstream of the cGcGcAcG appear to have a diminished effect on in vitro protein synthesis. However, the pentanucleotide analog cGcGcAcGcG did not interfere with protein synthesis. It is worth noting that

Figure 4-4 Effect of neu-genes on in vitro translation.

The results shown are for a typical experiment. The range of values for three sets of experiments was $\pm 15\%$. DNA analogs (40 μ moles) were added to an in vitro translation system derived from E. coli. The system contained ^3H -Lysine in addition to the other 19 unlabeled amino acids. Addition of analogs preceded the addition of mRNA (MS2). Reactions were incubated at 37°C for 30 min followed by termination by the addition of 1 ml of 10% TCA and heating at 75°C for 15 min. The reactions were filtered through GF/C glass fiber filters which were then dried and incorporation of ^3H -Lysine into protein were estimated from the precipitable radioactivity.

Control reactions contained all the components of in vitro translation except DNA analog. Results are expressed as percent of control. Only those analogs which had effect are presented in the graph. *Indicates that PEG1000 was attached.

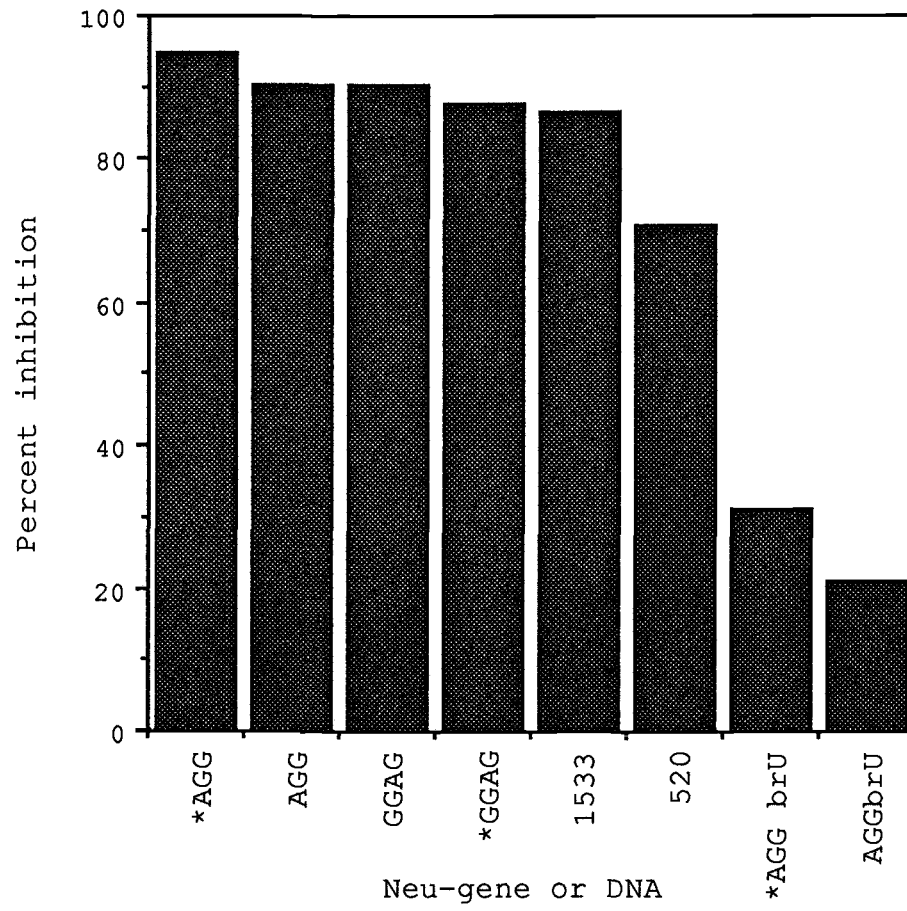


Figure 4-4

3' ← 16S rRNA — — — 5' 5' → DNA → 3'	% Inhibition (<u>in vitro</u>)
<u>AU</u> UCCUCCACUA	
AGGAGG ₁	90-94
<u>GGAG</u> ₁	87-90
<u>AGGbrU</u> ₁	37
<u>AGGT</u> ₁	0
<u>GGAGG</u> ₁	0
<u>AAGG</u> ₁	0

Figure 4-5 Alignment of some neu-genes with the antiSD sequence of 16S rRNA and their effect on in vitro translation. The sequence 5'-AGG-AGG-3' indicates the stacking of two trimers of 5'-AGG-3'.

this was the longest analog DNA complementary to the antiSD sequence which was tested.

4.4.2 In vivo evaluation using permeable cells

None of the test DNAs which possessed normal phosphodiester bonds was an effective inhibitor of colony formation by the permeable E. coli ML308-225 cells (Plate set 4-1). Of the analog DNAs, only cAcGcG, cGcGcAcG and cAcGcGcbrU inhibit in vitro protein synthesis (Figure 4-4) and manifest antibacterial activity in the permeable E. coli ML 308-225 cells (Figure 4-6, and Plate set 4-2).

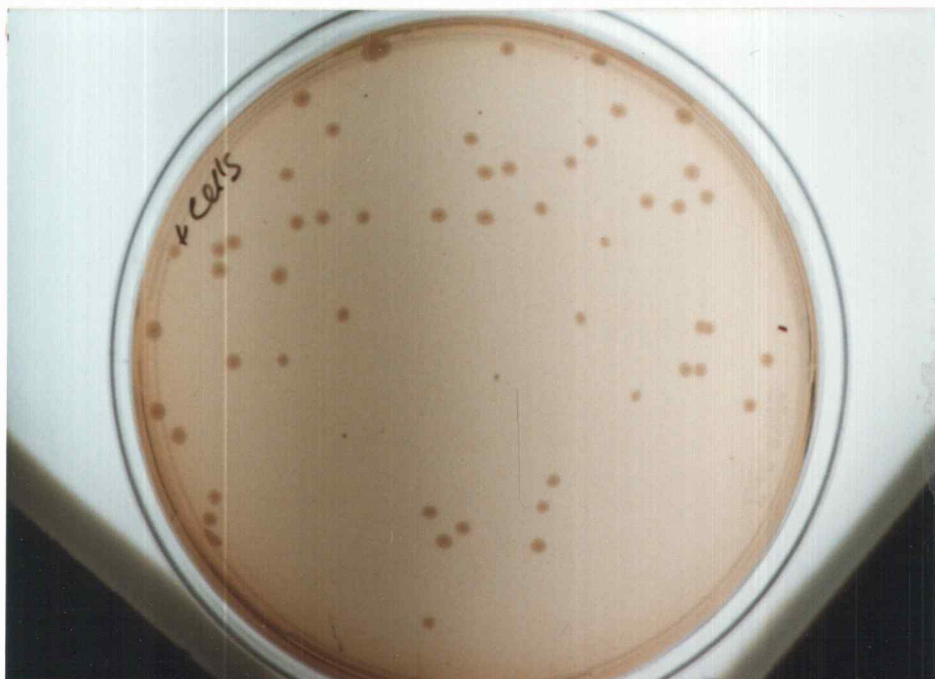
Surprisingly, though the five base sequence cGcGcAcGcG did not impair in vitro translation but manifested a modest effect (about 27% inhibition) on colony formation. The inhibition of cell growth by the test sequences AAAAAAA, and *AAAAAAA was observed (about 45%) and is not obviously correlated as an inhibitory mechanism associated with protein synthesis. The effect is, nevertheless, substantially lower than that observed for GGAG and AGGbrU. The remaining ten test oligonucleotide analogs which were complementary to the antiSD sequence gave virtually no effect on colony formation by the permeable ML308-225 cells.

Plate set 4-1 Effect of DNA 520 (B) and 1533 (C) on colony formation by the permeable E. coli ML 308-225 cells.

E. coli ML308-225 cells were initially grown in M9 liquid medium supplemented with L-methionine for 4-5 hours at 37°C in a shaking water bath. The cells were then diluted to a concentration of about 0.05 A560/ml in the same medium. Ten µl of this suspension was then aliquoted to 90 µl of M9 medium containing 160-200 µmoles of DNA analog. The cells were allowed to grow for 2 hours at 37°C. The growth medium were then diluted to 300 µl with M9 medium and mixed well. One third (100 µl) of the reactions were then transferred and layered onto 10 cm plates containing 1.5% bacto agar in M9 medium. The plates were incubated at 37°C for 48-72 hours and colonies counted. The control (A) was treated the same way except without DNA.

Plate set 4-1

A



B

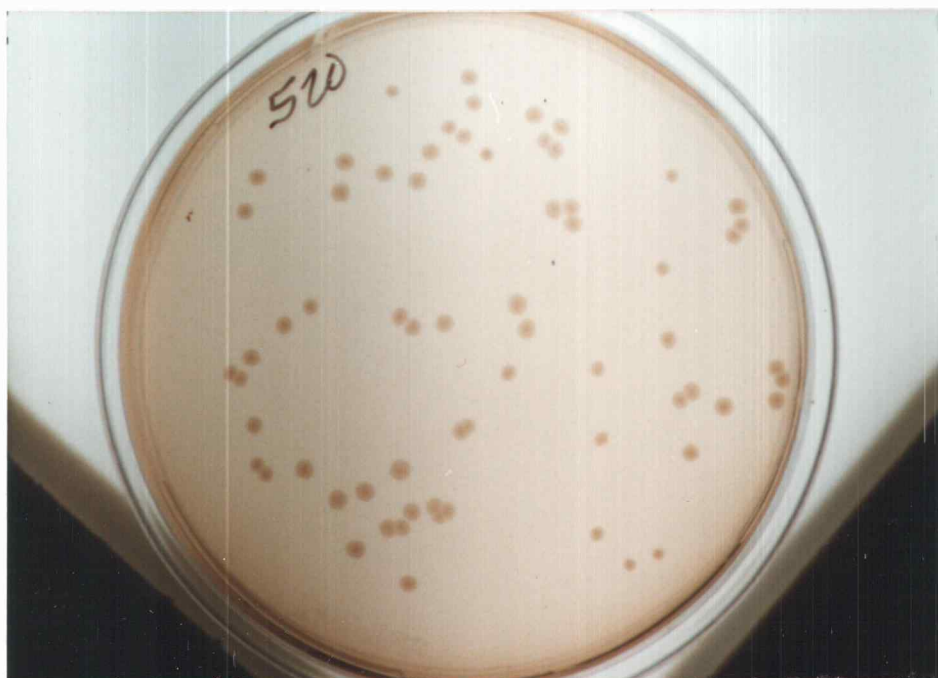


Plate set 4-1 continued

c

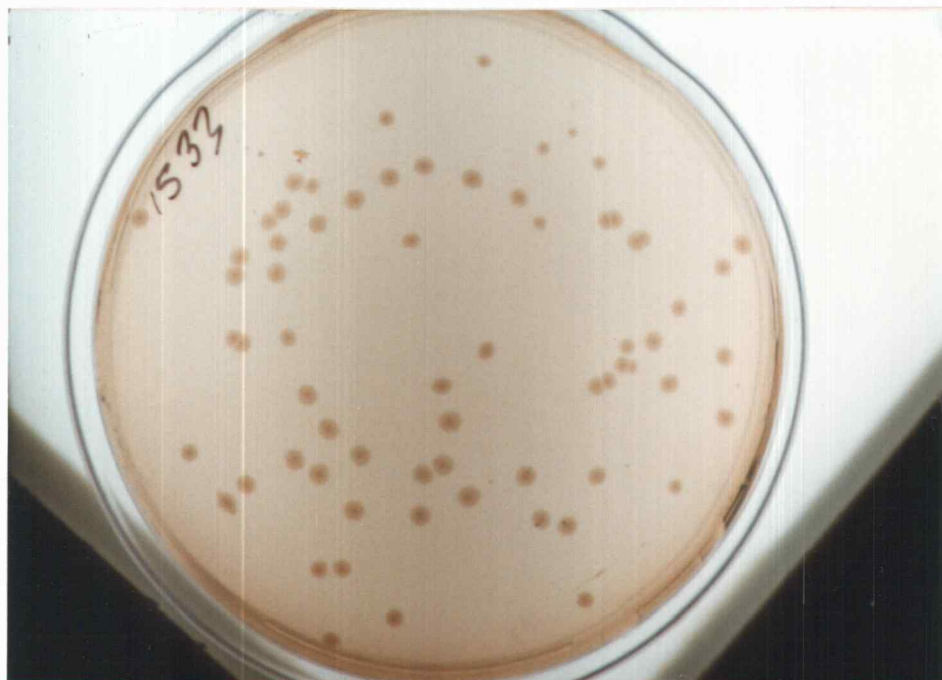
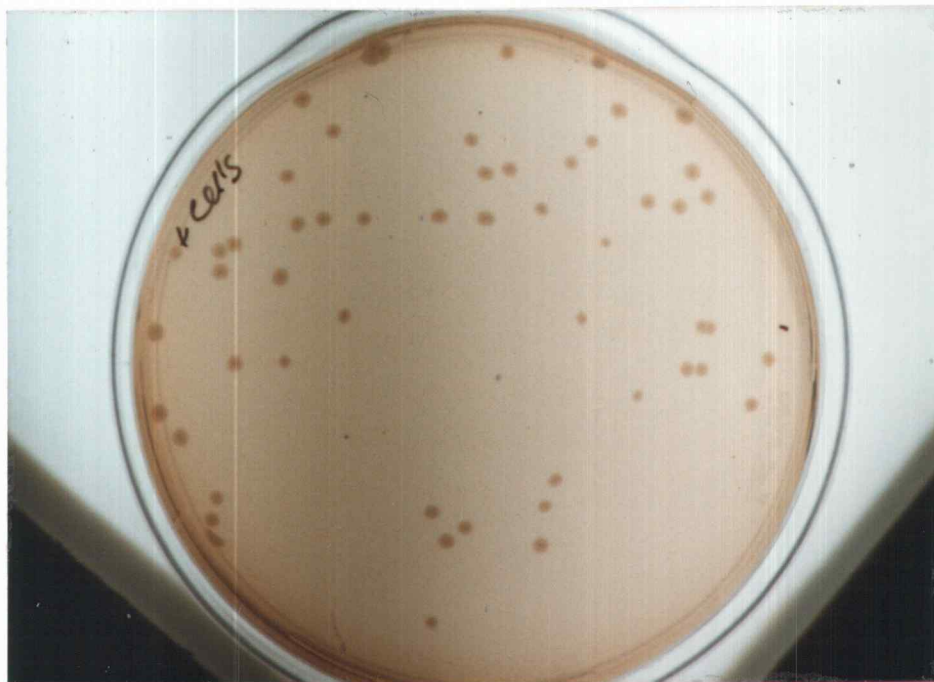


Plate set 4-2 Effect of neu-genes cGcGcAcG (B) and cAcGcGcbrU (C) on colony formation by the permeable E. coli ML308-225 cells. The growth medium and other conditions are as described in the legend of Plate set 4-1. The control (A) contained no DNA analog.

Plate set 4-2

A



B



Plate set 4-2 continued

C

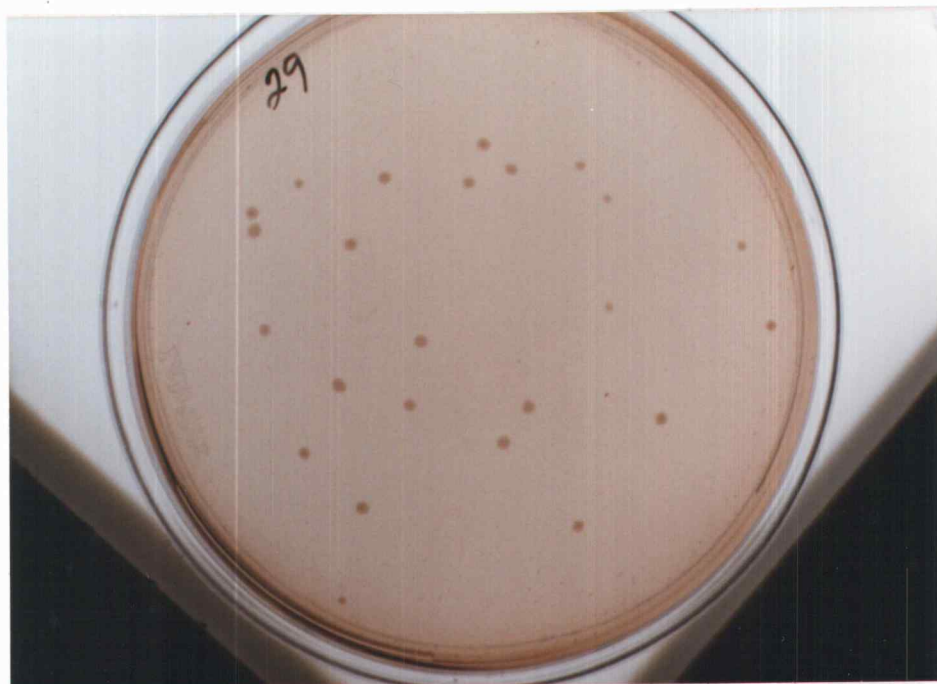


Figure 4-6 Effect of neu-genes on colony formation by the permeable E. coli ML308-225 cells.

The average number of colonies from six experiments as described in Plate set 4-1 and Plate set 4-2 were counted and compared with controls. The results are expressed as percent inhibition of colony formation. The range of values was $\pm 16\%$. Only those which have inhibited colony formation are presented.

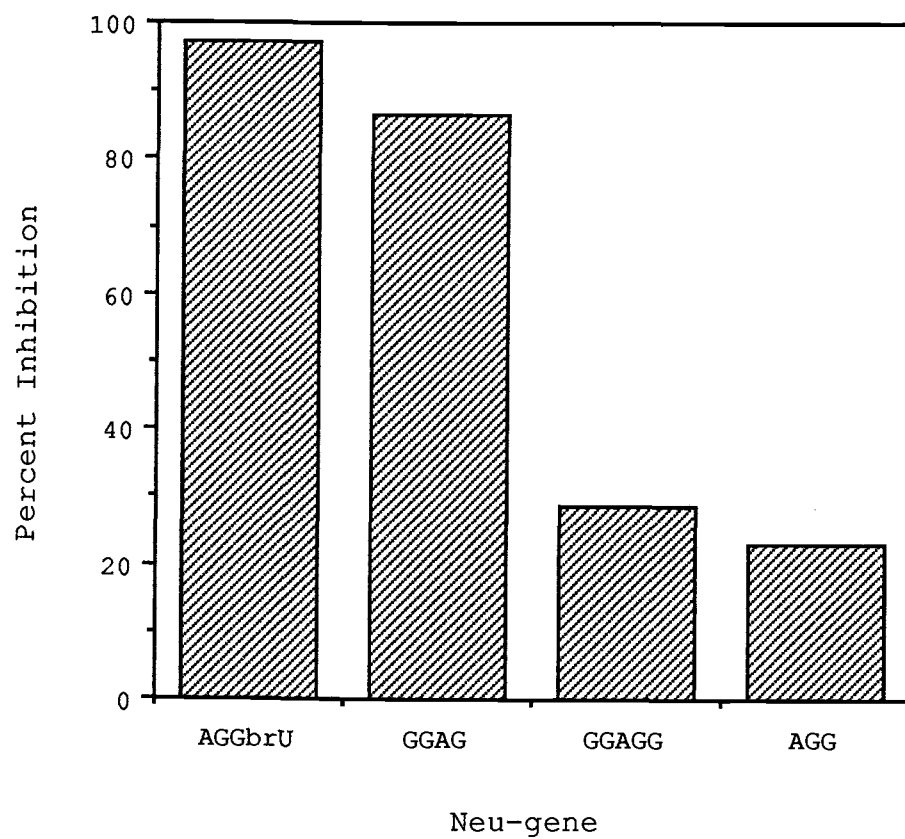


Figure 4-6

4.4.3 In vivo evaluation using nonpermeable cells

The experimental DNAs were also tested for antibacterial activity using a nonpermeable E. coli D-10 strain. The experiments were conducted using the same protocol applied in the permeable cell analysis. Analogs *mGmGmAmG, *mAmGmG and *AmAmGmGm had a substantial inhibitory effect on colony formation by E. coli D-10 cells (Plate set 4-3). The overall inhibition by these analogs calculated over ten experiments using 160-200 μ M of DNA was $75 \pm 6.5\%$. Surprisingly, the analog mAmGmGm_{br}U appears to have enhanced colony formation by E. coli D-10 cells. This result was reproducible using different preparations of the DNA analog. The same DNA with PEG attached showed no effect.

The DNA oligomers 520 and 1533 had no effect on colony formation by E. coli D-10 cells (data not shown).

Plate set 4-3 Inhibition of colony formation by E. coli
D-10 cells in presence of neu-gene *cAcGcG
(B), *cGcGcAcG (C) and cAcAcGcG (D). The
growth medium and other conditions are same
as described in the legend of Plate set 4-1.
The control (A) contained no DNA.

Plate set 4-3

A

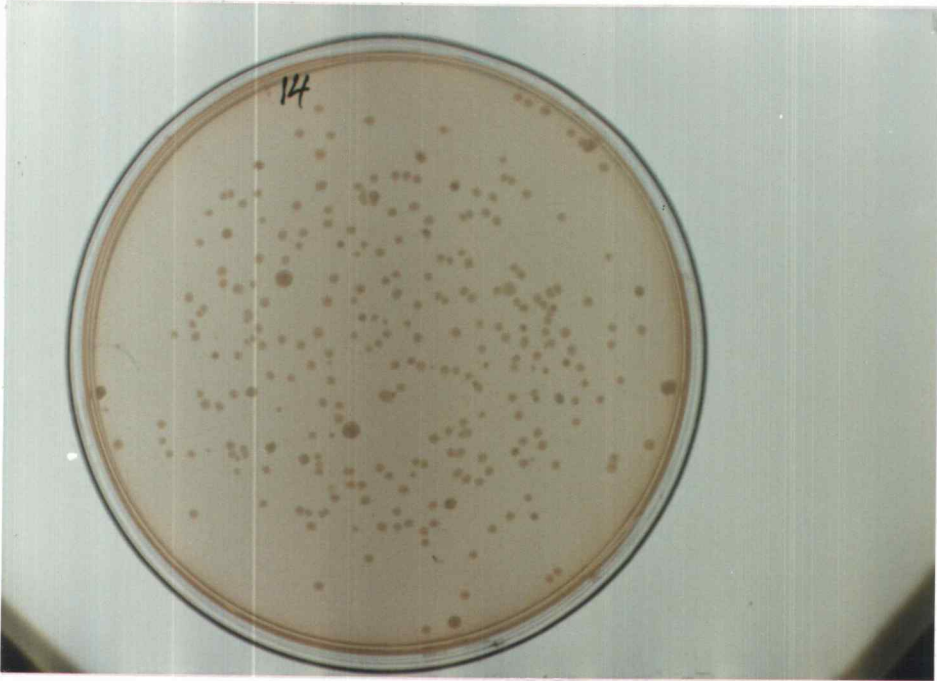


B

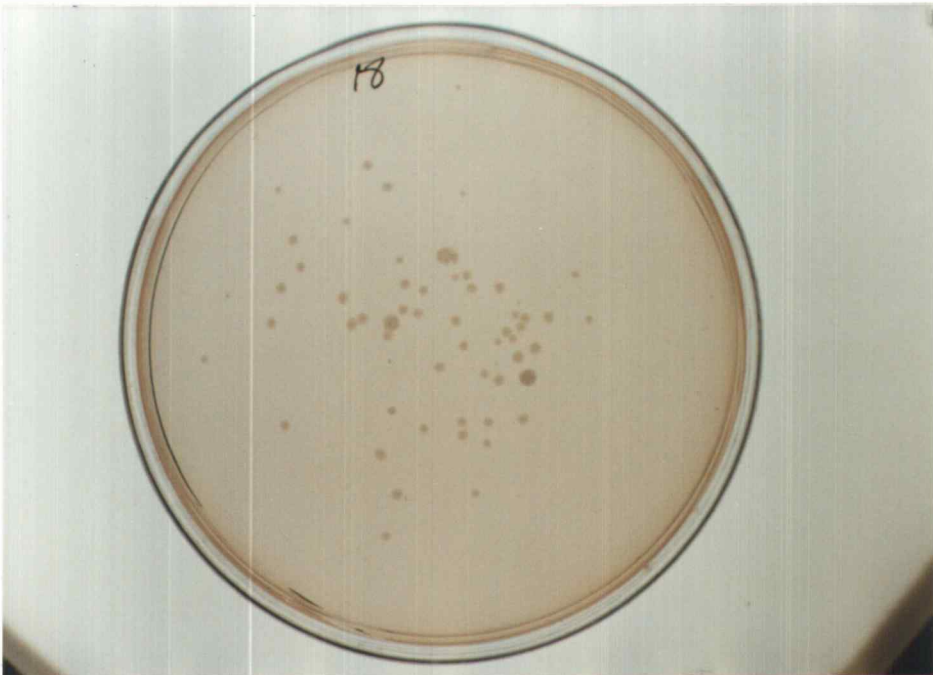


Plate set 4-3 continued

C



D



4.5 Discussion

DNA oligomers with methyl phosphonate bonds complementary to the antiSD sequence of 16S rRNA have previously been shown to inhibit prokaryotic in vitro protein translation systems. Unfortunately, this class of DNA analogs has proved to be ineffective as antibiotics when they were incubated with normal *E. coli* (Jayaraman et al., 1981). The DNA analogs with antibacterial activity which were used in this investigation had methylcarbamate linkages and were further modified by the 5' terminal addition of PEG₁₀₀₀. The PEG₁₀₀₀ was added to enhance the solubility of the oligonucleosides. The methylcarbamate linkages, as with methyl phosphonate bonds, were employed because of their neutrality and resistance to nucleases which attack phosphodiester bonds. In addition to PEG, the stereochemistry renders the glycosidic linkages resistant to glycosidases. The stereoregular methylcarbamate coupling desirable for durability may well limit the size for effective DNA analogs because of the rigidity of this back bone. For example, we observed inhibition of in vitro translation and *E. coli* D-10 colony formation by the oligonucleotide cGcAcGcG but not cGcGcAcGcG. Size alone cannot account for this observation because we found that in vitro translation was inhibited by an antiSD decanucleotide (5'-GTGGAGGAAT-3') which had normal phosphodiester bonds. Normal oligonucleotides which are

structural constraints. That is, normal oligonucleotides are flexible and more likely to form stable complements with a nucleic acid such as 16S rRNA which is structurally constrained within a particle such as a ribosome. We did observe a small effect for GGAGG in vivo with E. coli ML308-225. This may reflect GGAGG interaction with 16S rRNA when its complement is more accessible during the biosynthesis of 16S rRNA and the assembly of ribosomes.

The methylcarbamate linked tetramers cGcGcAcG and *cGcGcAcG were both very effective inhibitors of in vitro protein synthesis (Figure 4-4). However, they differed substantially in their inhibitory effect on colony formation by E. coli ML308-225 cells in that only the analog without PEG₁₀₀₀ attached was an effective inhibitor of colony formation (Figure 4-6). In contrast to this, with E. coli D-10 cells only the tetramer cGcGcAcG with PEG₁₀₀₀ tail inhibited colony formation (Plate set 4-3). The general observation for this work was that antiSD complements to the central CU and on the 5' side of the sequence are the most effective inhibitors of in vitro translation and colony formation. Of these complements only GGAG and AAGG interfere with colony formation in normal E. coli D-10. We think the data are reconciled if the PEG tailed analogs are viewed as binary antibiotics much like diphtheria toxin which inhibits protein synthesis in eukaryotes. We believe that the PEG directs insertion of the DNA analog into the outer cell wall system. The

analog is either placed into or transported to the periplasmic space where the PEG tail is enzymatically removed. The paradigm, with the free analog in the periplasmic space, approximates the situation with E. coli ML308-225 which is permeable to these compounds. Hence the oligonucleotide inhibits colony formation presumably through the inhibition of protein synthesis. The proposal then, is that the PEG prevents uptake of the analogs in cells that lack the periplasmic space with a high concentration of enzymes. Examination of the bond coupling the PEG to the analog (which resembles a peptide bond) and noting the extended nature of PEG itself leads us to speculate that a proteolytic enzyme may be responsible for the cleavage. Similar recognition of the carbamate bonds between nucleotides is most likely precluded by the bulk of the groups in the immediate vicinity of the bonds. A binary design for these types of antibiotics assures safety and specificity at two levels: cell surface and sequence specificity.

Curiously, we have observed that the sequence cAcGcGcbrU stimulated cell growth and colony formation by the normal E. coli cells. This stimulatory effect manifested by AGGbrU on D-10 cells contradicts the findings of in vitro result as well as in vivo results with the permeable cells where inhibitory activity was observed. An explanation may be that when this sequence comes in contact with the nonpermeable cells there may be an interaction

with one or more surface receptor(s) which triggers metabolic events that ultimately enhance DNA replication and cell division. Bacteria are known to have surface receptors which sense environmental conditions and cause intracellular cascades that alter the metabolic activity of the cell (Igo et al., 1989). Beyond this we have no reasonable explanation for this response. It does, however, raise a cautionary note when considering using antisense DNA analogs for therapeutic purposes. Similarly the hexanucleotides (cA)₆ with or without tail showed a modest inhibition of colony formation in the permeable cells, but they did not have any effect on in vitro translation or the normal E. coli cells.

All the evidence presented here indicate that DNA oligomers may prove to be potent antibacterial agents if they can be targeted on and introduced successfully into bacterial cells. The neu-genes used in this study were constructed to readily penetrate the bacterial cell membrane. As noted earlier, there may be a size limit for successful cell penetration as well as a size constraint dictated by the disposition of the target sequence.

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