

AN ABSTRACT OF THE THESIS OF

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Title: Modulation of Translational Fidelity by Small Subunit

Ribosomal RNA from Escherichia coli

Redacted for Privacy

Abstract approved: _____

Henry W. Schaup

Ribosomes are ribonucleoprotein particles that are essential for the process of protein synthesis in all living systems. Escherichia coli, eubacterial organisms, have ribosomes which display a 70S sedimentation coefficient. They are formed by the association of two ribonucleoprotein subunits which sediment at 50S and 30S. The 30S subunit is a construct made of 21 different proteins and 16S ribosomal RNA. The 3'-terminus of this rRNA was the focus of the research recounted in this dissertation. This tract of 10 nucleotides in rRNA is absolutely conserved in eubacterial organisms and is asserted to be crucial for proper initiation of protein synthesis in eubacteria.

Here I report the results of experiments done with 16S rRNA missing a part of the conserved 3'-terminus. These experiments were undertaken to provide a refinement of our understanding of the requirement for and function of this conserved segment of 16S rRNA. The deletions were made directly within mature 16S rRNA using RNase H and a 10 nucleotide synthetic DNA complementary to the 3'-terminus of

16S rRNA. The synthetic DNA was hybridized to 16S rRNA and then treated with RNase H. RNase H will only attack RNA when it is basepaired with DNA. This permits site-directed mutagenesis on the mature RNA. This deletion strategy efficiently yielded a 3'-terminal nucleotide deletion in E. coli 16S rRNA.

This 3'-terminal deletion did not impair in vitro 30S subunit assembly. Therefore, the conservation of the sequence is not necessary for ribosome assembly. To investigate the functional properties of the modified particles an in vitro protein translation system primed with a natural mRNA was employed. The mRNA was viral MS2 messenger. Modified particles did translate the MS2 mRNA but the fidelity with which the translation occurred was diminished.

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Ribosomal RNA from Escherichia coli

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DEDICATION

This thesis is dedicated especially to my father, my mother and the rest of my family, Along, Atan, Idah, Ani, Anwar, Ana and Nadia. I am so fortunate to have such wonderful people to encourage and support me to achieve my ambition.

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MODULATION OF TRANSLATIONAL FIDELITY BY SMALL SUBUNIT
RIBOSOMAL RNA FROM ESCHERICHIA COLI

1. INTRODUCTION

1.1 General Aspects of Protein Synthesis

Ribosomes are ribonucleoprotein particles found in all living cells. They play a fundamental role in the biosynthesis of proteins. They serve to bring a single mRNA and charged tRNA molecules into the proper position for translation. They are enzymatically active in forming peptide bonds during the polymerization process which yields proteins.

The molecular composition of the bacterial ribosome is well known and most of its macromolecular components are well characterized in terms of physical and primary structural characteristics. An Escherichia coli ribosome contains three species of ribosomal RNA (23S, 16S, 5S) and approximately 52 proteins. It is difficult to assign specific functions to each of the components since they appear to operate cooperatively in both ribosome assembly and translation. The two events involve protein-protein as well as protein-RNA interactions (1,2,3).

The overall process of protein synthesis can be divided into three stages; initiation, elongation and termination. The process in general is essentially the same in bacterial and eukaryotic systems.

However, at the molecular level there are major differences in the initiation phase. In eubacterias the initiation event takes place prior to the completion of transcription while in eukaryotes the mRNA is process post-transcriptionally and the initiation process takes place in cytoplasm (4). This difference has resulted in radically variant molecular requirements for initiation.

1.1.1 Initiation of Protein Synthesis

Figure 1 shows a schematic diagram for the initiation of protein synthesis in eubacteria. In eubacterial systems, initiation of polypeptide chain synthesis requires the binding of 30S subunits to a specific region of mRNA presumably involving Watson-Crick base pairing of the Shine-Dalgarno sequence with 3'-end of 16S rRNA. This is followed by fMet-tRNA binding to the initiator AUG codon. This process requires GTP and three initiation factors, IF-1, IF-2 and IF-3. IF-1 serves to increase the rate of dissociation of 70S ribosomes, while IF-2 functions to direct fMet-tRNA binding to the complex. IF-3 acts as an 'anti-association' factor which prevents premature 50S binding. All the initiation factors are released when 50S subunit binds. This forms the final 70S initiation complex and sets the system for the elongation phase (5,6).

Some primary differences between eubacterial and eukaryotic initiation are as follows: 1. In eukaryotes the initiator Met-tRNA is not formylated. 2. The 5' end of the mRNA is posttranscriptionally modified to generate what is called a cap structure. This latter feature is associated with ribosome attachment to the mRNA in a way

Figure 1-1 Schematic diagram showing initiation of protein synthesis
in eubacteria.

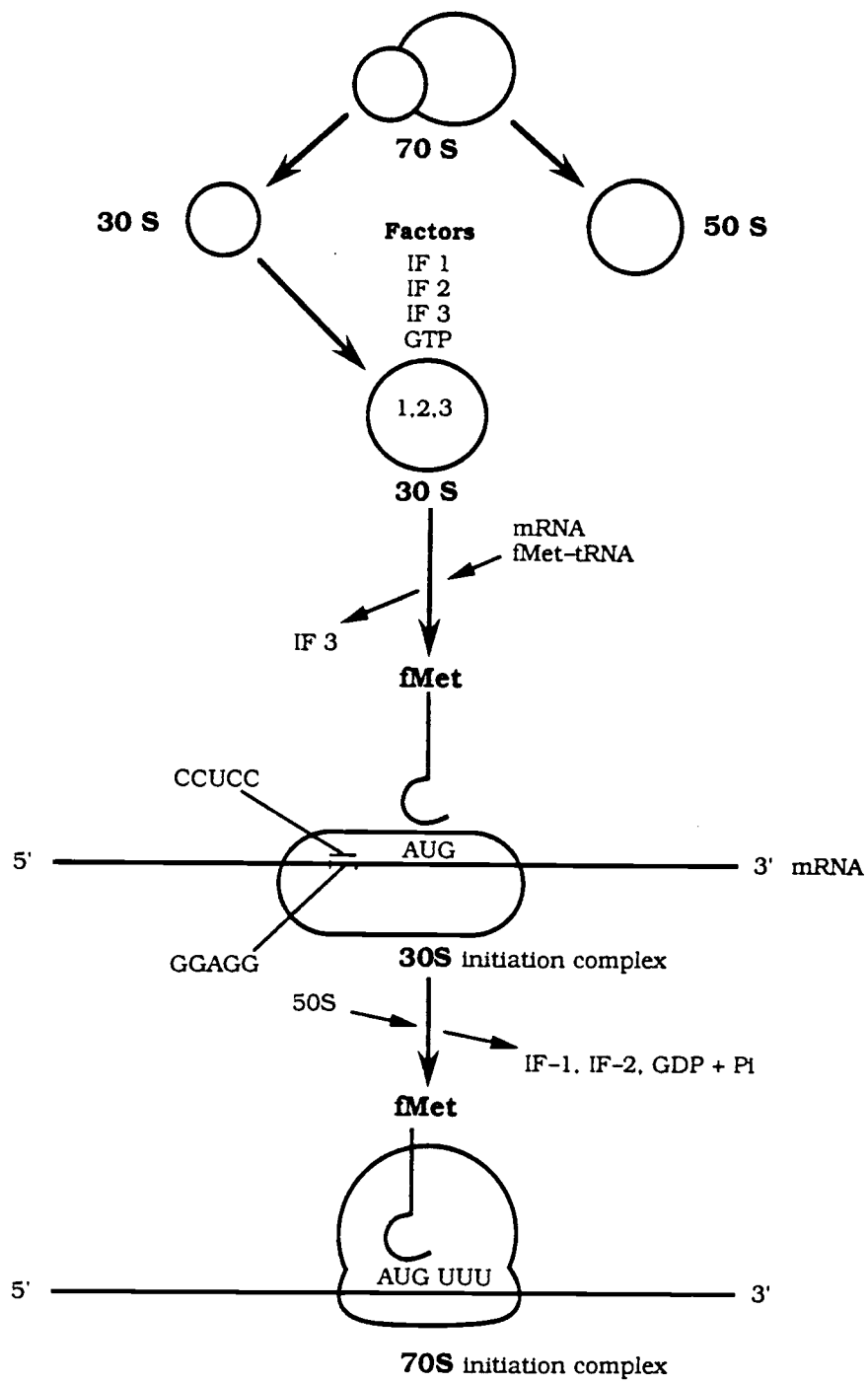


Figure 1-1

that allows it to scan for the initiator AUG (7). The initiation step involves complex formation of 40S subunit with the initiator tRNA before binding to 5'-end of the mRNA. The Shine-Dalgarno sequence is absent in eukaryotes. The actual binding of this complex is between the correct initiation codon on the mRNA with the anti-codon on the initiator tRNA. This is followed by the binding of 60S subunit to form 80S initiation complex. These events require GTP and involve many more initiation factors, eIF-1, eIF-2, eIF-3, eIF-4 (A,B,C,D,F), eIF-5, and eIF-6.

1.1.2 Elongation Step of Protein Synthesis

Once the initiation complex is formed, amino acids are added to the growing polypeptide chain one at a time in a sequence determined by base sequence of the mRNA. Figure 2 shows a schematic diagram of the elongation process of protein synthesis in eubacteria. In eubacterial systems elongation factor Tu participates in the placement of aminoacyl-tRNA into the A site and peptidyl transfer occurs from the P site to the amino acyl-tRNA in the A site. The translocation of the extended peptidyl tRNA from the A site to the P site requires elongation factor EF-G. The corresponding factors in eukaryotic systems are EF-1 and EF-2.

1.1.3 Peptide Chain Termination

Figure 3 shows a schematic diagram of the termination process of protein synthesis in eubacteria. In termination of protein synthesis,

Figure 1-2 Schematic diagram showing elongation of protein synthesis
in eubacteria.

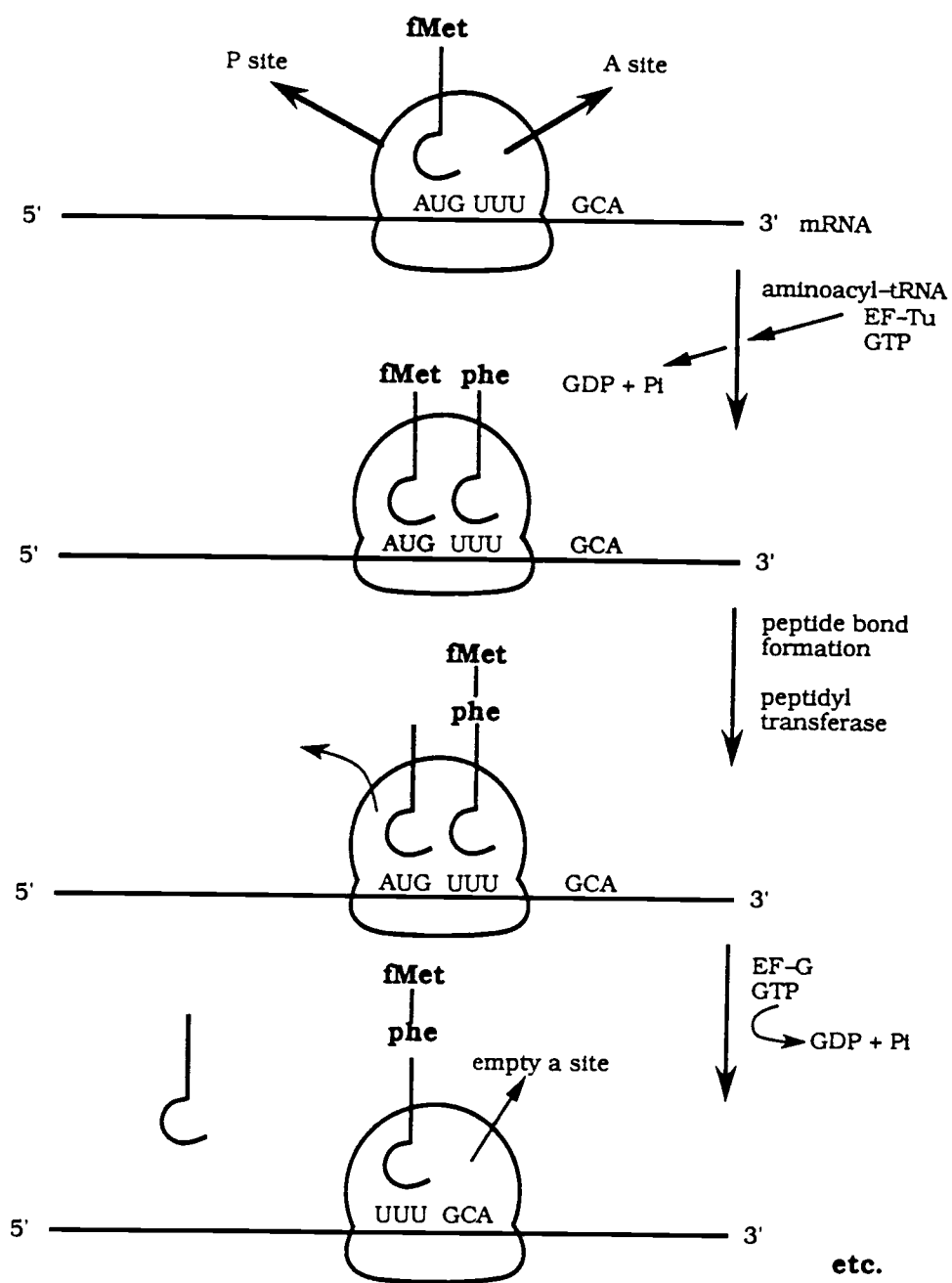


Figure 1-2

Figure 1-3 Schematic diagram outlining termination of protein synthesis in eubacteria.

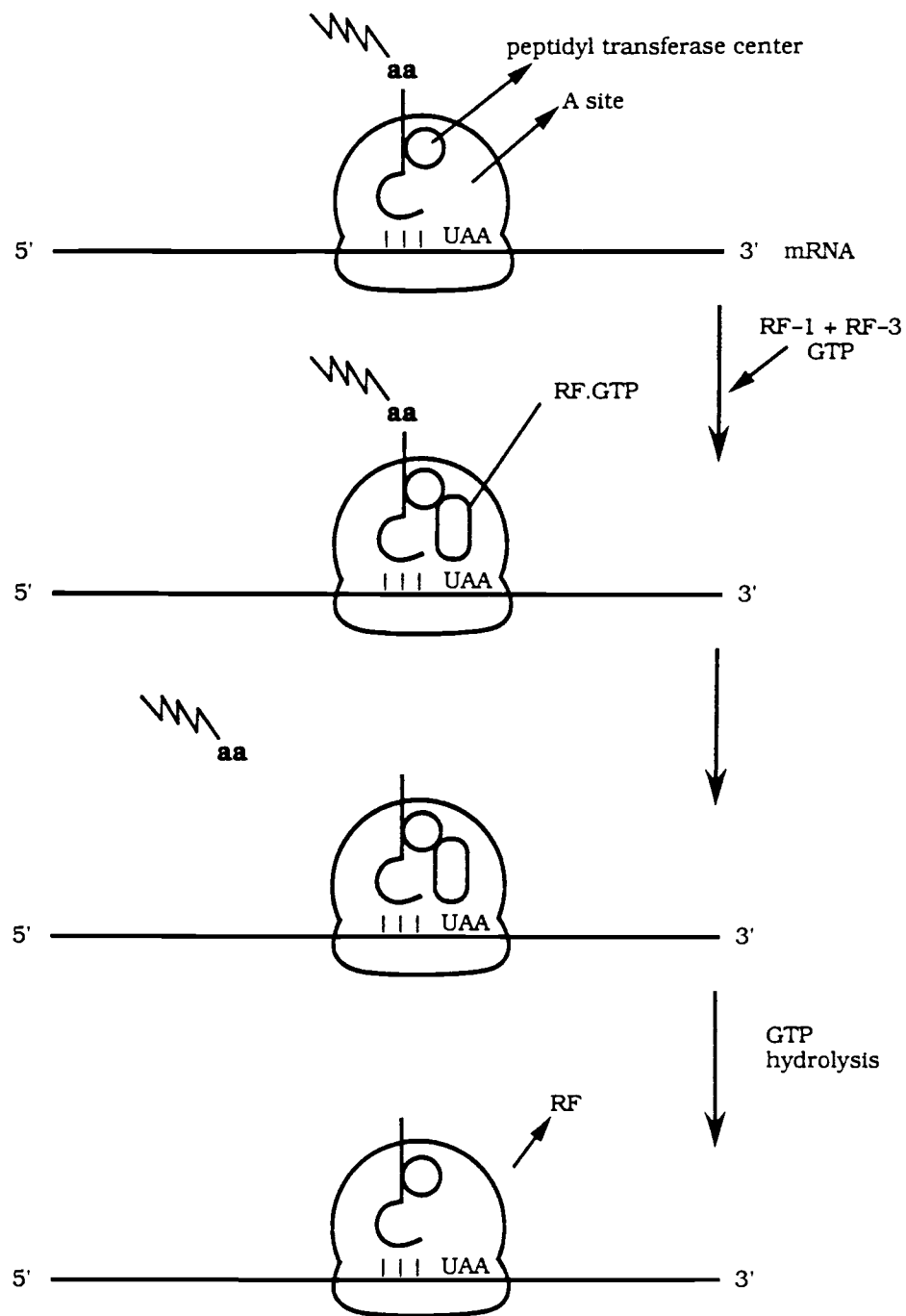


Figure 1-3

the process is directed by three specific codons UAA, UAG, and UGA. When any of these codons are found in the A site peptidyl transferase cleaves the nascent peptide from the tRNA in the P-site. This causes the release of the completed peptide from the ribosome. In eubacterial systems, this process is mediated by three protein release factors, RF1, RF2 and RF3. RF1 promotes termination in response to UAA, UAG, RF2 to UAA or UGA. RF3 stimulates the action of RF1 and RF2. A single protein termination factor, RF is operative in eukaryotic protein synthesis.

1.2 Regulation of Translation During Eubacterial Initiation

Ribosomal RNA, which is the major component of the ribosome, plays an important role in directing assembly of the particles. It is also likely to participate directly in the process of protein synthesis (8,9,10). An important question concerning the process of initiation concern how the ribosome finds the correct starting places on mRNA, and how this process is controlled. The initiation step is one of the major points at which protein synthesis is controlled at the translational level in bacteria (4).

In E. coli, at least two base-pairing interactions with mRNA are thought to be involved in specific initiation of protein synthesis. These are at the AUG initiation codon loop of fMet-tRNA, and a sequence to the 5'-side of the AUG (Shine-Dalgarno sequence) pairing with a sequence near the 3'-end of the 16S ribosomal RNA (11). The degree of complementarity of Shine-Dalgarno sequence to the 3'-end of 16S rRNA on the 30S subunit will affect the stability of the

initiation complex formed, and therefore regulate the amount of protein produced (12).

The initiation of protein synthesis can be negatively controlled by the binding of a specific protein within or near the initiation zones on mRNA. Such an event will interfere with the formation of ribosome initiation complexes. For example, if ribosomal protein S8 accumulates it will bind to the initiation region on the mRNA which codes for ribosomal protein L5. This will repress the translation of the corresponding downstream cistron on the mRNA (13,14). This type of regulation has been called autogenous regulation and appears to play an important role in balancing ribosomal protein synthesis with rRNA synthesis.

The structure of the mRNA also plays an important role in regulating the initiation process. Successful initiation occurs only when the zone required for proper initiation is largely single stranded. MS2 viral messenger RNA provides a good example of this type of passive regulation. The portion of the mRNA coding for the coat protein is preferentially translated while other proteins encoded on the mRNA are not. Secondary structural models of this mRNA clearly show that the coat protein initiation zone is much more single stranded than initiation regions for the other coded proteins (15,16,17).

1.3 The Significance of the Shine and Dalgarno Sequence in Eubacteria

The 30S ribosomal subunit from E. coli is an assemblage made of 21 different proteins and a 16S ribosomal RNA. The primary structure of

16S rRNA is now available and the primary structures and properties of many of the proteins have been reported (18). The 30S subunit's employment in protein synthesis and sensitivity to antibiotics are well documented (19,20). Although much progress has been made in understanding the functional role of the proteins in this particle, little is known of the functional attributes of the RNA with the exception of the 3'-terminal sequence centered around CCUCC. This sequence known as the anti Shine and Dalgarno sequence has a functional role in the initiation of protein synthesis (21,22,23). The accepted view is that this pyrimidine-rich sequence can base pair with a complementary messenger RNA sequence centered approximately 5 to 10 nucleotides behind the initiation codon. Shine and Dalgarno proposed that the formation of Watson-Crick base pairs between the mRNA and the 16S rRNA in the 30S subunit allows the ribosome to detect the initiation area (21,24). This proposal is supported by the results of investigations on bacteriophage T7 mRNA where two mutations in the initiation zone of the 0.3 protein were analyzed. In one mutation the AUG initiation codon was changed to an ACG. This mutation did not alter the binding of ribosomes to the message. The second mutation was a G-to-A transition located 11 bases to the 5'-side of the initiation codon and this caused the ribosome binding to shift 15 bases to the 3'-side of the initiation codon. As a result both mutations abolished the translation of 0.3 protein (11).

The functional interactions of the 3'-terminal portion of 16S rRNA with the Shine-Dalgarno sequence on the mRNA message are consistent with other reports concerning the initiation of protein synthesis. For example, colicin E3 treatment of 30S subunits blocks initiation.

This is attributable to the cleavage of a single phosphodiester bond at position 50 near the 3'-end of 16S rRNA (25). Kasugamycin blocks initiation of protein synthesis. Sensitivity to this antibiotic is mediated by the presence of two highly conserved dimethyl-adenosine located 23 and 24 bases from the 3'-end of 16S rRNA. It has also been reported that copolymers rich in A and G are the effective competitive inhibitors of initiation. This observation underscores the importance of polypurines in ribosome binding to the initiator regions on mRNAs (26,22).

Watson and Crick base-pairing of 3'-terminal parts of 16S rRNA with the Shine-Dalgarno sequence on mRNA appears to be dependent on the functional state of the 30S subunit. For example, 30S subunits lacking protein S21 do not bind deoxyoctanucleotide which is complementary to the 3'-terminal end of 16S rRNA. The addition of protein S21 restores the binding and in vitro initiation on MS2 mRNA requires the presence of S21 (27,28).

More recently, other investigators have presented evidence, using an altered 16S rRNA anti-Shine and Dalgarno sequence, which suggest that only maintenance of the base pairing properties are required for translation of the growth hormone messenger (29). Replacement of the Shine-Dalgarno sequence of the mRNA sequence essentially abolishes mRNA translation (30).

A single base change in the anti-Shine Dalgarno sequence at position 1538 has a dramatic effect on the level of synthesis of most cellular proteins. A single base changing from a cytidine to a uridine has been constructed using a synthetic oligodeoxynucleotide, and the presence of a mutated ribosome retards cell growth

dramatically (31).

Messenger RNAs produced from cloned heterologous human growth hormone genes altered in the initiation zone are preferentially translated by a subpopulation of ribosomes that have been modified within the 16S anti-Shine Dalgarno sequence to restore base pairing (30). The changes in the mRNAs were GGAGG to CCTCC or GTGTG. Translation of the modified mRNA by a wild type ribosome was observed but it was inefficient. Further work by these investigators showed that these specialized ribosomes, which poorly translate the endogenous mRNA, are unable to produce feedback regulation of ribosomal RNA synthesis (32). This conclusion was based on the observation that repression of the induction of rRNA and ribosome synthesis from the λ P_L promoter/operator by temperature shift normally observed with the wild type gene is essentially abolished by the mutation in the anti-Shine Dalgarno.

The anti-Shine Dalgarno sequence has also been reported to be associated with the mRNA during elongation of protein synthesis. Recently a group of investigators reported that Watson-Crick base pairing occurs during the elongation phase of protein synthesis in E. coli (33). This basepairing function appears to increase the level of frameshifting by a process called string-decoding tRNA. A single nucleotide insertion between the Shine-Dalgarno sequence and the shift site distrupts the high level of frameshifting.

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2. ESCHERICHIA COLI 3'-TERMINAL 16S rRNA SEQUENCE
MODULATED FIDELITY DURING TRANSLATION

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2.1 Abstract

The ribosome is a central component of the protein synthetic apparatus. Although progress has been made in characterizing the functional role for many of the ribosomal proteins, the properties of ribosomal RNA and its role in ribosome structure and function are not well understood. To investigate the working properties of the highly conserved 3'-end of 16S rRNA, a site-specific deletion was made directly within the 16S rRNA molecule. The terminal deletion did not impair in vitro 30S subunit assembly, but the particles produced lost translational fidelity in an in vitro translation system primed with natural mRNA.

2.2 Introduction

There are portions of eubacterial small subunit ribosomal RNAs which are highly conserved [1]. Clear functions for these areas within rRNA have not been denoted with the exception of the 3'-terminal sequence centered around CCUCC. The accepted view is that this pyrimidine-rich sequence can base pair with a complementary sequence located approximately 5 to 10 nucleotides upstream from the initiation codon in mRNAs. This sequence is often referred to as the Shine and Dalgarno sequence. In base pairing, this part of rRNA participates in the initiation of protein synthesis [2,3,4,5]. That the 3'-end of 16S rRNA plays a functional role in the initiation of protein synthesis is consistent with several different observations [6,7,8]. For example, crosslinking and chemical modification experiments have been used to show that this part of rRNA provides a binding zone for initiation factors, and within this region binding sites for ribosomal proteins S1, S2, S11, S12, S13, S14 and S19 are localized [9,10,11]. All of these proteins have been shown in one way or another to be involved in the initiation process [12]. In addition to this, mRNAs lacking or with altered sites in the region required for the complementary interaction with rRNA are inefficiently translated [13].

Although there can be little doubt that the 3'-terminal 16S rRNA sequence enhances initiation, it is not entirely evident why the interaction is necessary. Is it just for stabilization of the initiation complex or is it necessary for initiation factor interactions? Is it necessary for setting the proper reading frame or

an unidentified ribosome-mediated step associated with initiation? Other workers have presented evidence, using an altered 16S rRNA anti-Shine-Dalgarno, which suggest that only maintenance of the base pairing properties are required for translation of the growth hormone messenger [14]. To approach the problem from the perspective of rRNA function and to evaluate the absolute requirement for the CCUCC sequence zone the in vitro procedure, shown in Fig. 1, was used to remove the relevant nucleotides from 16S rRNA. The RNAs which were modified by the removal of this sequence were used for in vitro reconstitution and translation studies to appraise the effect of the alterations on ribosome assembly as well as function. In vitro reconstitution procedures for 30S ribosomal subunits are well established and reconstituted particles are functional in in vitro protein synthesis [15,16,17]. Removal of the sequence did not impair in vitro ribosome reconstitution. In vitro translation was observed using the bacteriophage MS2 mRNA, but the fidelity and efficiency of translation were diminished.

Figure 2-1 Schematic showing the strategy for RNase H 3'-terminal deletions in 16S rRNA.

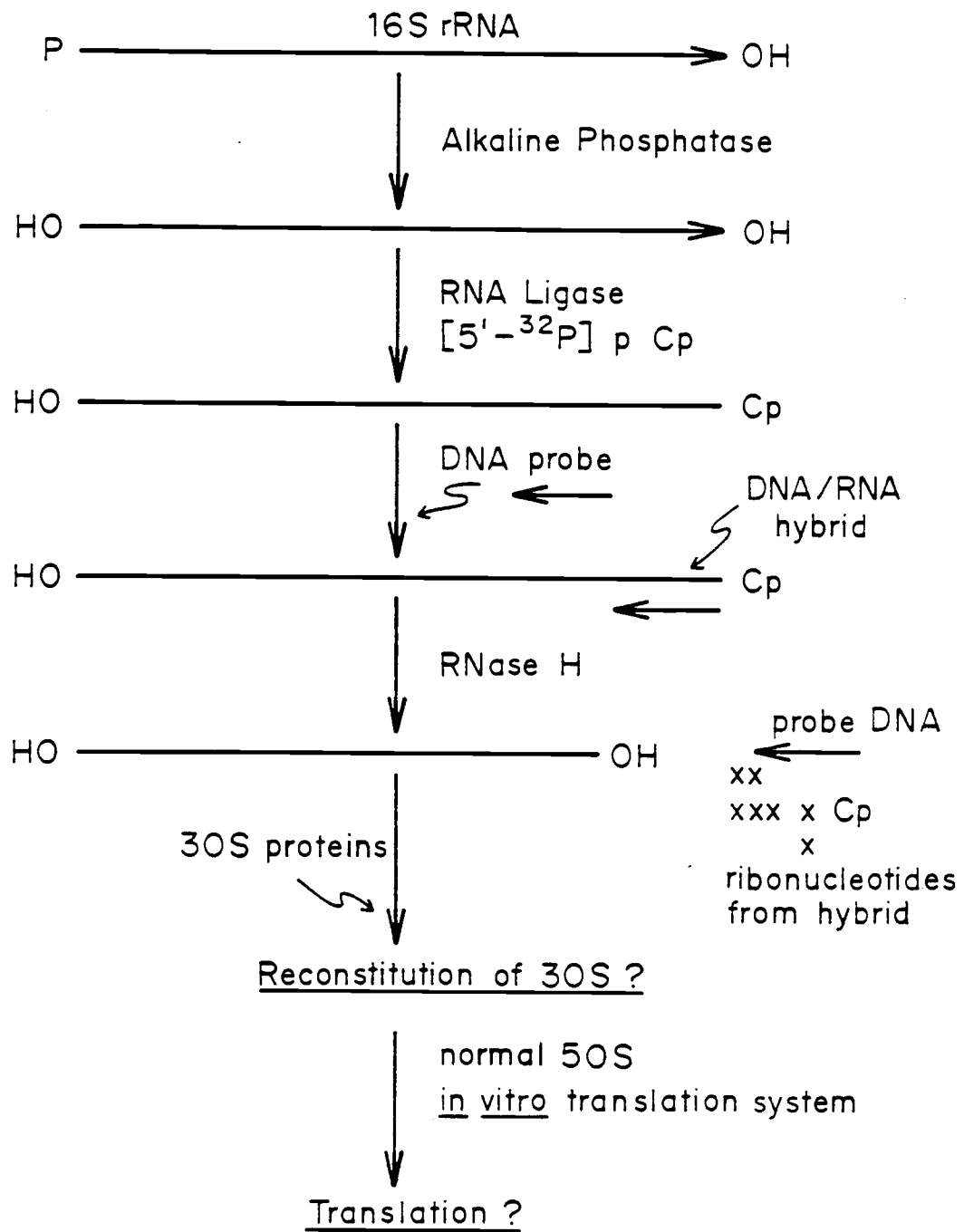


Figure 2-1

2.3 Materials and Methods

Mid-log phase E. coli D-10 cells disrupted with a French Pressure Cell were the source of ribosomal components. Ribosomal proteins were prepared from 30S subunits isolated by zone ultracentrifugation and extracted with two volumes of acetic acid [18]. RNA was prepared by phenol extraction of cell lysates and 16S rRNA was isolated by zone ultracentrifugation [19]. The RNA was treated with calf thymus alkaline phosphatase (E.C. 3.1.3.1) at 0.0079 units/ μ g RNA; the enzyme was purchased from New England Nuclear. T4 RNA ligase (E.C. 6.5.1.3) procured from P & L Biochemicals was used to label the 3'-terminus of the RNA with [5'- 32 P]pCp [20]. The DNA used to target the terminal deletion (pTAAGGAGGTG) was synthesized with an Applied Biosystems synthesizer which uses phosphoramidite chemistry [21]. Before use, the DNA was purified by high pressure liquid chromatography or polyacrylamide gel electrophoresis [22,23]. A portion of the purified DNA was 5'-end labeled and then analyzed for purity in a polyacrylamide sequencing gel [23].

RNase H [E.C. 3.1.4.34] from E. coli or calf thymus were used to digest the RNA component of RNA-DNA hybrids and both gave the same cleavage patterns. RNase H will only attack RNA which is hybridized to DNA [24]. The two enzymes were respectively present at either 1.5 or 0.7 units/ μ g RNA. The buffer used for hybridization and subsequent digestion was a modification of that used for calf thymus digestions and was as follows: 50 mM Tris-HCl (pH 8.3), 25 mM MgCl₂, 50 mM KCl, 1 mM DTT and 0.03 μ g/ml BSA [25]. A 1:10 molar ratio of RNA to DNA was routinely used in the hybridization procedure. The mixture was

preincubated at 50°C for 20 minutes and then at 37°C for 30 minutes. The RNase H was then added and the sample was incubated an additional 50 minutes at 37°C. The digestion products and the DNA probe were separated from the modified RNA by zone ultracentrifugation and the appropriate fractions were ethanol precipitated.

To determine that the predicted cleavage was made by RNase H samples of the altered 16S RNA were digested with RNase T1 which cleaves after guanines and analyzed by polyacrylamide gel electrophoresis [26]. This was done to analyze for 3'-end sequence homogeneity. For this type of analysis the digestion products from the altered RNA were labeled at the 3'-terminus with the (5'-³²P)pCp using T4 RNA ligase. Prior to electrophoretic analysis the 5'-termini of these fragments were rephosphorylated with nonradioactive ATP using T4 kinase to give them the same charge characteristics as the marker sets. For markers, 16S rRNA was completely digested with RNase T1 and these digestion products were 5'-end labeled using [³²P]-γ-ATP and polynucleotide kinase. This gives a set of markers within the anticipated size range for the experimental samples.

Ribosome reconstitutions were performed using one A₂₆₀ unit of 16S RNA and 1.2 molar equivalents of total 30S proteins. The reconstitution mixture was as follows: 10 mM Tris-HCl (pH 7.6), 20 mM MgCl₂, 0.3 M KCl and 6 mM β-mercaptoethanol [27]. The mixture with the RNA was preincubated at 42°C for 30 minutes and then the ribosomal proteins were added. The samples were then incubated for another 30 minutes at 42°C. For RNase T1 analysis, reconstituted 30S subunits which incorporated altered 16S rRNA were purified by zone ultracentrifugation, and recovered from the appropriate fractions by

ethanol precipitation. Protein concentrations were determined with the Bio-Rad protein assay system and protein composition was analyzed by polyacrylamide gel electrophoresis at pH 4.5 of extracted protein [28]. The proteins were visualized by silver staining after electrophoresis [29].

In vitro translation experiments using purified initiation factors and a high-speed supernatant S100 were accomplished as previously described [30,31,32]. The 0.10 ml reaction mixture contained in addition to the purified factors and S100: 40 mM Tris-acetate (pH 7.6), 5 mM magnesium-acetate, 50 mM KCl, 12 mM NH₄Cl, 6 mM β -mercaptoethanol, 1 mM ATP, 0.12 mM GTP, 5 mM phosphoenolpyruvate, 0.1 μ g pyruvate kinase, 17 μ g tRNA, 1.8×10^{-3} μ mol Phe unlabeled, 0.2×10^{-3} μ mol ¹⁴C-Phe (450 Ci/mol) and 2×10^{-3} μ mol of each of the other unlabeled amino acids. The system was programmed with MS2 mRNA. Misincorporation assays were carried out with ¹⁴C-Phe (450 Ci/mol), ³H-Phe (25 Ci/mmol), and ³H-His (15 Ci/mmol).

2.4 Results

2.4.1 Deletion of the 3'-Terminal Nucleotides on 16S rRNA

The sequence designated for deletion was pCACCUCUUUA, corresponding to bases 1533 to 1542 in 16S rRNA. The sequence of the DNA probe used was d(pTAAGGAGGTG). The DNA was hybridized to 16S rRNA and then the RNA component of the hybrid was specifically digested by E. coli RNase H. The enzyme selectively attacks the DNA/RNA hybrid, leaving the DNA and the remainder of the RNA intact [25,26]. To monitor the efficiency of the digestions, 3'-end [^{32}P] labeled RNA was used as a substrate. The cleavage should remove only a few of the 3'-terminal bases leaving the larger portion of the 16S rRNA undamaged. The efficiency of digestion can be determined on the basis of a reduction in TCA precipitable labeled RNA or by a decrease in label in the 16S zone on sucrose gradients (Figure 2). Typically, the cleavage efficiency was greater than 90%.

Conventional sequence analysis does not allow an accurate assessment of the precision of the enzymatic attack in these types of experiments. For example, if 80% of the cleavages were at position 1535, and the remaining 20% were at position 1538 within the rRNA, then in autoradiographs of sequencing gels it is likely that the shadows which would represent the minor cleavage would be difficult to interpret. Even if the presence of the minor component were recognized, it would not be possible to determine the actual amount present from such autoradiographs. To accurately determine the precision and the location of the enzymatic attack, RNase H cleavage

Figure 2-2 Sedimentation profile of RNase H Cleaved 16S rRNA. The RNA was 3'-end [32p]-labeled as described in materials and methods. (A) Control 16S rRNA. (B) Profile of 16S rRNA subjected to the terminal deletion procedure. Sedimentation was from left to right in 15% to 30% sucrose gradient and the separation was performed with a Sorvall AH 650 rotor at 49,000 rpm for 2.75 hrs.

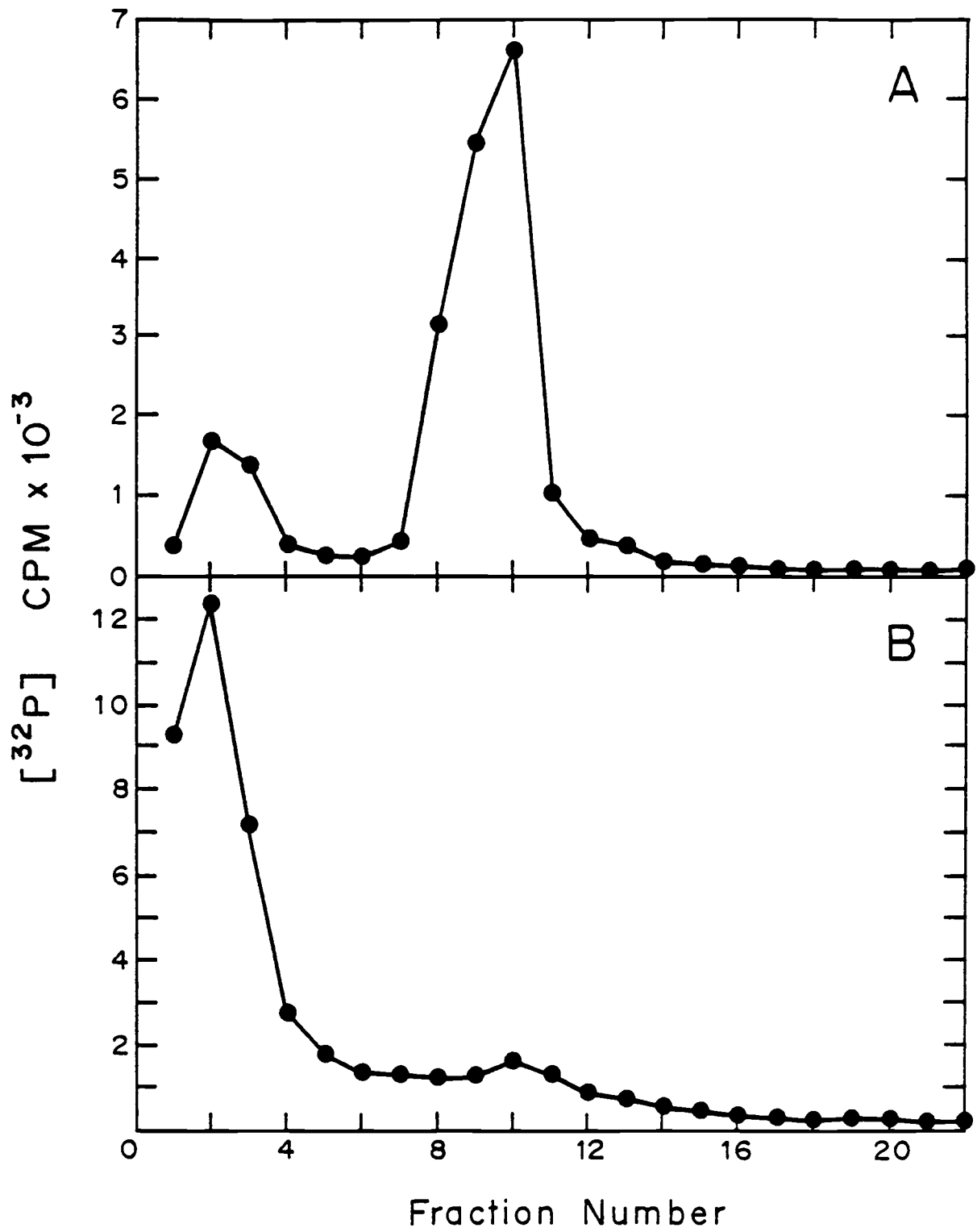


Figure 2-2

product found in the 16S zone of sucrose gradients was recovered and digested completely with RNase T1. These secondary digestion fragments were labeled with (5'- ^{32}P)pCp at the 3'-terminus. RNase T1 cleaves after guanines yielding digestion products with 3'-phosphates. The only exception will be the fragment generated from the 3'-terminus. It will be the sole product which has a free 3'-OH and therefore it will be the only digestion component that could be labeled with [^{32}P]-pCp. Knowledge of the primary structure of 16S RNA permits the prediction of the size of the 3'-end labeled digestion product. The last G in 16S rRNA is at position 1530; therefore, the 3'-end [^{32}P]-pCp labeled T1 digestion product from intact 16S rRNA would be 13 nucleotides in length (Figure 3A). The size of the labeled digestion product can be used to infer the cleavage point on the RNA. When the altered RNA was digested and labeled only a single trinucleotide was found (Figure 3B). This analysis indicates that only a single cleavage was made and that 10 bases had been removed from the 3'-end of the rRNA.

2.4.2 Reconstitution of 30S Particles

The altered 16S rRNA was tested for effectiveness in in vitro reconstitutions [16,17]. Reconstitution was judged successful if 30S particles containing the same amount of each of the proteins found in normal 30S subunits were recovered when the altered RNA was used in the reconstitution. The altered 16S rRNA was [5'- ^{32}P]pCp labeled with T4 RNA ligase and then phenol extracted to remove the enzyme and BSA from the sample. Following ethanol precipitation, the RNA was

Figure 2-3 Autoradiograph of complete RNase T1 digestion of 16S rRNA and 16S subjected to RNase H targeted digestion. A: (lane 1) 5'-end [^{32}P]-labeled 16S RNase T1 digestion products used for calibration. The bands respectively preceding and behind the lane 2 band are 12 and 14 nucleotides in length. (lane 2) RNase T1 digestion of 3'-end [^{32}P]-pCp labeled 16S rRNA. B: (lane 1) RNase T1 digestion of 3'-end [^{32}P]-pCp labeled RNase H cleaved 16S rRNA. (lane 2) RNase T1 digestion as in A: (lane 1). The band opposite the experimental in lane 1 is 3 nucleotides in length. The gel was a 20% acrylamide denaturing gel (25).

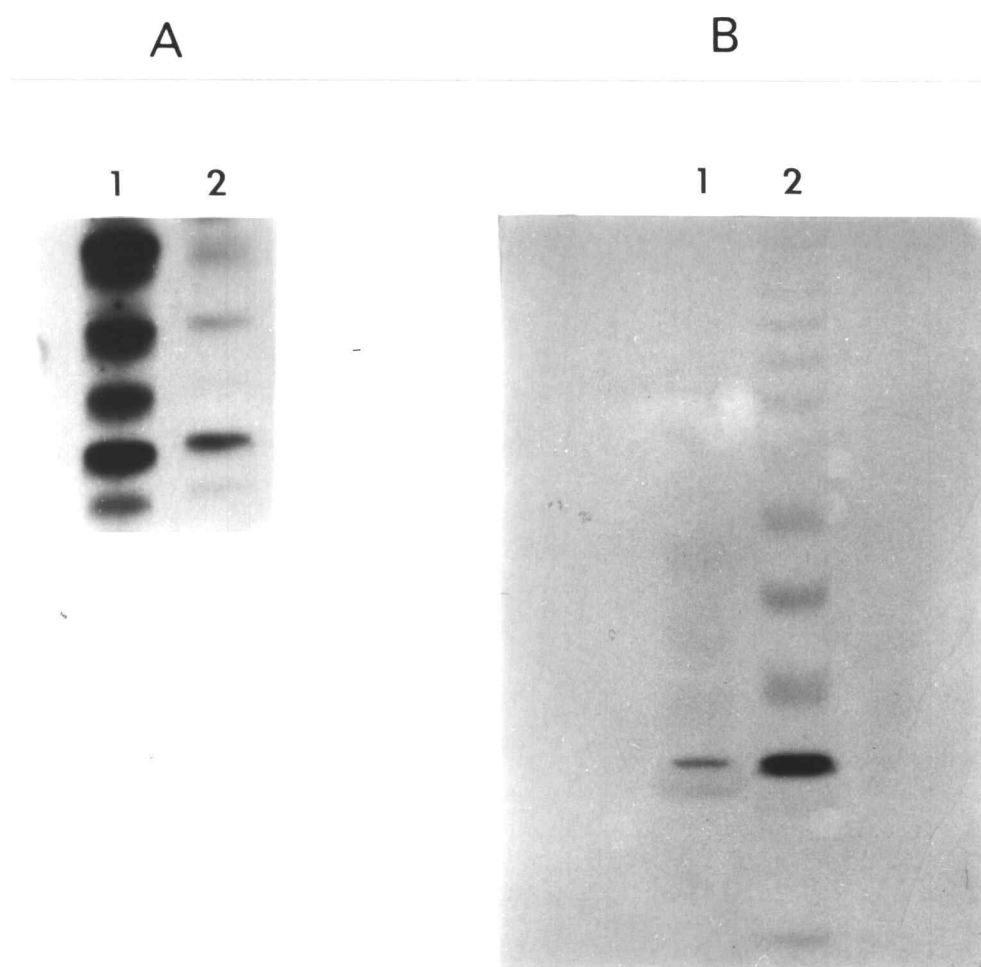


Figure 2-3

purified by zone ultracentrifugation. The purified ^{32}P -labeled 16S rRNA was tested for reconstitution competency. Analytical experiments were performed using altered 3'-end labeled 16S rRNA in the presence of nonlabeled normal 16S rRNA as an internal marker to monitor the efficiency of the reconstitution. The results shown in Fig. 4 indicate that the altered RNA will yield 30S particles comparable to those obtained with normal 16S rRNA. Analysis of the RNA as described in Figure 3 confirmed that the particles contained the altered RNA (not shown).

2.4.3 Protein Composition of Reconstituted Particles

To determine the protein composition of 30S particles derived using altered 16S rRNA, preparative amounts of this RNA were isolated. Reconstitutions were done as before but only modified RNA was present. A portion of the reconstituted 30S particles were purified by zone ultracentrifugation to assess the efficiency of the reconstitution. The remaining material was used for protein composition analysis. An equal volume of ethanol was added to the reconstituted particles and they were placed at -20°C for at least two hours to precipitate the newly formed 30S particles and separate them from proteins which were not associated with 30S particles. Free 30S proteins at protein concentrations used do not precipitate. Analysis of the particles prepared in this fashion showed that there were no observable differences between the protein composition of these particles and normal 30S subunits (Fig. 5).

Figure 2-4 Sedimentation profile of 30S particles reconstituted with RNase H altered [^{32}P]-pCp labeled 16S rRNA.

Sedimentation was from left to right and conditions were as described in Figure 2 except the centrifugation time was reduced to 2.25 hours. The [^{32}P] label in the upper zone of panels A and B is free [^{32}P]-pCp which was not consumed in the labeling reaction. (A) Reconstituted particles. The A260 measurements show the position of the internal marker 30S subunits (O-O). (B) RNase H altered [^{32}P]-pCp labeled 16S rRNA used in reconstitution shown in panel A. (C) [^{32}P]-pCp labeled 16S rRNA standard purified by polyacrylamide gel electrophoresis.

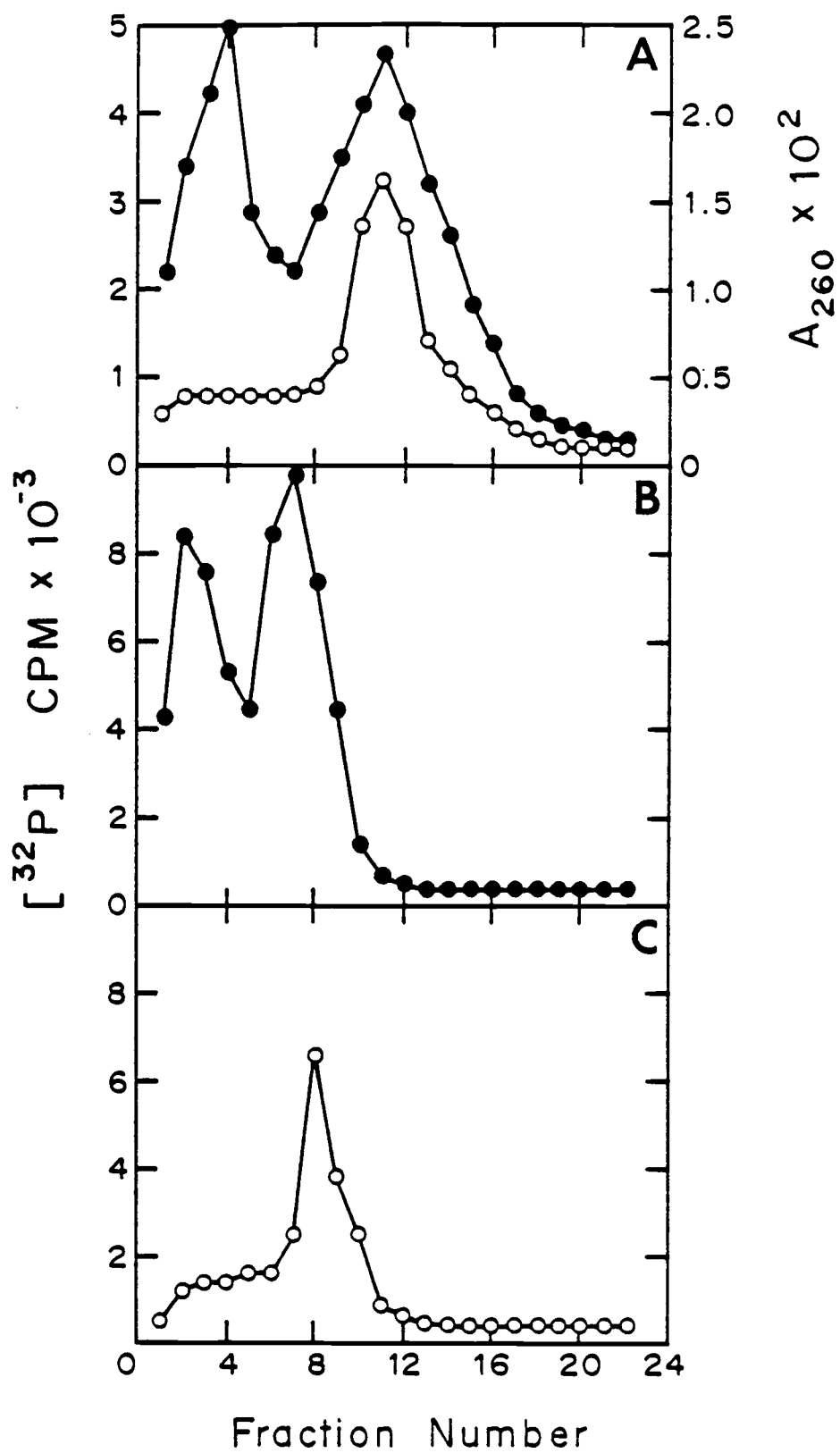


Figure 2-4

Figure 2-5 Laser densitometer scans of polyacrylamide separations of 30S protein from normal 30S particles and particles reconstituted using 3'-terminal deleted 16S rRNA. A. Protein from normal 30S subunits. B. Protein from particles reconstituted with deleted 16S rRNA. C. A photograph of the silver staining gels scanned with the control placed above the experimental. The location of the 30S proteins is indicated by the vertical bars.

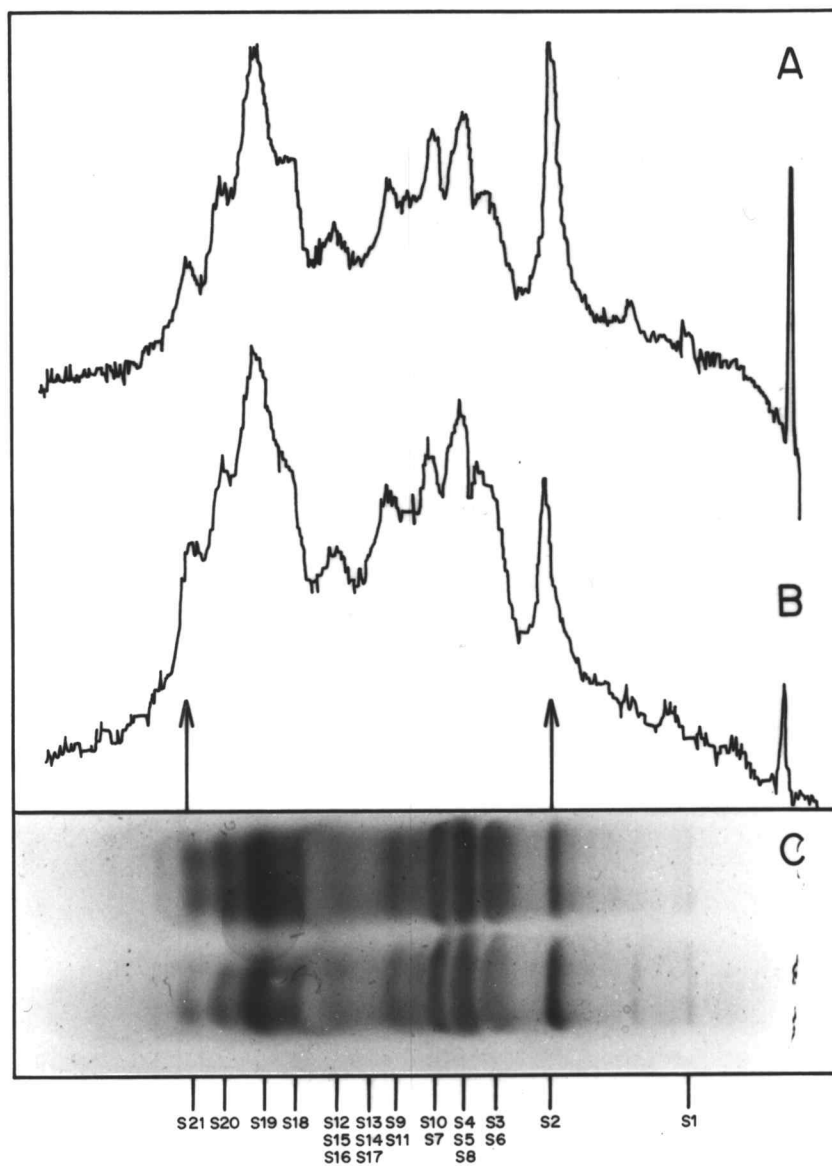


Figure 2-5

2.4.4 In Vitro Translation Analysis

To establish the functional properties of the particles containing the modified 16S, their activity in an in vitro translation system was examined. The natural mRNA used was an MS2 phage mRNA and incorporation of [^{14}C]-Phe into TCA precipitable counts was used as an index of synthetic activity [30,31]. In these experiments the systems using particles containing the altered rRNA incorporated [^{14}C]-Phe at levels consistently one-third of that observed for controls. This may have been due to inefficient initiation or premature termination of translation.

To test for the maintenance of fidelity in translation, the analysis was repeated using [^3H]-His as well as [^{14}C]-Phe in the in vitro system. The principal protein produced when MS2 mRNA is translated in vitro is coat protein which contains no histidine and 4 phenylalanines [33,34]. A small amount of other encoded protein is also synthesized for example, the A protein which has 5 histidines and 16 phenylalanines [35]. Therefore, if the accuracy with which the mRNA is translated was effected by the rRNA alterations then a comparison of the [^{14}C]/[^3H] for control and experimental will give an indication of misincorporation [36]. This assumes that the alteration induces a general disruption in the fidelity of the translation system. For a control, 16S rRNA was taken through the entire procedure including an incubation with RNase H but without the probe DNA present. This rRNA was then used to reconstitute 30S subunits. The [^{14}C]/[^3H] obtained when these particles were used in the translation system was divided by the [^{14}C]/[^3H] determined for 30S

subunits containing sucrose gradient purified 16S rRNA. This median ratio was 0.98 ± 0.04 for six separate experiments. For convenience we call these ratios reconstituted fidelity values. The comparable ratio when 30S particles containing the altered 16S rRNA was 0.77 ± 0.07 , supporting the notion that translational fidelity has been altered.

The misincorporation experiments do not permit a quantitative evaluation of the extent of fidelity disruption because both phenylalanine and histidine are subject to erroneous incorporation. To address this problem and evaluate the quality of newly synthesized protein, control ribosome translations were undertaken in the presence of [^{14}C]-Phe. After a 30 min. incubation at 37°C , samples were diluted to 1 ml with 0.1 M Tris-HCl, 0.01 M EDTA, pH 7 and incubated with pancreatic ribonuclease (20 $\mu\text{g}/\text{ml}$) at 37°C for 5 min. This was followed by the addition of sodium dodecyl sulfate (final concentration 0.35%) and further incubation for 10 min at 45°C . The samples were dialyzed into 0.1 M phosphate buffer (pH 7.2) and concentrated. This mixture was solubilized and the products were separated on a polyacrylamide gel (Figure 6). The distribution of the newly synthesized protein shows that, relative to the control, most of the material synthesized in the presence of the altered ribosomes is smaller in size. The relative mass fraction of protein in each gel slice was calculated using the respective [^3H]/[^{14}C] ratios. When the experimental and the control are identical, the mass fraction for each gel slice should be 1 ± 0.1 [37]. This is demonstrated in Figure 7B for a situation where two separate controls were mixed and analyzed when one translation was done in the presence of [^3H] and the other in

Figure 2-6 Polyacrylamide gel electrophoresis of bacteriophage MS2 proteins synthesized in vitro by altered ribosomes. A. Migration is from left to right with [^{14}C]-Phe and [^3H]-Phe incorporation indicating synthetic protein patterns for the control (X) and the experimental ribosomes (O). B. The relative mass fraction of protein synthesized in the reaction when products produced by altered ribosomes are compared to those synthesized on normal ribosome (O) and when normal ribosome products are compared to normal ribosome products (X). The calculations were based upon $^3\text{H}/^{14}\text{C}$ for the experimental profile shown in A and a separation of normal vs. normal in a neighboring lane. The method of mass fraction analysis is described elsewhere (37). The gel was 10%, and separation required 150 mA for 4.5 hr at 22°C. Gel slices were 5 mm x 2 mm x 0.8 mm and were prepared for liquid scintillation analysis as previously described (37). Bio-Rad protein standards were separated in a bordering lane and used to appraise molecular weights. These were visualized by protein-specific staining after the lanes containing radiolabeled material had been removed.

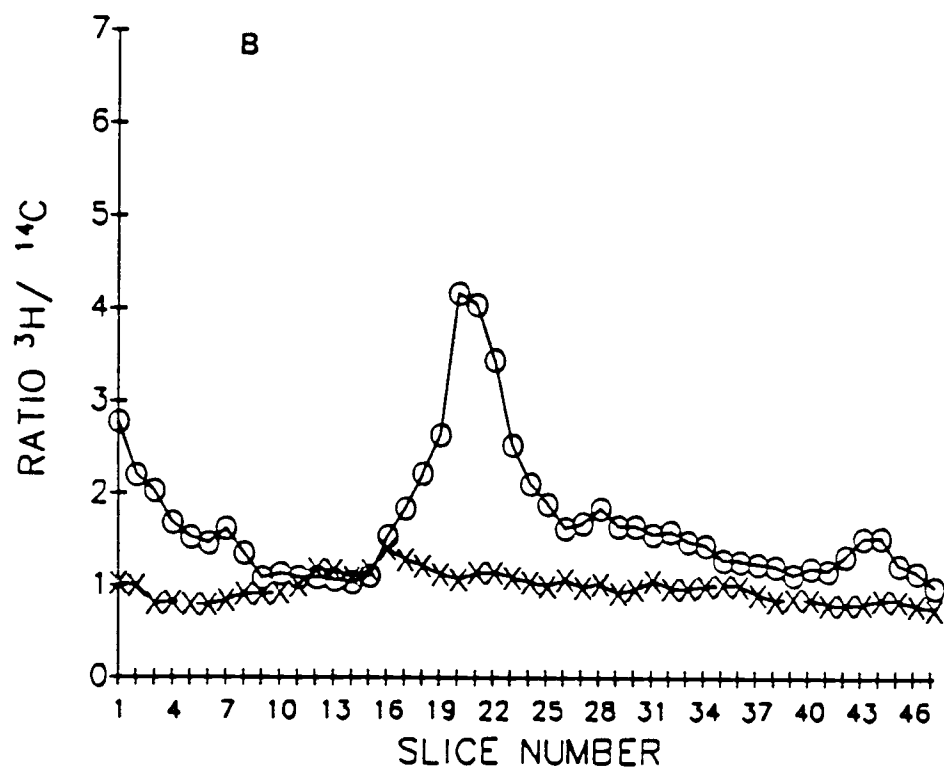
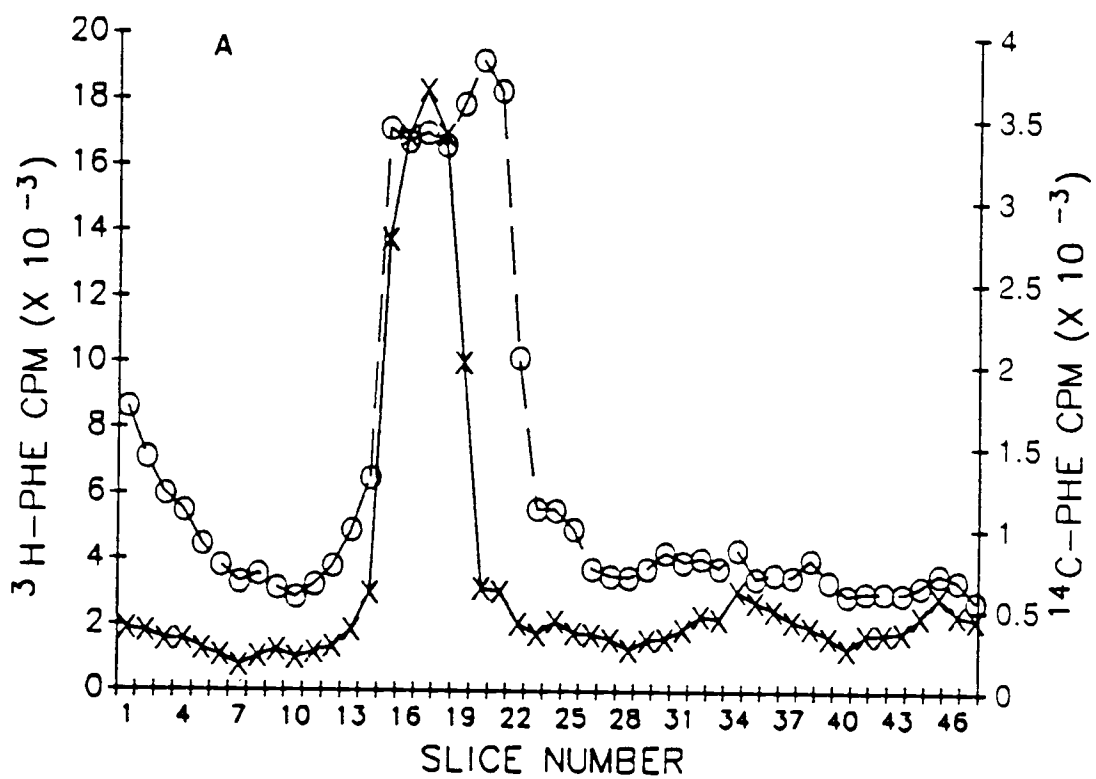


Figure 2-6

the presence of [^{14}C]. The extent to which the value deviates from 1 is a direct measure of misincorporation. The observed relative mass fractions when the experimental ribosomes are compared to controls shows about a four-fold increase in phenylalanine incorporation into the lower molecular weight zone of the gel, but little or no misincorporation in the zone associated with normal coat protein. These experiments were repeated with three different preparations with virtually no variation in the results. It is worth noting that a small amount of normal protein seems to be synthesized by the experimental ribosomes. Although the altered rRNA used in these experiments was judged to be at least 98% pure on the basis of RNase T1 digestion, the synthesis of normal product could be due to minor contamination of the preparation with normal 16S rRNA which would yield ribosomes that initiate more efficiently than altered ribosomes.

2.5 Discussion

2.5.1 30S Reconstitution

The anti-Shine and Dalgarno sequence in small subunit rRNA is a highly conserved sequence unique to prokaryotes. The removal of this sequence from the RNA using RNase H without impairing in vitro 30S assembly was a major concern. The reservation that the 16S rRNAs from which the terminal nucleotides had been removed might not be assembly competent was highlighted by the observation that when the modified RNA was compared to marker 16S RNA on sucrose gradients, the zone for altered rRNA was typically one to two fractions behind the 16S peak. Since only 10 nucleotides of 1542 had been removed, the difference could not be accounted for in terms of molecular weight. An unfolding of the modified molecule would increase the molecule's frictional coefficient and therefore account for the slower sedimentation. Such changes could have yielded molecules that would have been impaired in subunit reconstitutions [38,39,40]. The competency of the altered RNAs in in vitro reconstitution experiments to form particles with a full complement of proteins which sediment at 30S was not compromised by our procedures which remove ten 3'-terminal nucleotides from 16S rRNA. This observation is consistent with the results of experiments reported by other investigators in which 50 3'-terminal nucleotides were removed by colicin E3 digestion (41,42).

If the altered sedimentation properties of 16S rRNA from which the 3' ten terminal nucleotides have been removed does reflect tertiary structural changes in the molecule, these may well mirror

functional characteristics of the initiation process in protein synthesis. Van-Duin et al. (1981) have observed that 30S ribosomes which are protein S21 deficient are not able to properly bind MS2 mRNA during initiation of protein synthesis [43,44]. Addition of S21 to the particles restored functional initiation. The conclusion was that the S21 yielded a change in configuration that made the ribosome complement to the Shine and Dalgarno sequence available for interaction with mRNA. The question remained as to whether or not there was a direct interaction between S21 and the terminal nucleotides. Nomura et al. (1974) have reported that colicin E3 treated 16S rRNA does not bind S21 when the RNA is used in in vitro reconstitution experiments, but all of the other proteins do bind to shortened 16S rRNA [41,42,45]. Colicin E3 treatment removes the 3'-terminal 50 nucleotides from 16S rRNA. If there were a direct interaction involving the terminal 10 nucleotides, their removal could reduce S21 incorporation into reconstituted particles. S21 migrates uniquely on one dimensional polyacrylamide gels of the type used in this work. Analysis of the protein composition of particles generated using the modified 16S rRNA revealed no obvious difference between control and experimental particles with regard to protein S21. It is possible that the 3'-end interacts with other zones within the molecule that form part of the S21 binding region, and that the binding of S21 simply disrupts the interaction. In doing so, S21 would promote proper initiation by presumably making the 3' terminal sequence available for interaction with mRNA.

2.5.2 Fidelity in translation

Although 30S subunits reconstituted with altered 16S rRNA were competent in terms of promoting peptide bond formation the fidelity of the translation process, as judged by misincorporation of [^3H]-His, was impaired. The ratios of Phe to His represent not only variation in His incorporation, but also the inherent error in Phe incorporation. The percentage difference between controls and experimental then can only be taken as a conservative representation of the actual magnitude of the deletion induced error. The experiments which directly compare phenylalanine incorporation provide a much clearer assessment of the extent of misincorporation. Minimally one could interpret these data to mean that the error frequency is at least in the range of 1 in 4 and this could be due to misalignment of the 30S subunit and/or aminoacyl-tRNA on mRNA during the initiation and elongation event. Other explanations or combination of events could account for the loss in fidelity. For example, all of the ribosomal proteins are present in the modified subunits, but we do not know how these altered particles interact with initiation factors, tRNAs and so on. Investigation of these other interactions will be a logical extension of this work.

The apparent misincorporation could also be due to initiation at sites other than the usual AUG for the coat protein. For example, the start codon for the MS2 maturation protein is GUG [35,46]. If GUG is an allowed start signal, then a search for GUGs in single-stranded or partially single-stranded regions of the MS2 RNA should reveal potential open reading frames that would yield polypeptides of a size

comparable to that which we have observed for the low-molecular-weight protein peak seen in Figure 6. We estimate the mean size of peptides in this peak, based upon markers run in the gel, to be about 100 amino acid residues in length. A likely in-frame GUG initiation site in the coat protein region can be found 18 codons from the normal AUG initiation site. Proper translation from this point would yield a polypeptide of about 110 amino acid residues. Similar in-frame GUG sites which could yield peptides of about 180 and 80 amino acid residues can be found in the replicase gene. Out-of-frame GUG initiations would yield polypeptides in the range of 2 to 40 amino acid residues, but for the most part these starts are located in what appear to be very stable double-stranded zones of the MS2 RNA and thus would not be suitable for initiation. Polypeptides, initiated with GUG, produced from the replicase portion of the RNA would have four histidines compared to none for the coat protein, and 16 phenylalanines compared to four in the coat protein. These observations then suggest that we may, at least in part, be reducing the selection fidelity with which the initiation occurs at structurally open AUG and GUG initiation codons. If this is the case, then the anti-Shine and Dalgarno sequence in the ribosome may function to prevent random initiation. This would make the sequence absolutely essential when potential false initiation sites are not masked by secondary structure, or translating ribosomes.

2.6 Acknowledgments

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3. PROTEIN SEQUENCE ANALYSIS OF MS2 IN VITRO

TRANSLATION PRODUCT

3.1 Introduction

In these experiments we wished to pinpoint the exact initiation site for the polypeptides produced in the in vitro translation assay shown in Figure 6 in Chapter II. The major protein produced when MS2 is translated in vitro is the coat protein which contains 4 phenylalanines at positions 5, 8, 26 and 96 (1,2). In the previous experiments we found that deletion of anti-Shine Dalgarno sequence of 16S rRNA does not impair 30S subunit assembly, but the particles exhibited lost translational fidelity in an in vitro cell-free translation system primed with MS2 mRNA.

Relative to the controls, the altered ribosomal particles produced mostly polypeptide smaller in size than the coat protein. In addition to this, a larger polypeptide near the top of the SDS gels along with a polypeptide of the same size as the coat protein were observed. Details of the structure and sequence of MS2 mRNA have been published (3,4,5,6). This information can be used to predict possible initiation sites on the mRNA which could yield translation products of the size observed in these investigations. Translation out of reading frame cannot produce products much larger than 20 amino acid residues in length. The translation products observed here were 100 or more residues in length. This assumes that the modified particles recognize termination codons. Only an in frame product would then be possible. Assuming a requirement for an AUG or GUG initiation codon

and the placement of the codon in a largely single stranded region then one can predict likely translation products. All of the products observed here can be accounted for in this way. The synthesized product were labeled with ^3H -phe and was analyzed using a gas-phase protein sequencer. If the hypothesized initiation sites were correct, one would expect in the control translation product that after sequencing in 10 amino acids we would get ^3H -phe residues at positions 5 and 8. The location of the ^3H in the experimental would be a predictor of initiation sites. If initiation were random then there would be ^3H found in all cycles of the sequencing reactions.

3.2 Materials and Methods

In vitro translation experiments were done as described (7,8,9) in the presence of ^3H -phe (25 Ci/mmol). After 30 minutes incubation at 37°C the reaction mixture were diluted to 1 ml with buffer containing 0.1 M Tris-HCl and 0.01 M EDTA (pH 7.0) and incubated with pancreatic ribonuclease (20 $\mu\text{g}/\text{ml}$) for another 5 minutes at 37°C.

This sample were then dialyzed 3X in 1 liter of water containing 1 mM DTT and 0.1% SDS. Then the sample were dried in the lyophilizer and resuspended in 25 μl water for analysis in the protein sequencer. In another set of experiments, the dried samples were treated with 100% trifluoroacetic acid (TFA) for 2 hours at 60°C. Sequencing was carried out using an Applied Biosystems 470A protein sequencer. The samples were in water when applied to a glass-fiber membrane, which was dried to sequencing. After 10 cleavage cycles, each fraction containing the cleaved amino acid residues was dried and redissolved in 50 μl 33.5% acetonitrile. The fractions were counted in a liquid scintillation spectrometer using TX-100 scintillation cocktail.

3.3 Results and Discussion

In these experiments we did not succeed in determining the sequence of the polypeptide produced in either the control or experimental in the in vitro translation assay. We observed that about 90% of the counts, newly synthesized protein, remained bound to the glass-fiber membrane in the protein sequencer. We concluded that the protein was not sequencable because the N termini were blocked with an N-formyl group. Therefore, we treated the sample with 100% trifluoroacetic acid for 2 hours to remove N-formyl blocking groups (10). This treatment did not yield products that could be sequenced in either the control or experimental. Eight attempts were made to obtain sequence data.

We thought that the samples might be contaminated with RNAs molecules and other unlabeled protein which might interfere with the sequencing reactions. In order to overcome this problem, prior to sequencing the sample was processed through a centricon column which has a cut off MW of 30,000 daltons. In this treatment the larger RNAs and protein would be separated from the coat protein (MW 14300). The results were still disappointing.

From these attempts, I conclude that in order to sequence the product synthesized in the in vitro translation system, the translation products will have to be purified. A suitable approach would be to use the electroblotting technique reported by L. Hood's group (11) fractionated and then eluted on the membrane before analyzed in the protein sequencer.

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4. PHENOL BORONATE COLUMN FRACTIONATION OF
HIGH MOLECULAR WEIGHT RNA

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4.1 Abstract

The interactions of borate ion with 1,2-cis-diol groups are useful in fractionating mixtures of biological molecules. Separation of RNAs, up to the size of tRNAs, from other biomolecules using phenyl boronate agarose (PBA) columns has been shown. In this work, we used phenyl-boronate agarose columns to separate normal E. coli 16S ribosomal RNA from 16S ribosomal RNA with a blocked 3'-terminus. The blocking group was a 3',5'-cytidine (5'- ^{32}P)-bisphosphate. Using a 30 μm boronate/ml of agarose (PBA-30) column we obtained reproducible separations at 22°C. The results show that this method is useful in preparing high molecular weight RNA of high specific activity for sequence analysis. It is also effective for separation of nuclease digestion products.

4.2 Introduction

The specific interactions of borate with cis-diols can be exploited for use in separating oligo and polyribonucleotides. Borate binds to a wide variety of ribonucleosides, ribonucleotides and ribonucleic acids because of the 1,2-cis-diol group at the 2' and 3' positions of the ribose (1). Borate gels are also used to separate RNA from DNA (2,3). Polynucleotides or nucleotides which are either 2' or 3' phosphorylated do not interact with boronate. This is attributable to blockage of one of the hydroxyl groups. The interactions of boronate with the cis-diol groups that form a tetrahedral complex can be disrupted by a competing cis-diol such as sorbitol. A shift in pH or monovalent ion concentration will also alter the stability of complex formation (4,5). Separation of RNAs of various sizes on phenyl boronate agarose columns depends upon the amount of the boron attached to the agarose. Sample concentration and ionic strength of buffers also affect the fractionation (1,4). Hydrophobic and hydrogen bonding are important because they also modulate the strength of bonding.

Several different boronate containing supports are commonly used to separate RNAs (4). Boronated polyacrylamide has been employed as an affinity support for electrophoretic analysis of RNA (6). Cellulose aminoethyl boronic acid column chromatography gives an effective separation of aminoacylated tRNA from uncharged tRNA (7,8). Boronate substituted agarose is also suitable for the separation of RNAs (4). In the present study we report conditions that permit separation on phenyl boronate agarose columns of large RNAs with free

2',3'-cis-diols from RNAs with a blocked 3'-OH. Our goal was to enrich for 16S rRNA that had been 3' end labeled using T4 RNA ligase and 3',5'-cytidine (5'-³²P)-bisphosphate (pCp). For RNA sequencing, enrichment of the labeled material is particularly useful since large RNAs often label poorly with T4 RNA ligase (9,10,11). The columns can also effectively separate RNase H digestion products. RNase H is useful in generating specific restriction like fragments of RNA (12).

4.3 Materials and Methods

4.3.1 Boronate Columns

Boronate derivatized agarose packed in columns was purchased from Amicon Corporation. Gel matrices with 10, 30, and 60 μm of boron/ml of agarose respectively were packed into 2 ml bed volume columns (0.9 x 3.0). These are designated as PBA-10, PBA-30 and PBA-60 columns. The columns were used at room temperature but stored at 4°C when not in use. Before use, the columns were allowed to equilibrate at room temperature for at least 1 hour. The columns were washed with 14 ml of application buffer prior to sample application: 50 mM Hepes, 10 mM MgCl_2 , 0.2 M NaCl pH 8.5. Samples were applied in 0.5 ml (1 A₂₆₀ RNA) to 1 ml (2 A₂₆₀ RNA) of application buffer and allowed to flow into the column matrix. Then 0.1 ml of application buffer was allowed to flow into the column. At this point the flow was stopped and the sample was allowed to equilibrate in the column for at least 30 minutes. The columns were then washed with 14 ml of application buffer and fractions were collected at a flow rate of 1.5 ml/min (PBA-10), 0.5 ml/min (PBA-30) and 0.3 ml/min (PBA-60). Bound RNA was eluted by washing the column with the following buffer: 50 mM Hepes, 10 mM MgCl_2 , 0.2 M NaCl, 0.1 M sorbitol pH 8.5. The columns can be reused up to 10 times. The columns were regenerated with 12 ml of 50 mM Acetate pH 5.0.

4.3.2 Sample Preparation

Mid-log phase E. coli D-10 cells were the source of 16S rRNA and 23S rRNA. Ribosomal RNAs were prepared from phenol extracts of cell lysates. Ribosomal 16S and 23S RNA was isolated by zone ultracentrifugation (13). The 3'-termini of 16S rRNAs were radio labeled with (5'- ^{32}P)pCp using T4 RNA Ligase (14). Uniformly labeled 23S rRNA was prepared from cells grown in the presence of (^{32}P)-orthophosphate.

4.4 Results and Discussion

Table 1 lists the buffers used in this work. PBA-60, PBA-30, and PBA-10 columns were tested. We have found that matrix gel PBA-30 columns are best for separating large RNAs like 16S ribosomal RNA (1542 nucleotides) based on the presence of 3'-terminus cis-diols. To demonstrate this separation a mixture of normal and (^{32}P)-3'-end labeled 16S rRNA was applied in the various sample application buffers (A,B,C,D) shown in Table 1. The 3'-phosphorylation on the labeled RNA should interfere with binding to the columns.

Buffers A and C have been reported to be good application buffers when RNA separations are required (1,4). However, we have found that only buffer D is suitable as an application buffer when large RNAs are applied to the columns. In contrast with previous investigators (1,4), we found that buffers A and B were not able to remove 16S RNA from the column, whether or not the 3'-end was blocked. Binding to the column occurred at either 4°C or 22°C. These application buffers did work as previously reported when low molecular weight RNAs, such as tRNA, were passed through the columns. Buffer D with 0.2 M NaCl reduced nonspecific binding of 3'-end blocked 16S rRNA which occurred when buffer B was used. Buffer E and F are elution buffers, but optimal recovery of cis-diol containing 16S rRNA with buffer E requires sorbitol. The sorbitol provides competing cis-diols. Low pH alone was sufficient to elute small RNAs.

Figure 1 shows a typical elution profile PBA-30, where 70% of the normal 16S rRNA which has free 3' cis-diols is retained and approximately 80% of the 3'-end blocked RNA passes through the column.

Table 4.1

Composition of Application and Elution Buffers

<u>Buffer</u>	<u>Composition</u>
A	50 mM Hepes, 10 mM MgCl ₂ , 20% v/v 95% EtOH, pH 8.5 with NaOH
B	50 mM Hepes, 10 mM MgCl ₂ , pH 8.5 with NaOH
C	50 mM Hepes, 10 mM MgCl ₂ , 20% v/v 95% EtOH, 0.2 M NaCl, pH 8.5 with NaOH
D	50 mM Hepes, 10 mM MgCl ₂ , 0.2 M NaCl pH 8.5 with NaOH
E	100 mM Tris pH 8.5 with HCl
F	50 mM Hepes, 10 mM MgCl ₂ , 0.2 M NaCl, 0.1 M Sorbitol pH 8.5 with NaOH
G	50 mM Acetate pH 5.0

Figure 4-1 Column chromatographic separation of 16S rRNA on PBA-30.

Two-milliliter bed volume PBA-30 was loaded with 2 A₂₆₀ unlabel 16S rRNA (●—●) and 8×10^3 cpm (5'-³²P)pCp 3'-end blocked 16S rRNA (■—■). The samples were applied in buffer D and eluted with buffer F. The change to elution buffer is shown by the arrow. Fraction volumes are 1.5 ml and the flow rate was 0.5 ml/min.

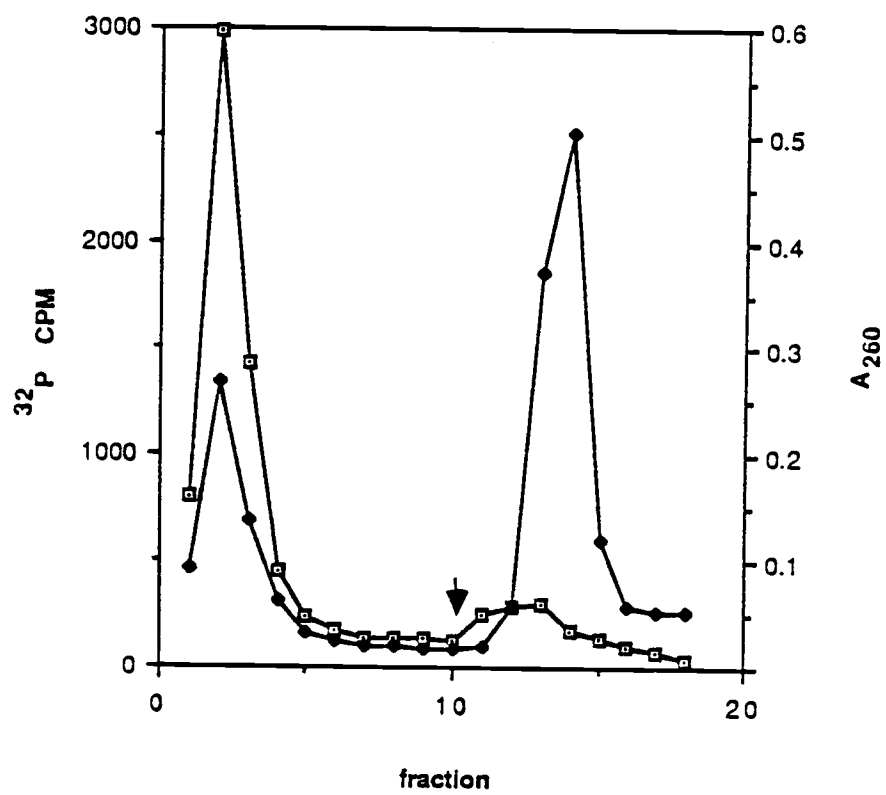


Figure 4-1

The material which was not retained on the column was reapplied to the column to determine if the sample would distribute again in the same manner. Again, approximately 20% of the blocked material was bound to the column suggesting that the retention observed in the first passage through the column was most likely because of nonspecific interactions. Furthermore, when the retained material was eluted, dialyzed into application buffer and reapplied approximately 80% of the previously retained blocked RNA eluted. We found 23S rRNA to have comparable elution and retention characteristics as 16S rRNA. PBA-10 columns (Figure 2) do not retain either free 3' cis-diol 16S rRNA or blocked 16S rRNA, while PBA-60 (Figure 3) retains both types of 16S rRNA independent of the application buffer used.

To show the utility of the columns in separating RNase H digestion products we prepared a synthetic DNA which was complementary to the 3'-end of 16S rRNA. The DNA (pTAAGGAGGTG) was hybridized with (5'-³²P)pCp 3'-end blocked 16S rRNA and then incubated with RNase H. The RNase H will only attack the DNA/RNA hybrid. The digestion product for the larger rRNA fragment has a free 3'-end and was bound to the column. This RNA was eluted, relabeled with (5'-³²P)pCp and found to migrate on polyacrylamide gels in a zone expected for a "16S" like rRNA molecule (Figure 4).

4.5 Acknowledgment

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Figure 4-2 Column chromatographic separation of 16S rRNA on PBA-10. Column was loaded with 1.5 A_{260} unlabeled 16S rRNA (●—●) and 5×10^3 cpm labeled 16S rRNA (■—■). The column conditions and fraction size were the same as Figure 1. The flow rate was 1.5 ml/min.

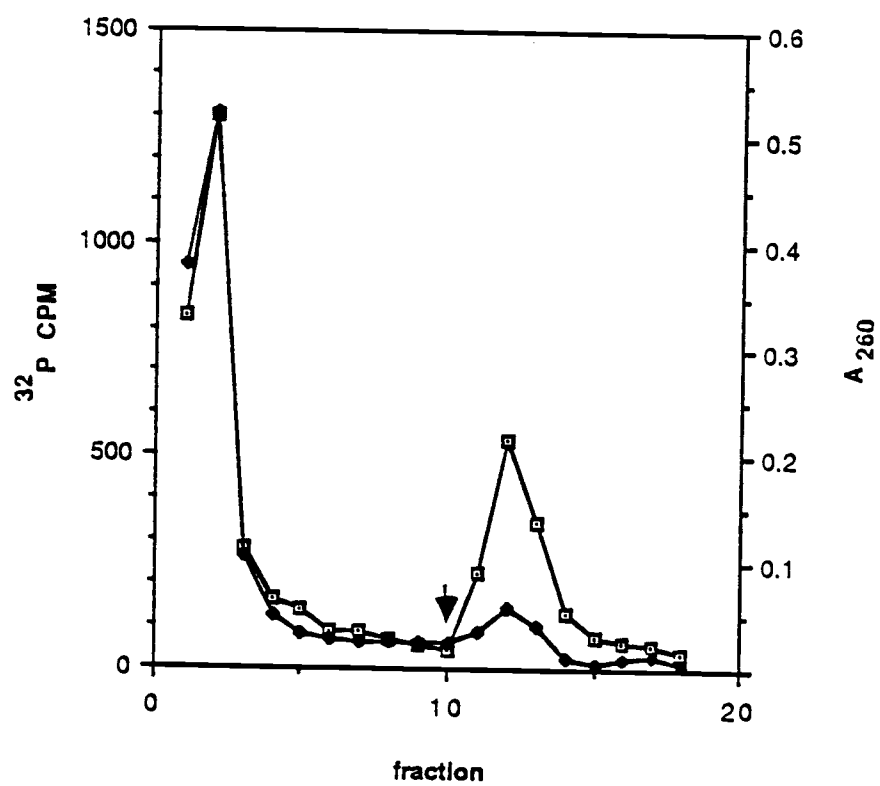


Figure 4-2

Figure 4-3 Column chromatographic separation of 16S rRNA on PBA-60.

The column was loaded with 2.5 A₂₆₀ unlabeled 16S rRNA

(●—●) and 8x10³ cpm labeled 16S rRNA (■—■) . The

apparently conditions were the same as in Figure 1. The flow rate was 0.3 ml/min.

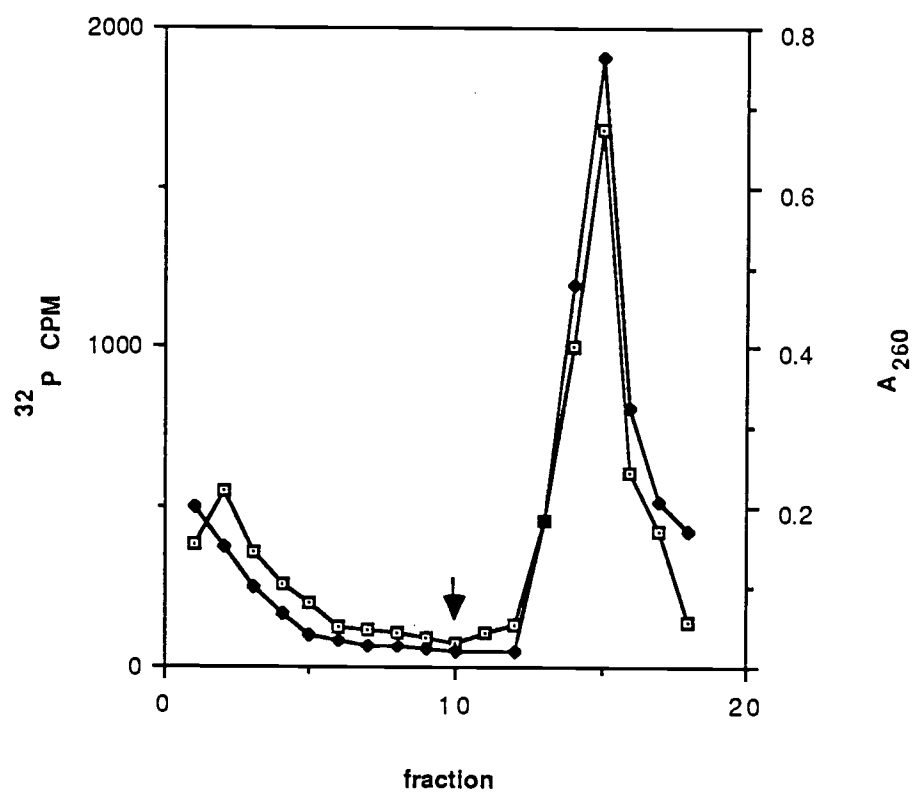


Figure 4-3

Figure 4-4 Autoradiograph of a polyacrylamide showing 3'-end labeled 16S rRNA. A. 3'-end [^{32}P]pCp labeled normal 16S rRNA purified by zone ultracentrifugation. B. 3'-end [^{32}P]pCp labeled RNase H digested 16S rRNA purified on a PBA-30 column. The polyacrylamide gel was a split 2.8% - 10% denaturing gel (15x10 cm, 0.75 mm thick) and the running buffer was 50 mM Tris-base, 1 mM EDTA pH 8.3. Running time was 2 hours at 5 mA.

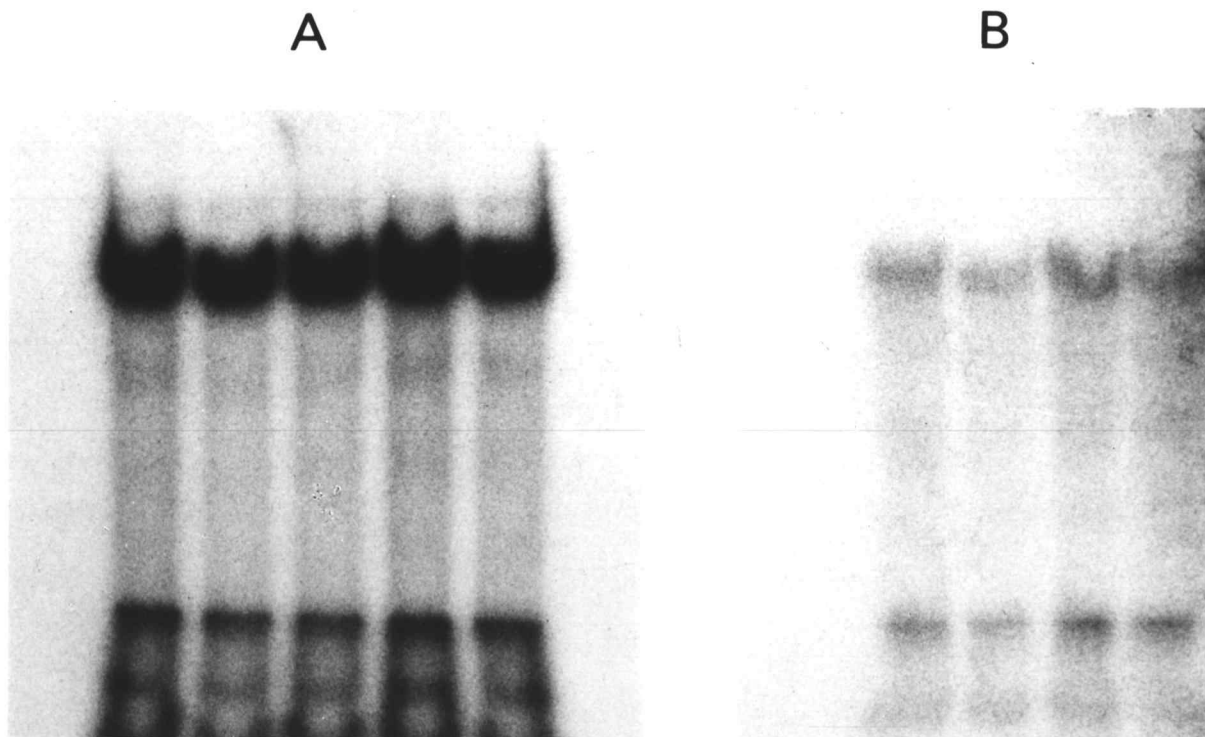


Figure 4-4

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APPENDIX

6. APPENDIX

6.1 In vitro translation assay primed with MS2 mRNA

The buffer and reaction mixture used in the in vitro translation assay are as described in the text. For each amount of 1 A₂₆₀ of 30S subunit and 2 A₂₆₀ 50S subunit used in the assay, we used high-speed supernatant S-100 containing 70 µg protein, 0.3 µg IF-1, 3.0 µg IF-2 and 0.6 µg IF-3. Protein concentration was determined by Bio-Rad protein assay (Figure 8). The amount of MS2 mRNA added are 4 µg/assay.

In order to monitor the incorporation of radioactivity in the product synthesized in the assay, after 30 minutes incubation at 37°C, the reaction was stopped with 1 ml 5% trichloroacetic acid. Then the mixture were heated for 20 minutes at 90°C, and the precipitate were cooled in ice for 10 minutes before collected on glass-fibre filters (Whatman GF/C). The sample were washed with 6 ml 5% trichloroacetic acid and 3 ml methanol. The membrane were dried in the oven for at least 40 minutes and then were counted in a toluene-based scintillation liquid.

High-speed supernatant S-100 and all the initiation factors were extracted from E. coli D-10 cells lysed with French Pressure cell. These S-100 supernatant and initiation factors were purified by centrifugation in buffer containing high salt (10 mM Tris-HCl, 20 mM NH₄Cl, 10 mM Mg acetate, 7 mM β-mercaptoethanol and 15% v/v glycerol, pH 7.4). Initiation factors were further purified by ammonium sulfate fractionation and run through phosphocellulose and DEAE-Sephadex G-50 column. This method yields about 98% purity of initiation factors.

Figure 6-1 A proposed secondary structure for 16S ribosomal
ribonucleic acid as described by Woese et al. (1983).

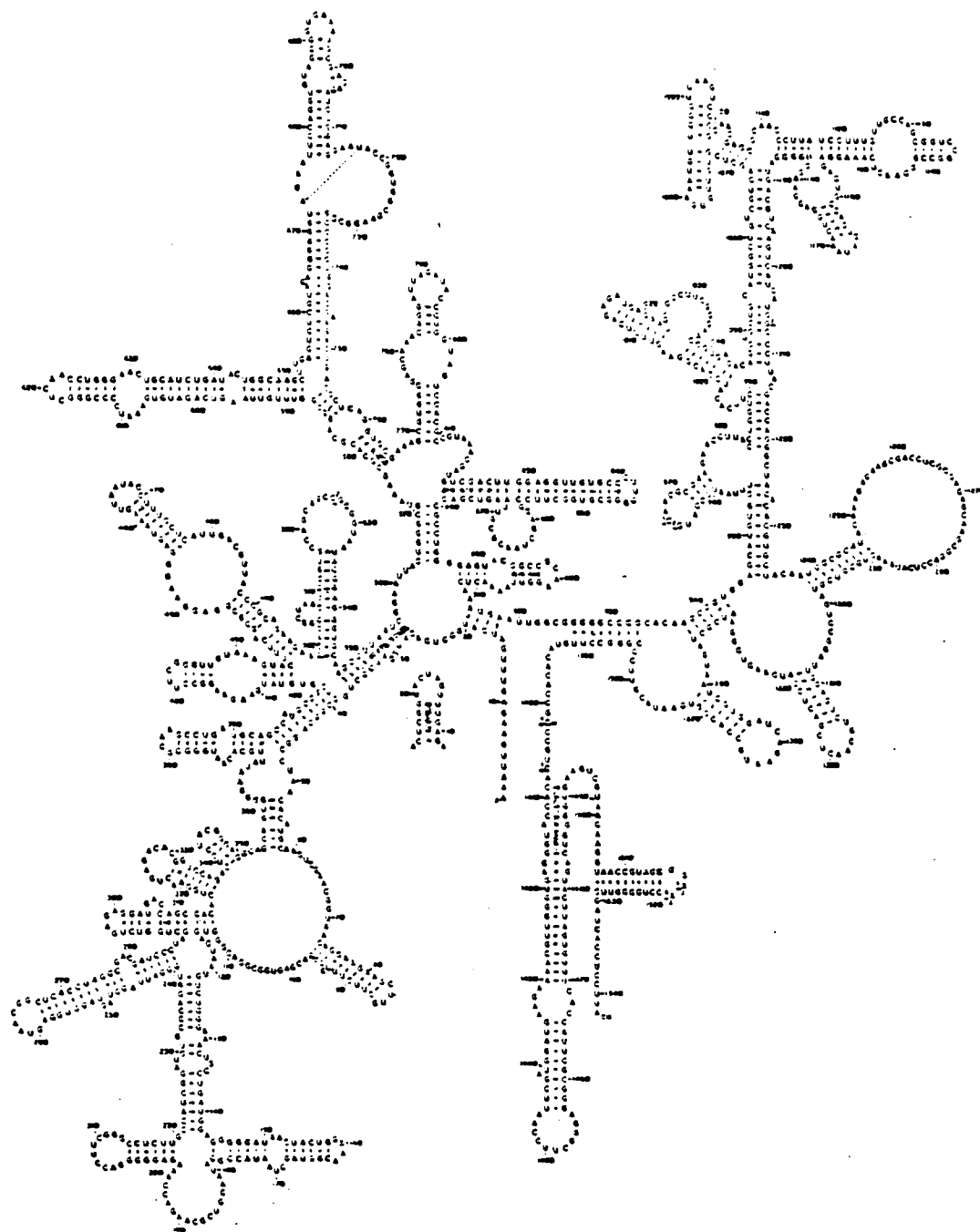


Figure 6-1

Figure 6-2 Autoradiograph of 5'-end ^{32}P -labeled 10 bases length of DNA probe (pTAAGGAGGTG). Lanes 1 and 3 are HPLC purified 5'-end ^{32}P -labeled probe. Lane 2 is total DNA probe 5'-end ^{32}P -labeled before purification.

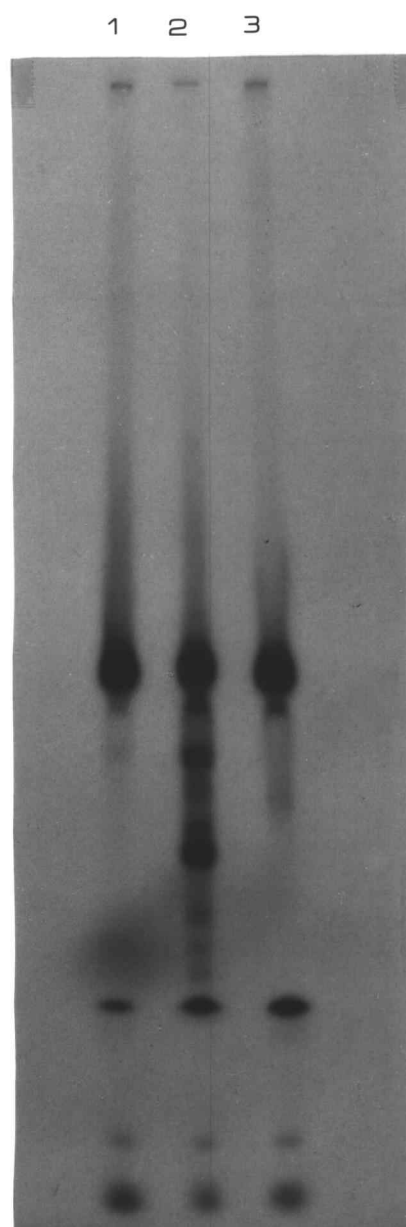


Figure 6-2

Figure 6-3 Sucrose gradient sedimentation profile of a total RNA extract from E. coli. The gradient was 15 to 30% in TSM buffer (10 mM Tris-HCl, 10 mM MgCl₂, 0.3 mM Succinic acid, pH 8). Sedimentation is from right to left, showing small RNAs, 16S, and 23S respectively. The separation was performed with a Sorvall AH 627 rotor at 24,000 rpm at 4°C for 21 hours.

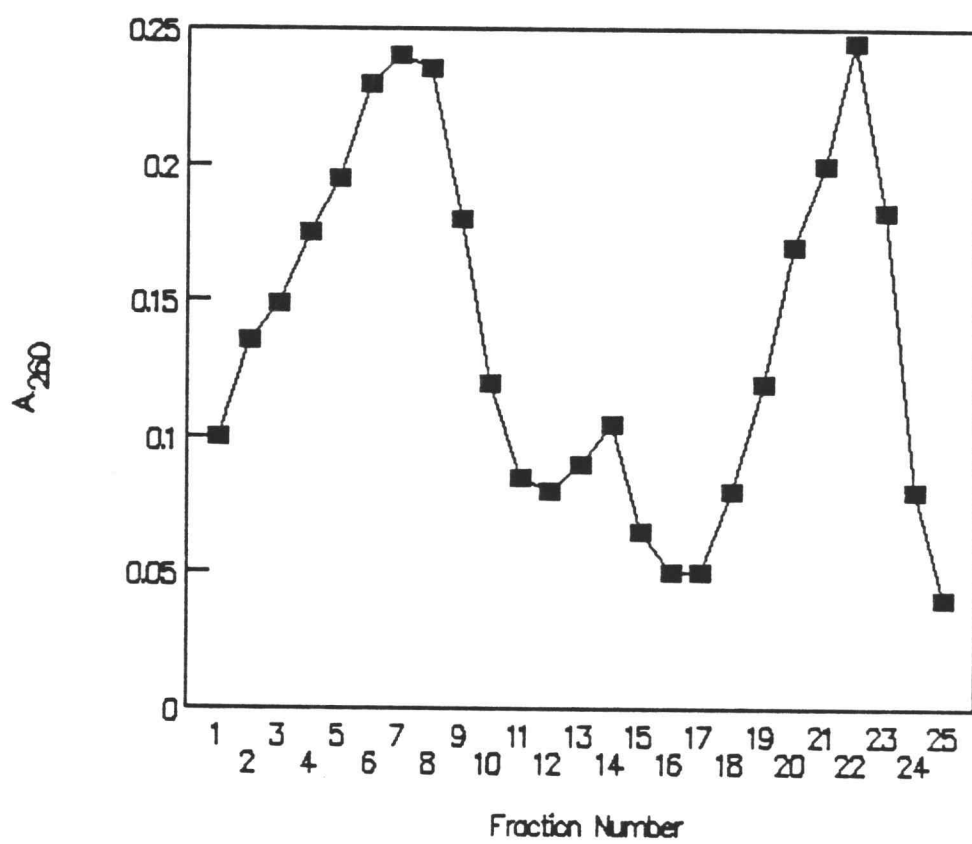


Figure 6-3

Figure 6-4 Autoradiograph of 3'-end ^{32}P -labeled 16S rRNA from sucrose gradient.

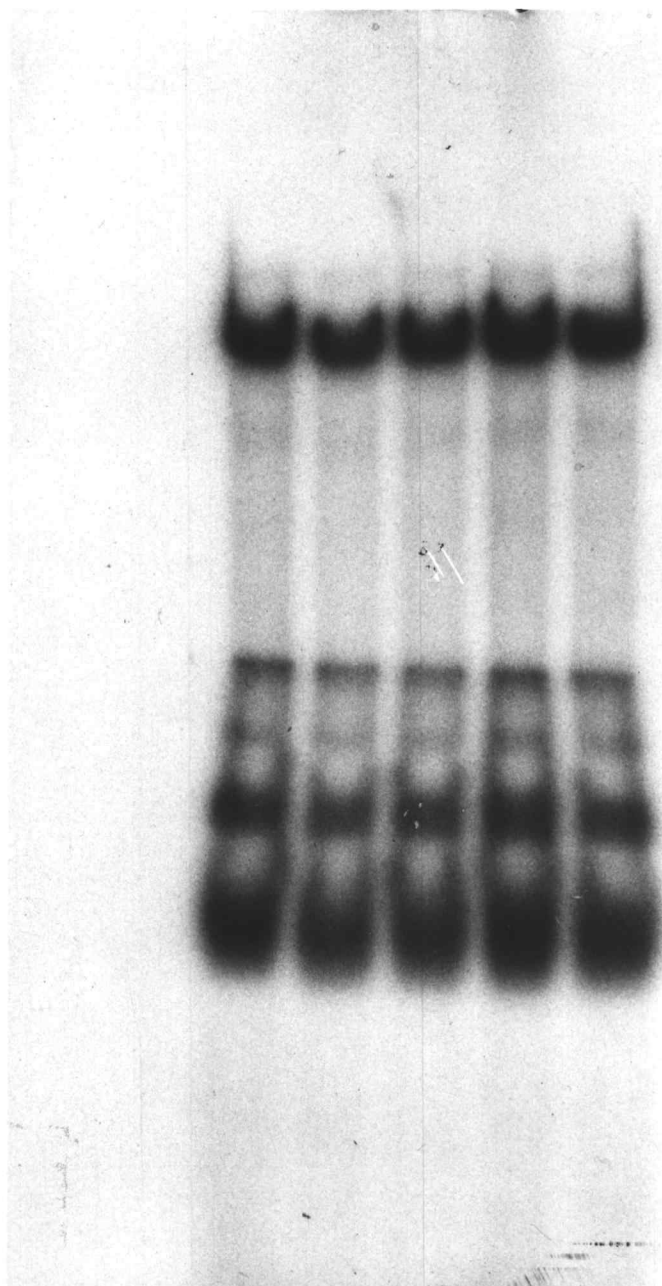


Figure 6-4

Figure 6-5 Sedimentation profile of T4 RNA ligase labeled (5'-³²P)pCp 16S rRNA on a sucrose gradients (15-30% sucrose in TSM Buffer). Sedimentation was from left to right and the separation was performed with a Sorvall AH 650 rotor at 49,000 rpm for 3 hours, 25 min at 4°C. Fraction 9 through 12 contain the 16S rRNA and unincorporated (5'-³²P)pCp remains in fractions 1 through 5.

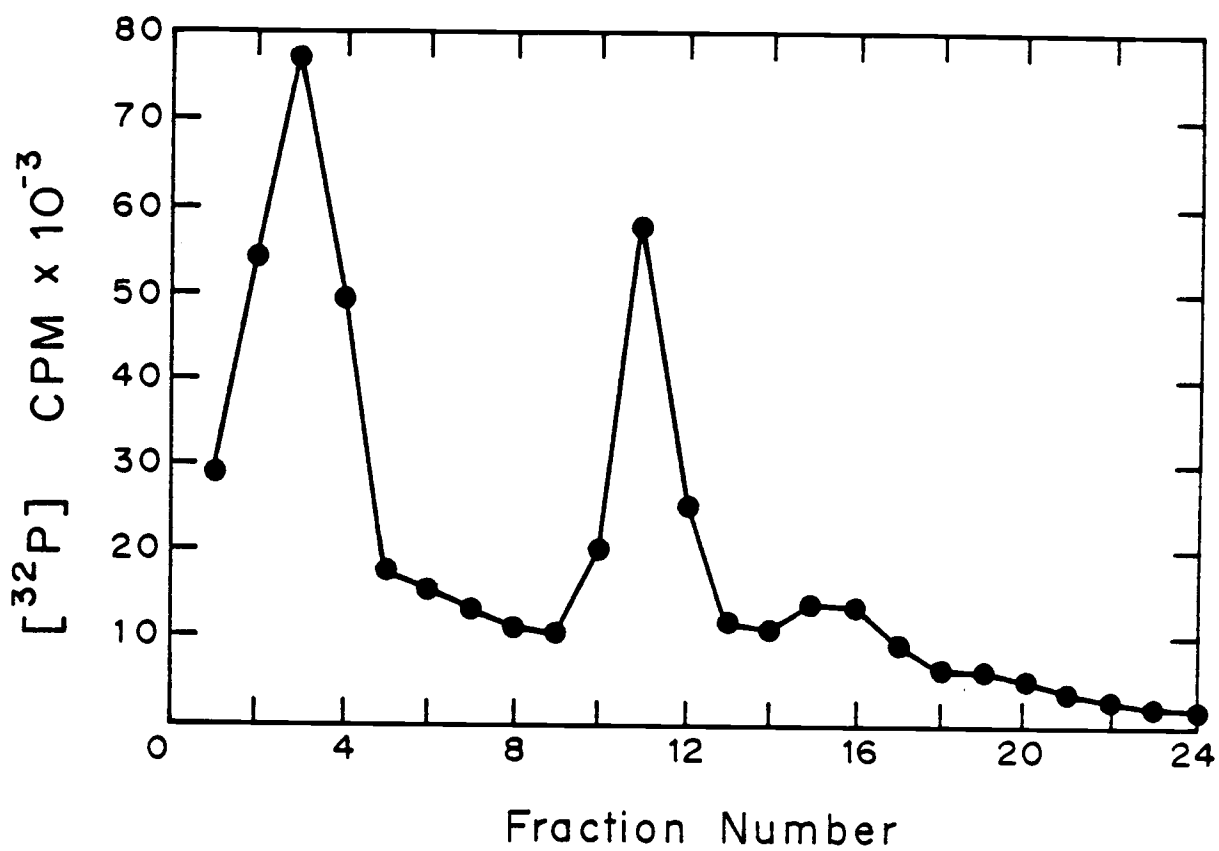


Figure 6-5

Figure 6-6 Sedimentation profile of (5'- ^{32}P)-labeled 16S rRNA subjected to the terminal deletion procedure. Sedimentation is from left to right and was performed as in Figure 5.

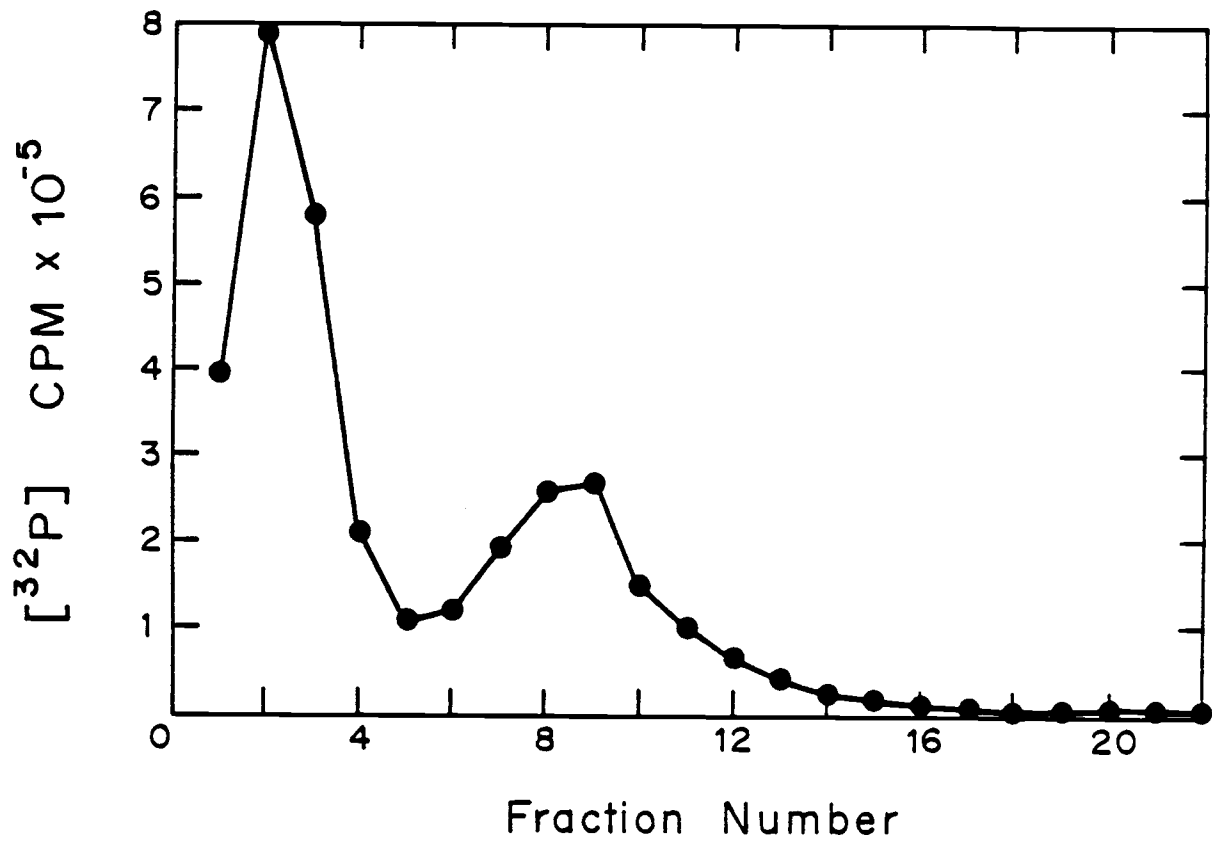


Figure 6-6

Figure 6-7 Sedimentation profile of 70S ribosomes in 15-30% sucrose gradient in gradient buffer (10 mM Tris-base, 50 mM KCl, 0.3 mM MgCl_2 , pH 7.6). Sedimentation is from right to left, shows the region of 50S subunit, 30S subunit and tRNA. The separation was performed with Sorvall AH 627 at 24,000 rpm, 4°C for 21 hours.

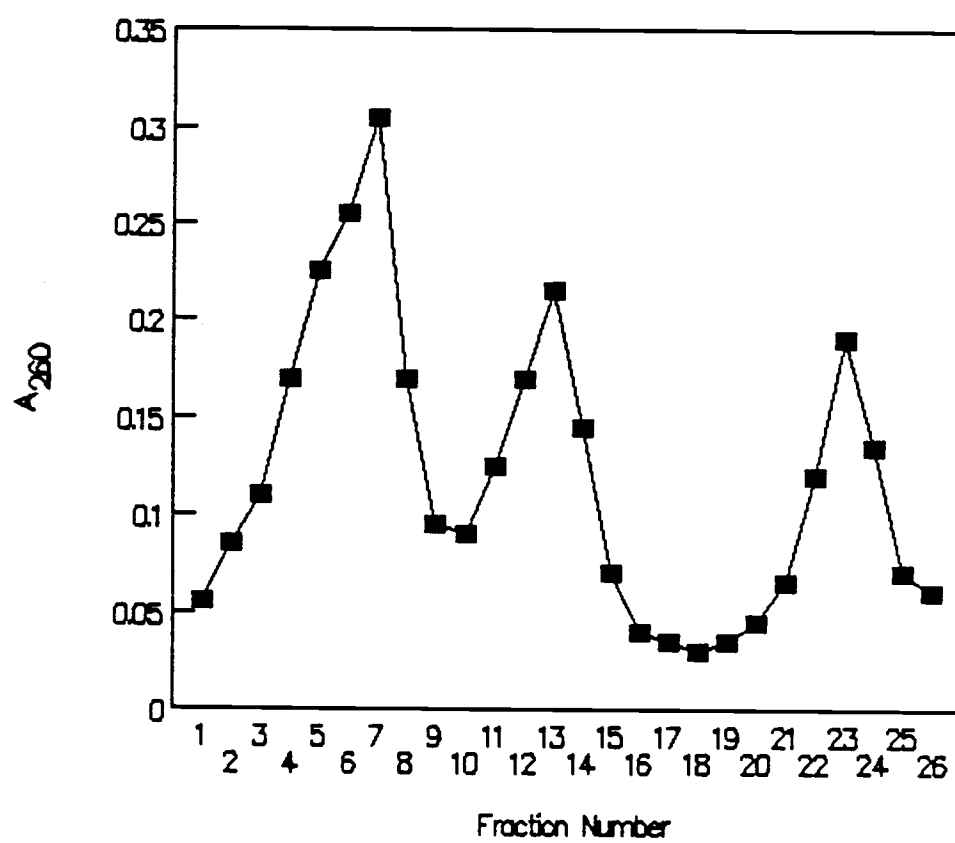


Figure 6-7

Figure 6-8 Standard curve for the Bio-Rad Protein Assay using
Bio-Rad protein standard.

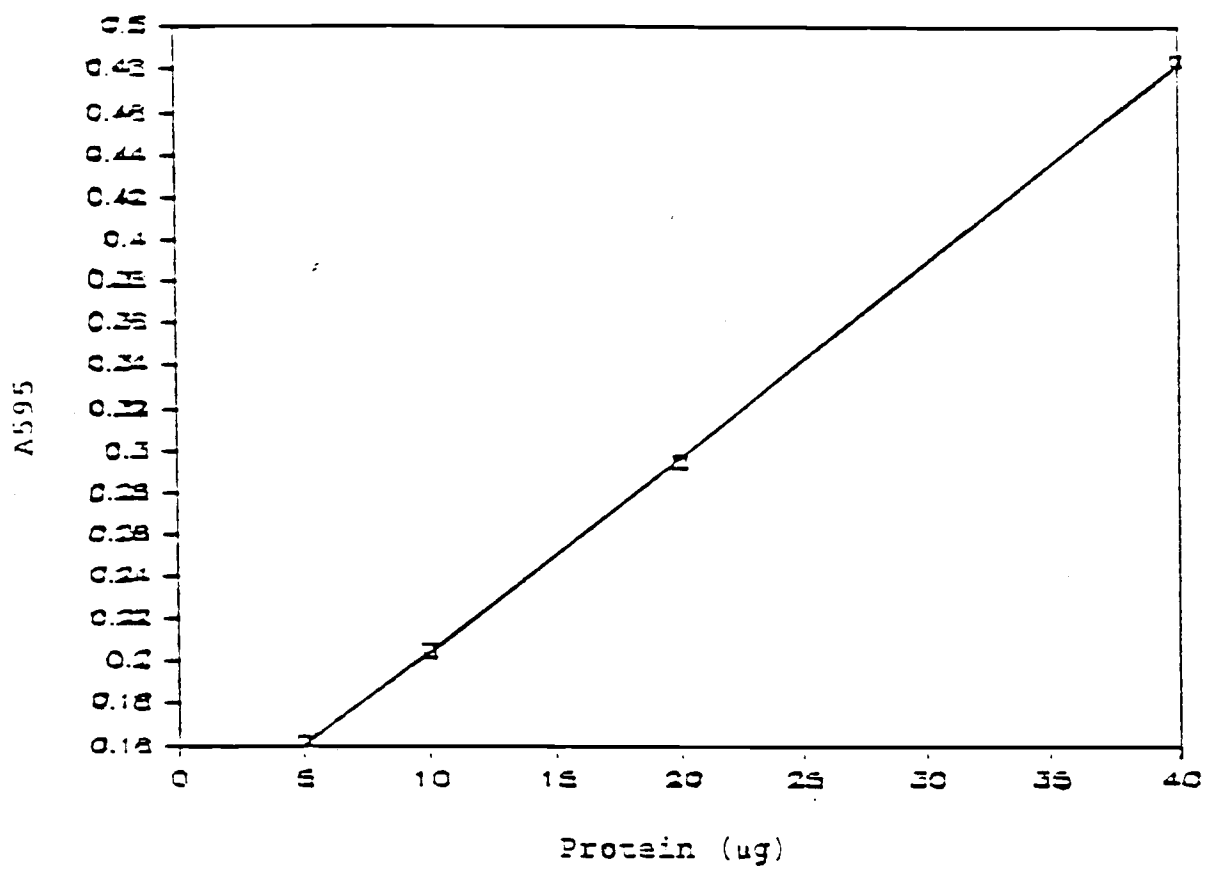


Figure 6-8

Figure 6-9 In vitro translation analysis. The experiments were repeated six times using three different altered 16S rRNA preparations to reconstitute 30S subunits. The bars show the misincorporation ratios as described in the text. Minimum and maximum show the range of values obtained. The open bars represent altered particles and the hatched bars show control data.

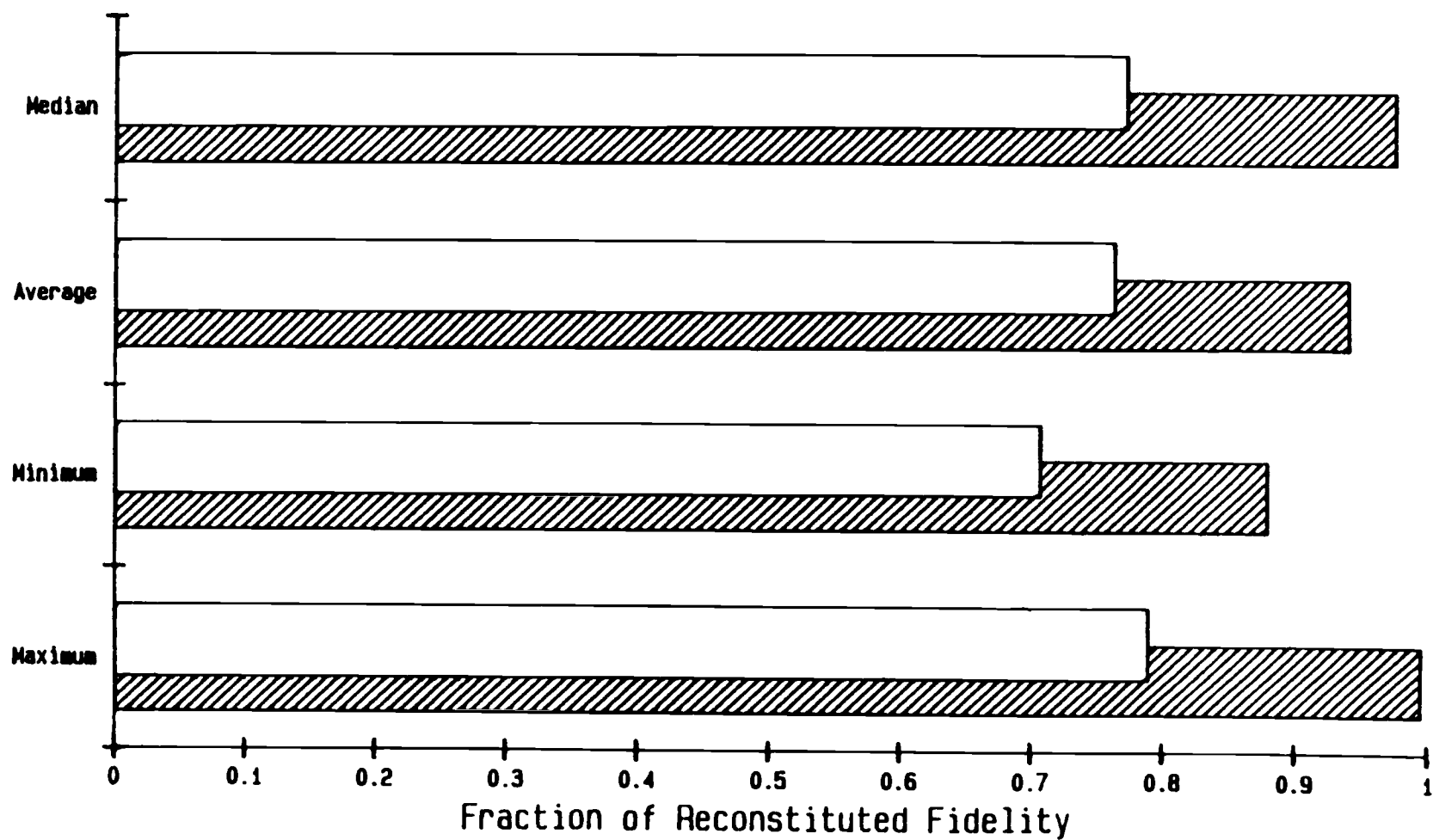


Figure 6-9