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Title: Site-Specific 3'-Terminal Deletions in Escherichia
coli 16S rRNA and the Effect on Assembly of 30S Ribosomal
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The ribosome is a central component of the protein
synthetic apparatus. It is a macromolecular complex of
protein and RNA. Although much progress has been made in
understanding the functional role of the proteins in this
particle, little is known of the functional role which
the RNA plays. Naturally occurring primary structural
mutations in rRNAs have not been reported. The work
described here focused on developing methodology for
generating site-specific deletions in rRNA directly to
explore the functional properties of the RNA.

The 3'-terminus of E. coli small subunit 16S rRNA was
chosen as the site to be deleted. This region of the RNA
is believed to be important in the initiation of protein

synthesis and could be essential for proper assembly of ribosomes. The deletions were achieved by synthesizing a DNA molecule of 10 bases in length which was complementary to the 3'-end of 16S rRNA. The DNA was hybridized to the RNA and then the RNA component of the hybrid was specifically digested with a combination of RNase H isolated from E. coli and calf thymus. This produced an equal mixture of "16S" RNAs missing either 10 or 8 terminal nucleotides. This RNA was shown to be functional in in vitro reconstitution studies indicating that this zone of the RNA is not essential for ribosome assembly.

SITE-SPECIFIC 3'-TERMINAL DELETIONS IN ESCHERICHIA COLI
16S rRNA AND THE EFFECT ON ASSEMBLY OF
30S RIBOSOMAL SUBUNITS

by

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INTRODUCTION

Ribosome Function

Ribosomes are ribonucleoprotein particles found in
all types of cells. They play a fundamental role in the
biosynthesis of proteins. Eukaryotic and prokaryotic
ribosomes are physically quite similar in design. The
general features are shown schematically in Figure 1.
Typically the eukaryotic ribosome is larger than the pro-
karyotic particle, and they contain additional proteins
and four distinct rRNAs. The proportion of RNA by mass in
eukaryotic ribosomes is 50% in the small subunit and 65%
in the larger subunit, and comparable values for the RNA
in prokaryotes are 60% and 70%, respectively (1,2,3).

In addition to the major rRNAs (18S, 28S in
eukaryotes and 16S, 23S in prokaryotes), the large sub-
units contain a molecule of 5S RNA and 5.8S RNA. The 5S
RNA in eukaryotes is an analog of the prokaryotic 5S,
while the sequence of 5.8S RNA may correspond to the 5'-
end of the prokaryotic 23S rRNA (4). The reasons for this
difference are not obvious, but may reflect, for example,
fundamentally different requirements for initiation of
protein synthesis. In prokaryotes, the initiation event
takes place prior to completion of transcription, while in
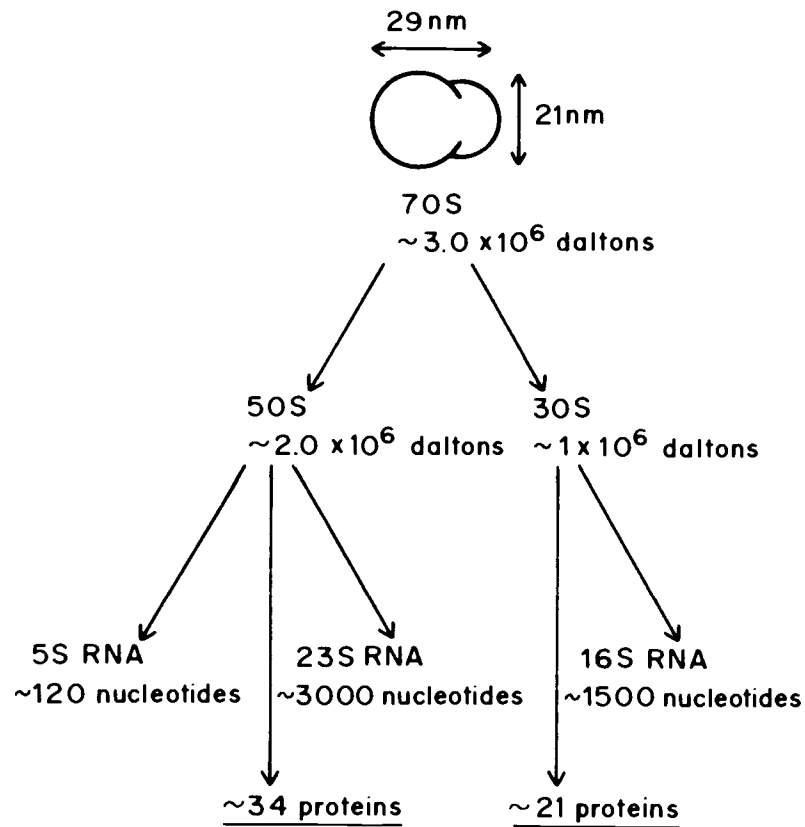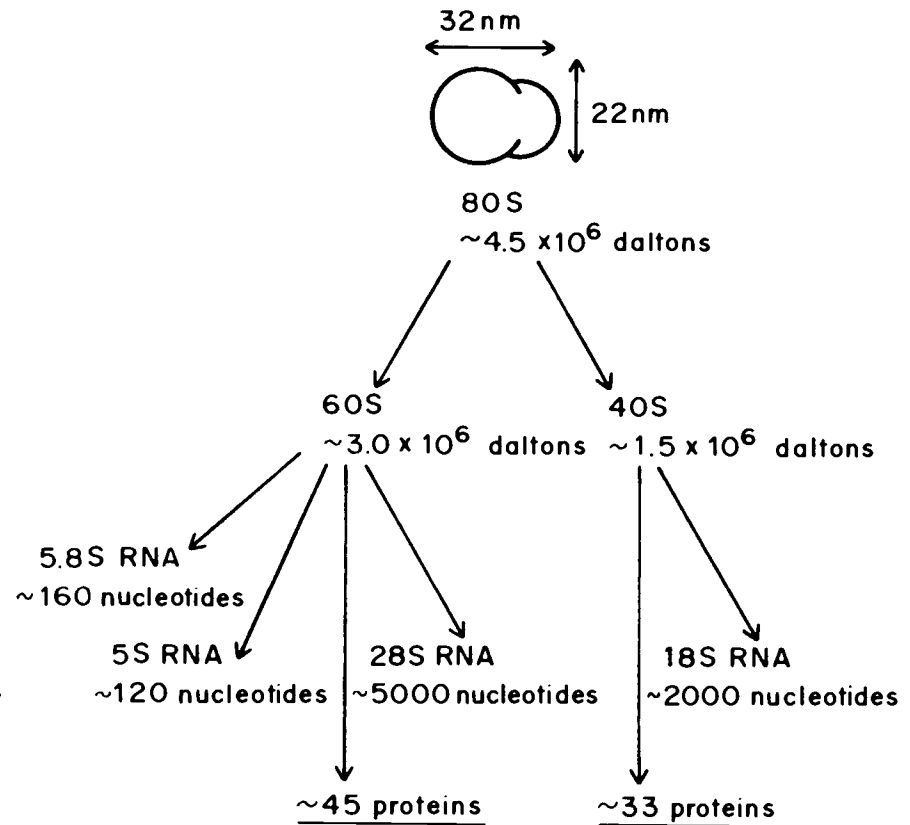
Figure 1. A comparison of the molecular components of prokaryotic and eukaryotic ribosomes.

eukaryotes the mRNA is processed post-transcriptionally and then in association with proteins transported to the cytoplasm (5). It is in the cytoplasm that initiation takes place.

Ribosomes have several enzymatic activities required for protein synthesis and they serve to bring together a single mRNA and charged tRNA molecules into the proper position for translation. Conceptionally, the ribosome can be divided into two domains (6). One part is called the translational domain and contains the apparatus for reading the message. The second domain is an exit domain which is the site of emergence of the nascent polypeptide chain and of membrane binding.

The molecular composition of prokaryotic ribosomes 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 rRNA (23S, 16S, 5S) and approximately 52 proteins. It is quite difficult to assign specific functions to each of the components since they appear to function cooperatively in ribosome assembly and in translation. Both events involve protein-protein as well as protein-RNA interactions (7,8,9).

The overall process of protein synthesis (translation) can be divided into three stages: initiation, elongation and termination of mRNA translation.

RNA is a major component of ribosomes and plays an important role in directing assembly of ribosomes and is also likely to participate directly in the process of protein synthesis (10,11,12). Available evidence implicates rRNA involvement in: 1) mRNA selection, 2) tRNA binding, 3) antibiotic sensitivity, and 4) subunit association (13,14). For example, there is very good evidence that 16S rRNA plays a central role, by forming base pairs with a site on mRNA just to the left of the initiation codon (12,15,16). Studies with several antibiotic inhibitors of initiation indicate that the site of action involves the 3' end of 16S rRNA. For example, kasugamycin inhibits the initiation phase of protein synthesis. Kasugamycin-resistant mutants lack the enzyme which methylates two adjacent adenosine residues 23 nucleotides from the 3'-terminus (17,18,19). Chemical modification of 16S rRNA with kethoxal which modifies guanines in single stranded regions have suggested a role for the RNA in subunit association (20,21,22).

Initiation of Protein Synthesis

Initiation of protein synthesis in bacterial systems involves the binding of 30S ribosomal subunits to specific regions of mRNA, accompanied by the initiation factor-dependent binding of fMet-tRNA. The final product is a 70S initiation complex containing the initiator tRNA in a position appropriate for the commencement of the elonga-

tion phase. Along with interaction of ribosomal subunits, mRNA and initiator tRNA, specific initiation factors are also involved. These three initiation factors are called IF-1, IF-2, IF-3 and they are required for the translation of natural mRNA. Initiation is believed to be the rate-limiting step in protein synthesis, during which a discrimination occurs between different mRNAs and different cistrons in mRNA (5). Initiation would therefore be expected to be an essential control point in translation.

An important question concerning the process of initiation is how ribosomes find the correct starting places on mRNA, and how is this process controlled. At least two base-pairing interactions with mRNA are thought to be involved in specific initiation of protein synthesis in E. coli: these are the AUG initiation codon pairing with the anticodon loop of fMet-tRNA, and a sequence to the 5'-side of the AUG pairing with a sequence near the 3'-end of the 16S ribosomal RNA (16,23).

The initial reaction involves the interaction of initiation factor 3 with the 30S subunit. IF-3 acts as an 'anti-association' factor, preventing 50S subunit complex formation until the mRNA has bound properly along with fMet-tRNA. This is followed by the binding of IF-1 and IF-2 to form a 30S initiation complex (24). The role of IF-3 which interacts with both 30S subunit and mRNA could be to potentiate or stabilize base-pairing between the 3'-

terminus of 16S RNA and the complementary region of the ribosome binding site. IF-1 serves to increase the rate constant for the dissociation of 70S ribosome, while IF-2 functions to direct fMet-tRNA binding (23). These three factors are released when 50S subunit of ribosomes bind. IF-2 is the only factor requiring GTP hydrolysis for its release.

The Elongation Step of Protein Biosynthesis

Once the 70S initiation complex is formed, amino acids are added to the growing polypeptide chain one at a time in a sequence determined by the base sequence of the mRNA. Steps involved in polypeptide chain elongation are as follows:

1) Elongation factor Tu guides an aminoacyl-tRNA to the A site.

2) Tu is reactivated by Ts following the release of Tu from the ribosome.

3) Peptidyl transfer occurs from the P site to the aminoacyl-tRNA in the A site.

4) EF-G mediates the extended peptidyl tRNA translocation from the A site to the P site.

The P site is defined as the peptidyl site in the 70S subunit complex which contains the growing polypeptide-tRNA (peptidyl-tRNA) attached to its codon on the mRNA. While the aminoacyl or A site contains the aminoacyl-tRNA attached to its respective codon on the

mRNA. It has been reported (25) that the only rRNA-mRNA interaction involved in the elongation phase is the interactions of tRNA in the P site which binds to the C_{1400} of the 16S RNA.

Peptide Chain Termination

Peptide chain termination is directed by one of the three specific codons (UAA, UAG, and UGA) and, when found in the A site, they facilitate release of the completed peptide from ribosomal bound tRNA. In prokaryotes, termination is mediated by three proteins, RF1, RF2, and RF3 (26,27). RF1 promotes termination in response to UAA or UAG, RF2 to UAA or UGA. RF3 stimulates the action of RF1 and RF2; it also has an intrinsic GTPase activity. A single protein termination factor, RF is operative in eukaryotic protein synthesis. No rRNA-mRNA interactions have been proposed for this final phase in protein synthesis.

The Shine and Dalgarno Sequence and Protein Synthesis in E. coli

The research described here was focused on bacterial (E. coli) 30S subunits of the ribosome and the initiation process. The 30S subunit is composed of 21 proteins and a 16S rRNA (13,28). The primary structure of 16S rRNA is now available and the primary structures and properties of many of the proteins have been reported (13,29). The 30S subunit role in protein synthesis and sensitivity to anti-

biotics are well documented (18,30).

The secondary structure of 16S rRNA, as shown in Fig. 2, can be split into four domains (31). The 5'-domain is defined by helix linking position 30 to 550, the central domain is defined by linking 565 and 885, the 3'-major domain by linking 930 to 1390, and the 3'-minor domain is the sequence beyond the 1390. Although much progress has been made in understanding the functional role of the proteins in this particle, little is known of the functional role which the RNA plays. This research was focused on the role of the so-called Shine and Dalgarno sequence located on the 3'-end of the 16S rRNA (bases 1532-1542). This sequence centered around CCUCC is highly conserved within prokaryotic 16S rRNAs.

The accepted view is that this pyrimidine-rich sequence can base pair with a complementary messenger RNA sequence centered approximately 10 nucleotides behind the initiation codon (32,33). Shine and Dalgarno proposed that the formation of Watson-Crick base pairs between the mRNA and 16S rRNA allows the ribosome to detect the initiation region (34). The Shine and Dalgarno proposal that the 3' end of 16S rRNA plays a functional role in the initiation of protein synthesis is consistent with several different observations. Crosslinking and chemical modification experiments have been used to show that the 3'-end of this RNA provides a binding zone for initiation fac-

Figure 2.   A proposed secondary structure for 16S ribo-
            somal ribonucleic acid as described by Woese
            et al. (1983) (31).   The arrow and bar show the
            central zone designated as the Shine and
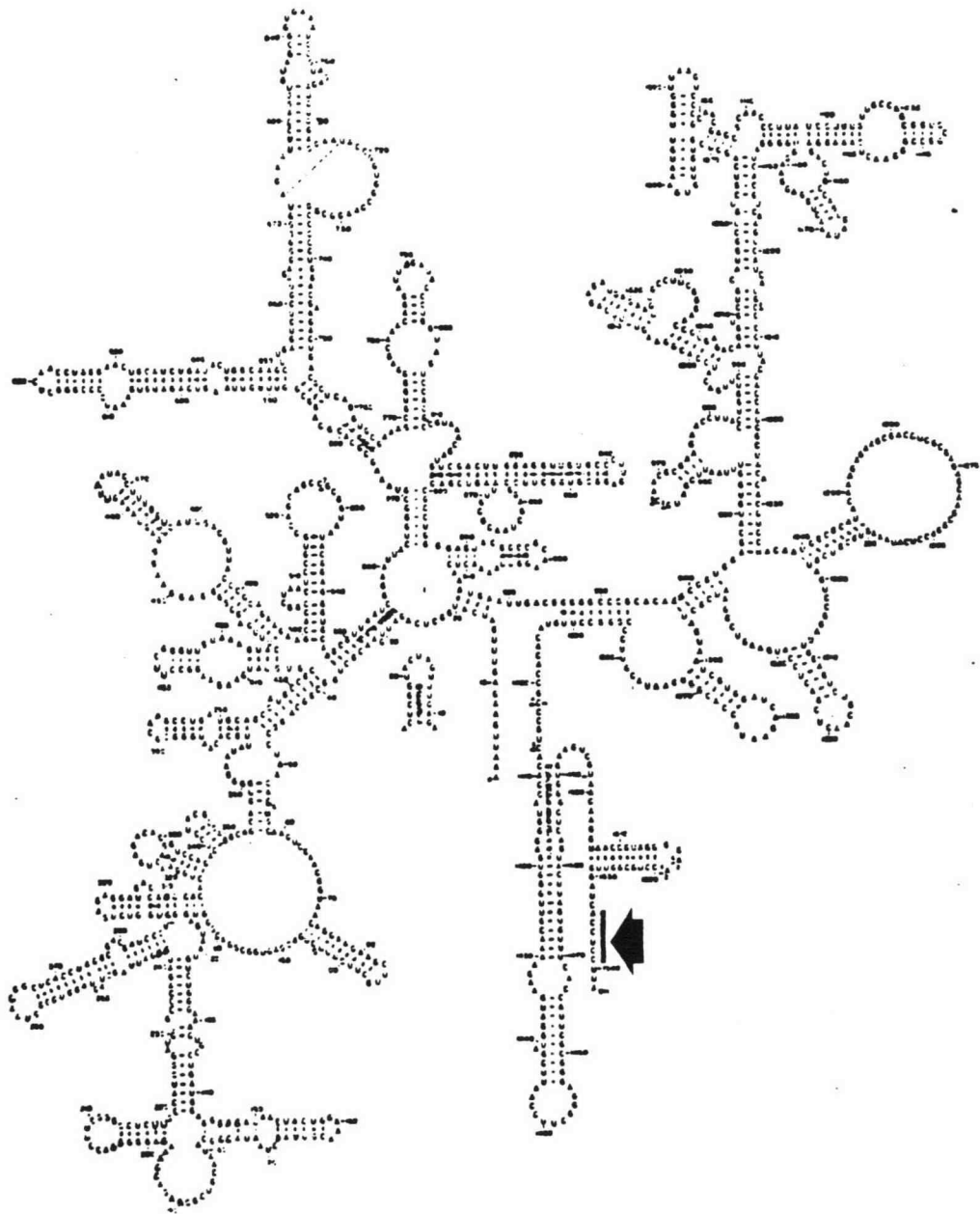   Dalgarno sequence.

Figure 2

tors, and within this region binding sites for ribosomal proteins S1, S2, S11, S12, S13, S14 and S19 are localized (35,36,37). All of these proteins have been shown in one way or another to be involved in the initiation process.

Kasugamycin sensitivity results in a disruption of initiation of protein synthesis and this is mediated by the presence of two highly-conserved dimethyladenosines located 23 and 24 bases from the 3'-end of 16S RNA (17,18). Colicin E3 inactivation of 30S particles is caused by a cleavage of a single phosphodiester bond at position 50 near the 3'-end (15). It has also been observed that copolymers rich in A and G are effective competitive inhibitors of initiation, underscoring the importance of polypurines in ribosome binding to initiator regions (33).

It has been reported (16) that base pairing interaction between 16S RNA and mRNA in E. coli is important in initiation of protein synthesis and this indicates that this interaction is important in selecting the site in mRNA at which the ribosomes bind. Two mutations have been examined on the mRNA of the 0.3 protein of bacteriophage T7. The initiation triplet in one codon involved an AUG to ACG change. This mutation was found to have little effect on the binding of ribosomes to the 0.3 mRNA in vitro, although 0.3 protein synthesis was greatly depressed in vitro as well as in vivo. The second muta-

tion was a G-to-A transition located 11 bases to the 5'-side of the initiator AUG. This change disrupts a possible five-base pairing with a sequence near the 3'-end of 16S ribosomal RNA. This mutation causes the site of ribosome binding to shift about 15 bases to the 3'-side, centering on an internal AUG, but this new site has only a poor potential interaction with 16S RNA.

A direct test of the role of the Shine and Dalgarno sequence has not been reported. A logical approach would be to isolate primary structural mutants of rRNAs. Unfortunately, naturally occuring primary structural mutations in rRNAs have not been reported. In vitro mutagenesis analysis could provide a means to test the assertion that the 3'-sequence is critical in the initiation phase of protein synthesis. In this work, an in vitro procedure, shown in Fig. 3, was used to remove the Shine and Dalgarno conserved sequence from 16S rRNA. The deletions were made directly within the RNA and do not involve recombinant DNA techniques or genetic manipulation of the bacteria.

The RNAs which were modified by the removal of this sequence were used in in vitro reconstitution studies to assess the effects of these alterations on ribosome assembly. In vitro reconstitution procedures for 30S particles from purified 30S ribosomal proteins and 16S rRNA are well established and the reconstituted particles are

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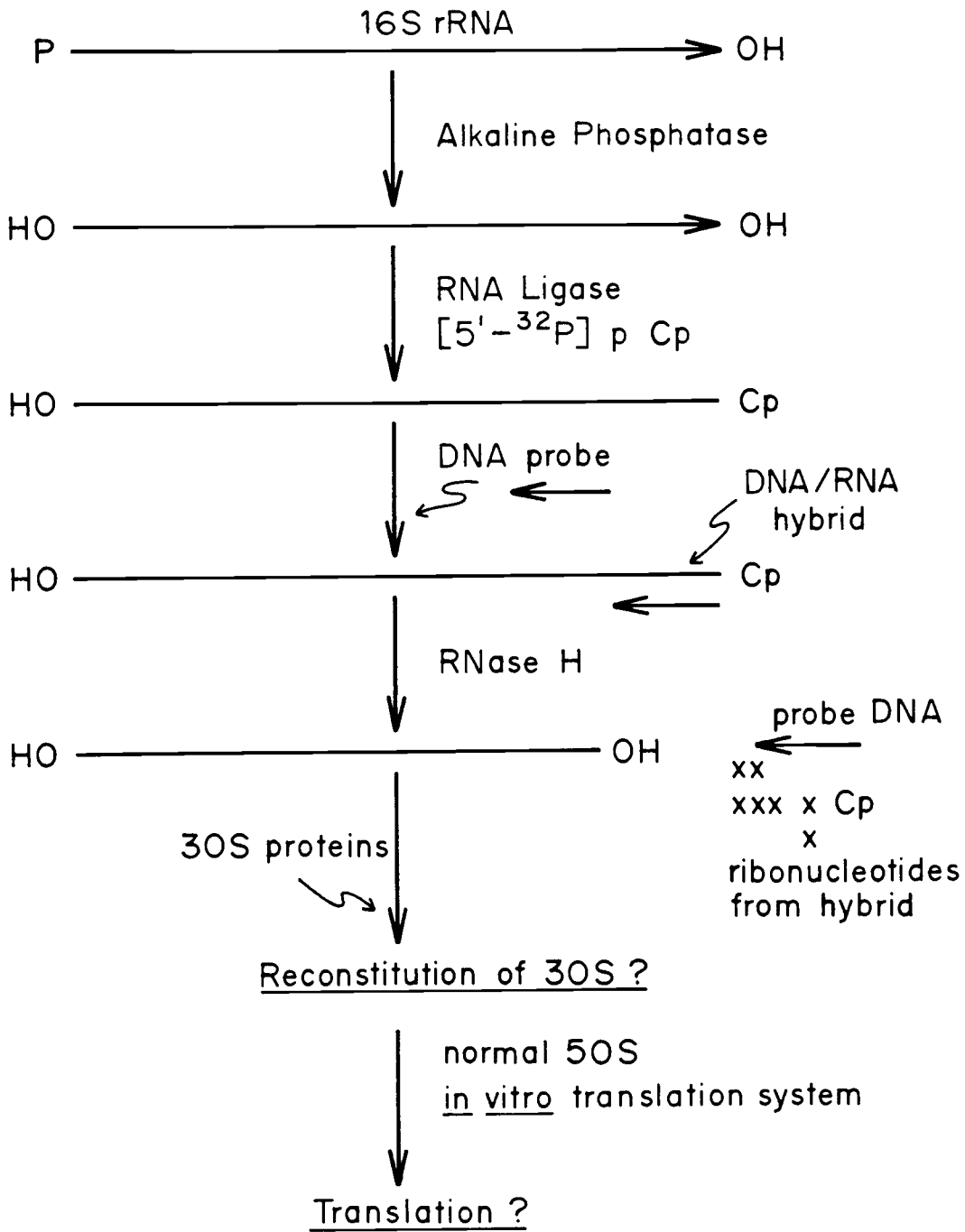
Figure 3

functional in <u>in</u> <u>vitro</u> protein synthesis (38,39,40).

The protein constituents of reconstituted particles were examined by polyacrylamide gel electrophoresis. The presence or absence of ribosomal protein S21 was of particular interest, since it has been reported that the base pairing potential of the 3'-terminus of 16S RNA is dependent on the functional state of the 30S subunit, which depends on the presence of protein S21 (41,42). It has been reported that 30S subunits lacking S21 do not bind deoxyoctanucleotides which are complementary to the 3'-terminus of the 16S RNA. The addition of protein S21 restores octamer binding and potential of the subunits to initiate translation of MS2 mRNA.

MATERIALS AND METHODS

Mid-log phase E. coli D-10 cells were the source of ribosomal components. Ribosomal proteins were prepared from 30S subunits isolated by zone ultracentrifugation and extracted with acetic acid as previously described (28). RNA was prepared by phenol extraction of cell lysates and 16S rRNA was isolated by zone ultracentrifugation (43). The cells were disrupted with a French pressure cell. When necessary, the RNA was treated with calf thymus alkaline phosphatase (E.C. 3.1.3.1) purchased from New England Nuclear. T4 RNA ligase (E.C. 6.5.1.3) purchased from P & L Biochemicals was used to label the 3'-terminus of the RNA with [5'-^{32}P]pCp from New England Nuclear (44). The labeled 16S rRNA was used as a substrate in deletion experiments using RNase H and a DNA probe complementary to the Shine and Dalgarno zone in the 16S rRNA. The DNA probe of ten bases in length (pTAAGGAGGTG) was synthesized on an Applied Biosystems synthesizer using the phosphoramidite chemistry. Before use, the DNA was purified by high pressure liquid chromatography (45). A portion of the purified DNA was 5'-end labeled using polynucleotide kinase and γ[^{32}P]-ATP. This material was then analyzed in a sequencing polyacrylamide gel for purity. Only material which was found to be homogeneous was used. RNase H from E. coli and calf thymus were used to digest the RNA component of RNA-DNA hybrids. The two enzymes were respectively present at 1.5 units/μg RNA and 0.7

units/μg RNA. The buffer used was a modification of that used for calf thymus digestions and was as follows: 50 mM Tris HCl (pH 8.3), 25 mM $MgCl_2$, 50 mM KCl, 1 mM DTT and 0.03 μg/ml BSA (46). A 1:10 molar ratio of RNA to DNA was routinely used in the hybridization procedure. The mixture was pre-incubated at 50°C for 20 minutes and then at 37°C for 30 minutes. The RNase H was added and the sample was then incubated an additional 50 minutes at 37°C.

The digestion products and the DNA probe were separated from the modified RNA by zone ultracentrifugation followed by ethanol precipitation of the appropriate fractions. The 'deleted' 16S RNAs were labeled at the 3'-terminus with [5'-^{32}P]pCp and purified by zone ultracentrifugation, after phenol extraction and ethanol precipitation. To assure proper cleavage by RNase H in the digestions, portions of the 'deleted' 16S RNA were treated with T1 RNase which cleaves after guanines to analyze for 3'-end homogeneity and its sequence (47).

Ribosome reconstitution experiments were carried out using one A_{260} 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 $MgCl_2$, .3 M KCl and 6 mM β-mercaptoethanol (39). The mixture with RNA was pre-incubated at 42°C for 30 min and then the ribosomal proteins were added. The sample was then incubated for another 30 min at 42°C. The reconstituted 30S particles for RNase T1 analysis were purified by zone ultracentrifu-

gation, followed by ethanol precipitation of the 30S zone. Protein concentrations were determined, using the Bio-Rad protein assay system and polyacrylamide gel electrophoresis at pH 4.5 was used to analyze the protein composition of extracted proteins as described (28,48). Silver-staining gel electrophoresis was carried out as described by Wray et al. (49).

RESULTS

Efficiency of 3'-End Labeling of 16S rRNA with [5'-^{32}P]pCp

The 5'-phosphate on purified 16S rRNA was removed by treatment of the samples with calf intestine alkaline phosphatase. This was done to prevent circularization of the RNA in subsequent steps. This 16S rRNA was labeled with [5'-^{32}P]pCp. The labeled 16S rRNA used in some experiments was further purified by polyacrylamide gel electrophoresis. However, we have found that preparations obtained by zone ultracentrifugation are sufficiently pure for these studies to be used directly (Fig. 4).

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

The sequence designated for deletion was (p-GAUCACCUCCUUA-OH) corresponding bases 1530 to 1542 in 16S rRNA. The sequence of the DNA probe used was d(pTAAGGAGGTG). Hybrids between the small DNA probe and 16S rRNA were formed and then specifically digested by a mixture of RNase H from E. coli and calf thymus. These enzymes selectively digest the RNA component of the hybrid, leaving the DNA probe and the remainder of the RNA intact (46,47). The efficiency of the digestion is typically greater than 80%, based on the reduction of label in the 16S RNA zone on the sucrose gradients (Fig. 5).

Although full sequencing would seem to be the best way to determine the location of the cleavage site or sequence of the 3'-end of the modified 16S, this would not allow an accurate assessment of the precision of the enzy-

Figure 4. Sedimentation profile of $[5'-^{32}P]$pCp 16S rRNA
on a sucrose gradient (15-30% sucrose in TSM
buffers). Sedimentation was from left to right
and the separation was performed with a Sorvall
AH 650 rotor at 49,000 rpm for 3 hrs, 25 min at
4°C. Fractions 9 through 12 contain the 16S
rRNA and unincorporated $[5'-^{32}P]$pCp remains in
fractions 1 through 5.

Figure 4

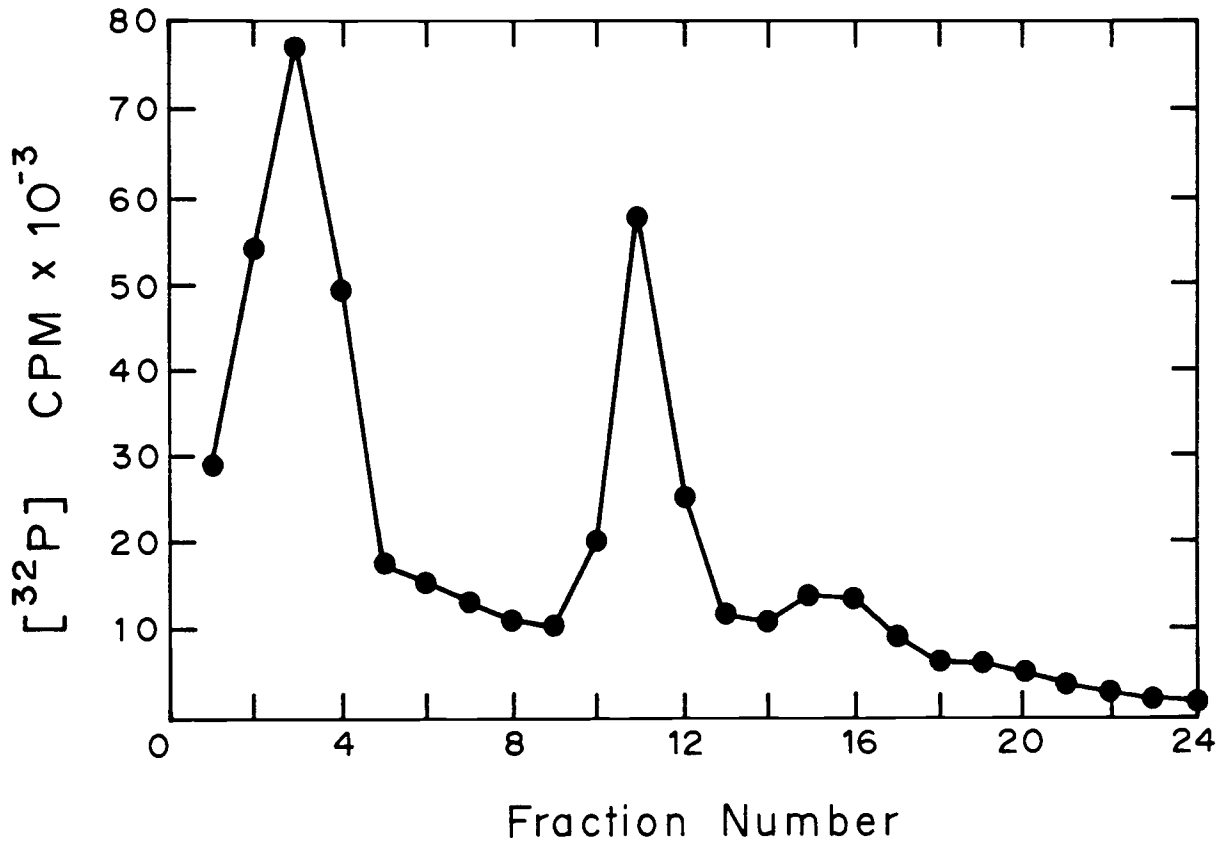Figure 5. Sedimentation profile of RNase H cleaved 16S
rRNA. The RNA was 3'-end [^{32}P]-labeled as
described in materials and methods. A. Control
16S rRNA. B. Profile of 16S rRNA subjected to
the terminal deletion procedure outlined in
Fig. 3 and detailed in the text. Sedimentation
was from left to right and the separation was
performed with a Sorvall AH 650 rotor at 49,000
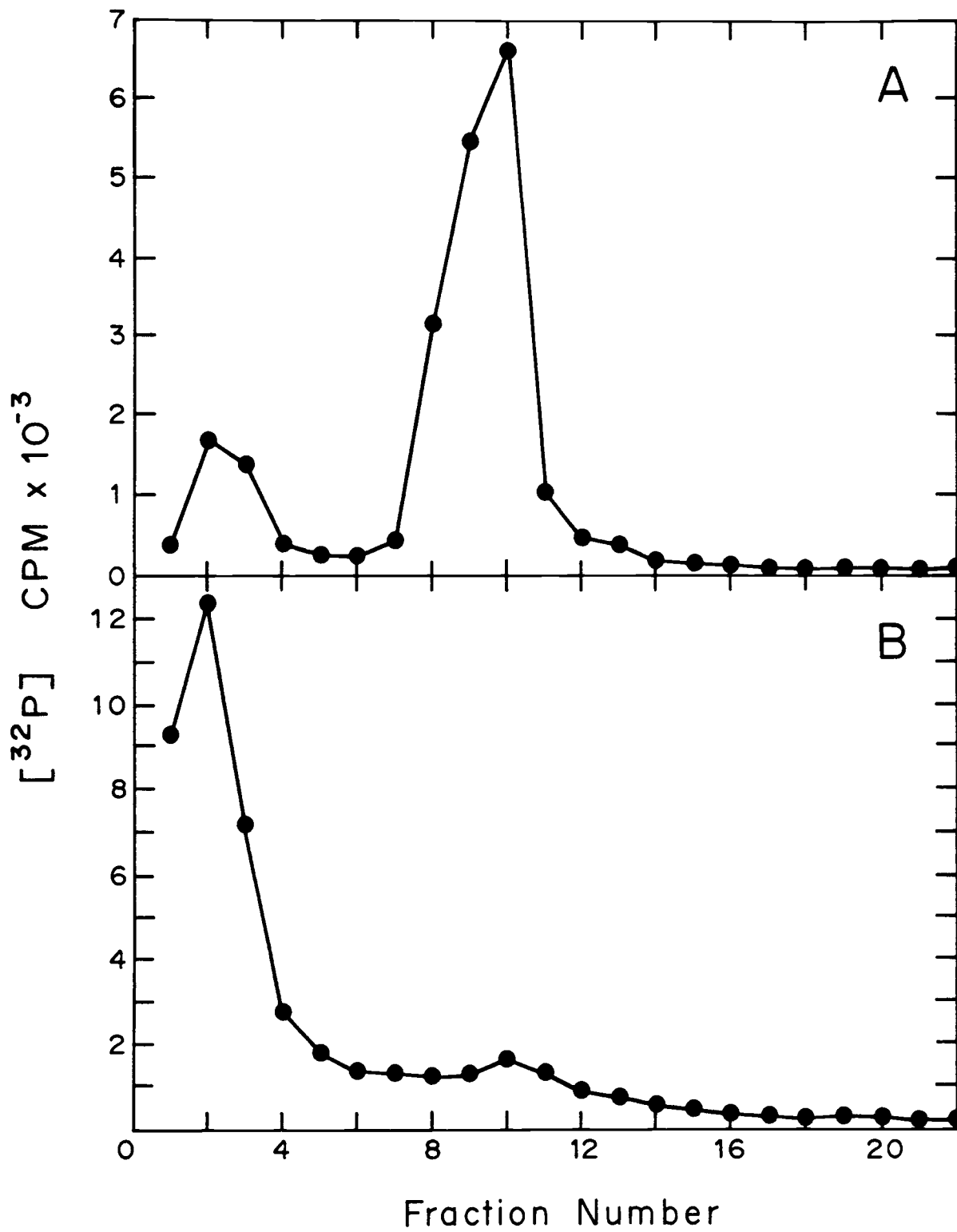rpm for 2.75 hours.

Figure 5

matic attack. For example, if 80% of the cleavages were at position 1535, and remaining 20% were at position 1538, in autoradiographs of sequencing gels, it is likely that the shadows which would represent the minor cleavage would be ignored. 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, 3'-end labeled samples were completely digested with T1 RNase. Knowledge of the primary structure of 16S rRNA permits the prediction of the size of the 3'-end labeled digestion product. That is, T1 RNase cleaves after guanines. If the cleavages are as predicted, then the size of the labeled digestion product should be as expected. If two products are found, the cleavage site can be determined on the basis of the size of the products. The distribution of the ^{32}P between the two products is also a direct measure of the amount of each variant 16S species present following the deletion procedure. Based on this type of analysis, 50% of the RNA has 8 bases removed from the 3'-end, the other 50% has 10 bases removed (Fig. 6).

Reconstitution of 30S Particles

Modified 16S rRNA was tested for effectiveness in in vitro reconstitution, using previously described procedures (39,40). Reconstitution was judged successful if 30S particles which contain the same amount of each of the

Figure 6. Autoradiograph of RNase Tl complete digestion
           of 3'-end labeled rRNA. 1. 16S rRNA digestion.
           The major labeled product is 13 nucleotides in
           length. 2. 16S rRNA subjected to the terminal
           deletion procedure. The principal labeled
           products are 3 and 5 nucleotides in length.
           3. As in 2 but recovered from reconstituted 30S
           particles. 4. A 5'-end labeled 10 nucleotide
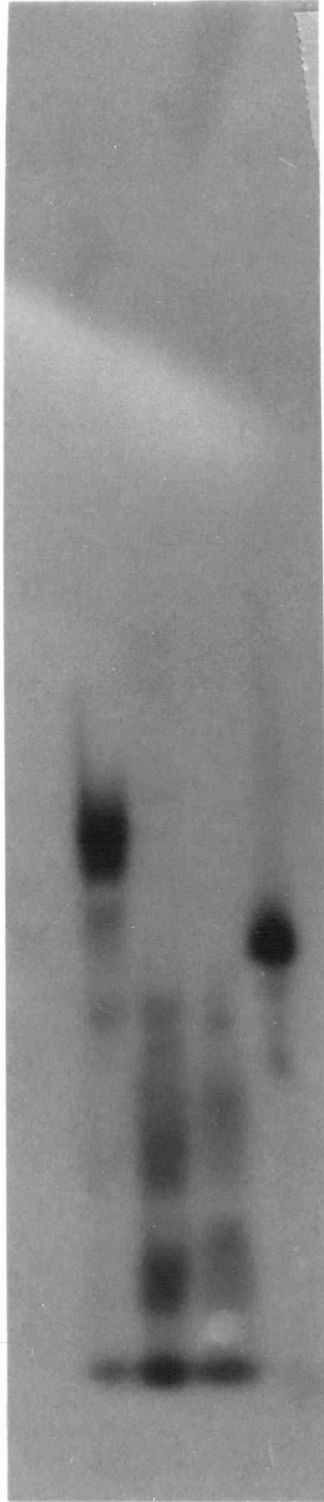   DNA marker.

1 2 3 4

Figure 6

proteins found in normal 30S subunits are recovered. The 'deleted' 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 repurified by zone ultracentrifugation (Fig. 7).

The purified labeled 16S was tested for reconstitution competency at a 1:1.2 molar ratio of RNA to proteins. Analytical experiments were performed using labeled [5'-^{32}P]pCp 'deleted' 16S rRNA in the presence of non-labeled normal 16S rRNA as an internal marker to monitor the efficiency of the reconstitution and location of 30S particles. The results shown in Fig. 8 indicate that the terminal deletion of 8 to 10 bases does not impair reconstitution competency of the RNA.

Determination of the Protein Composition of 30S
Reconstituted Particles

To determine the protein composition of 30S particles derived using deleted 16S rRNA, preparative amounts of this RNA were isolated. Reconstitutions were done as before but only deleted RNA was present. A portion of the reconstituted 30S particles was purified by zone ultra-centrifugation to assess the efficiency of the reconstitution and to recover the RNA in the particles for analysis with T1 RNase as described (46). The remaining portion was used for protein composition analysis. As shown in Fig. 9, proteins which are not associated with 30S particles do not precipitate. Therefore, an equal volume of

Figure 7.   Sedimentation profile of $[5'-^{32}P]$-labeled 16S
rRNA subjected to the terminal deletion pro-
cedure.   Sedimentation is from left to right
and was performed as described in Fig. 4.
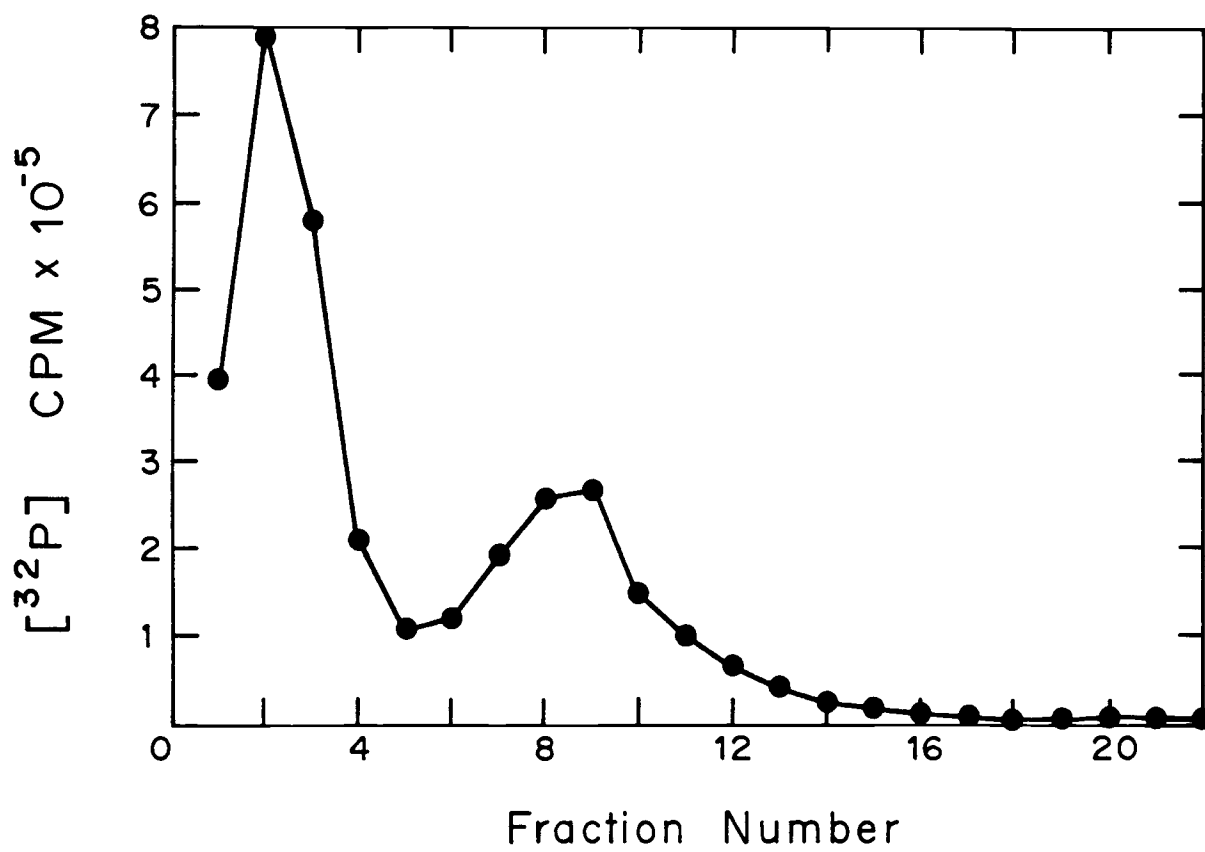
Figure 7

Figure 8.   Sedimentation profile of 30S particles
            reconstituted with 3'-end labeled 16S rRNA from
            which the 3'-terminal nucleotides had been
            removed. Sedimentation is from left to right
            and conditions are as described in Fig. 5.
            A. Profile of reconstituted particles. The
            A_{260} measurements are of the internal marker
            16S rRNA and is shown as open circles. A 1:1.2
            molar ratio of rRNA to r-protein was used.
            B. Profile of terminally deleted 16S rRNA.
            C. Profile of normal 16S rRNA.

Figure 8

Figure 9. Polyacrylamide gel analysis of reconstituted
           30S particles. The procedures were as
           described by Voynow et al. (1969) (28). The
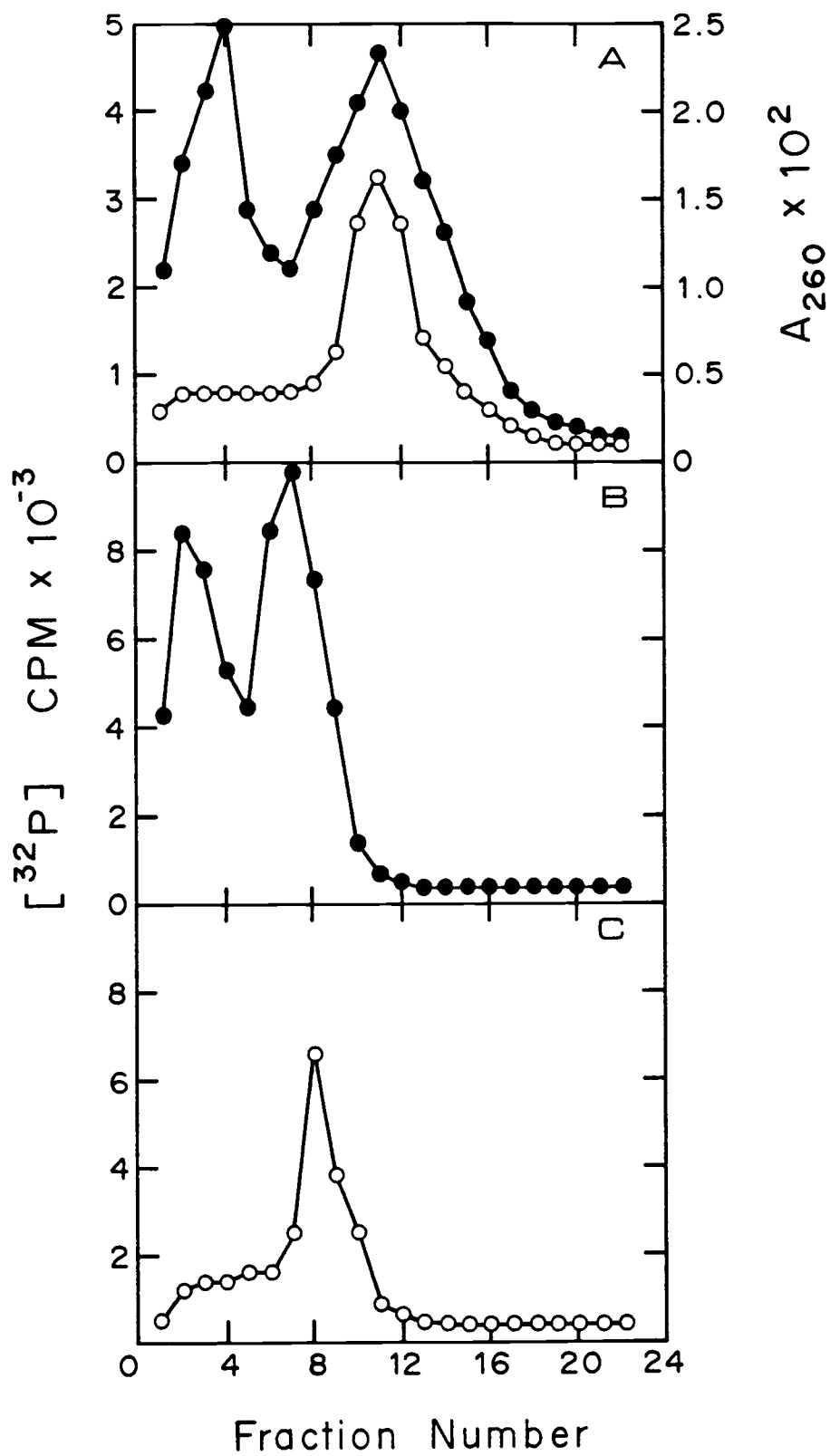   gels were 10% acrylamide, 1 mm thick and 10 cm
           x 8 cm. They were run at 7 mA for 2.75 hours
           at room temperature. Proteins were visualized
           by silver staining (49). Lanes 1 and 6. Pro-
           teins prepared by extraction of purified 30S
           subunits (4.4. μg and 2.2 μg, respectively).
           2. Ethanol supernate from reconstitution using
           3'-deleted 16S. 3. Ethanol supernate from
           purified 30S subunit proteins in the absence
           of normal 16S rRNA. 4. Protein recovered from
           precipitation of purified 30S subunit proteins
           in the absence of 16S rRNA. 5. Protein recov-
           ered from reconstituted 30S subunits in the
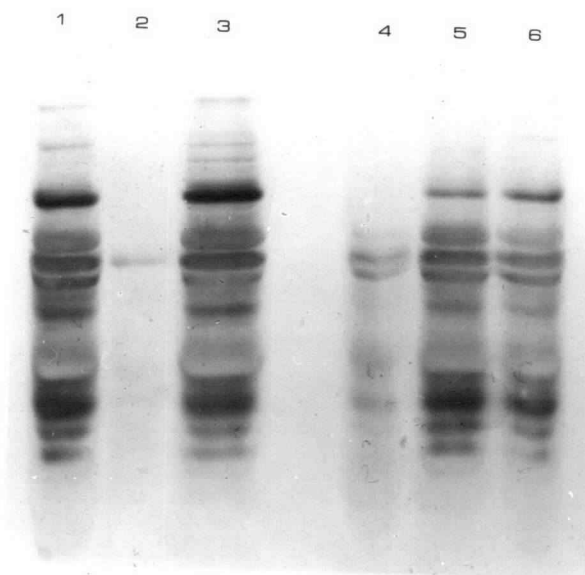   presence of 3'-deleted 16S rRNA.

Figure 9

ethanol was added to the sample and it was placed at −20°C for at least two hours to precipitate the newly formed 30S particles. Analysis of the particles prepared in this fashion shows that there is no observable difference between the protein composition of these particles and normal 30S subunits (Fig. 10).

Figure 10.   Laser densitometric scans of polyacrylamide
             separations of 30S protein from normal 30S
             particles and particles reconstituted using 3'
             terminally deleted 16S rRNA. A. Protein from
             normal 30S subunits. B. Protein from
             particles reconstituted with deleted 16S rRNA.
             C. A photograph of the gels scanned with the
             control placed above the experimental. The
             location of the 30S proteins is indicated by
             the vertical bars. The localization of the
             proteins on the gel is based upon previous
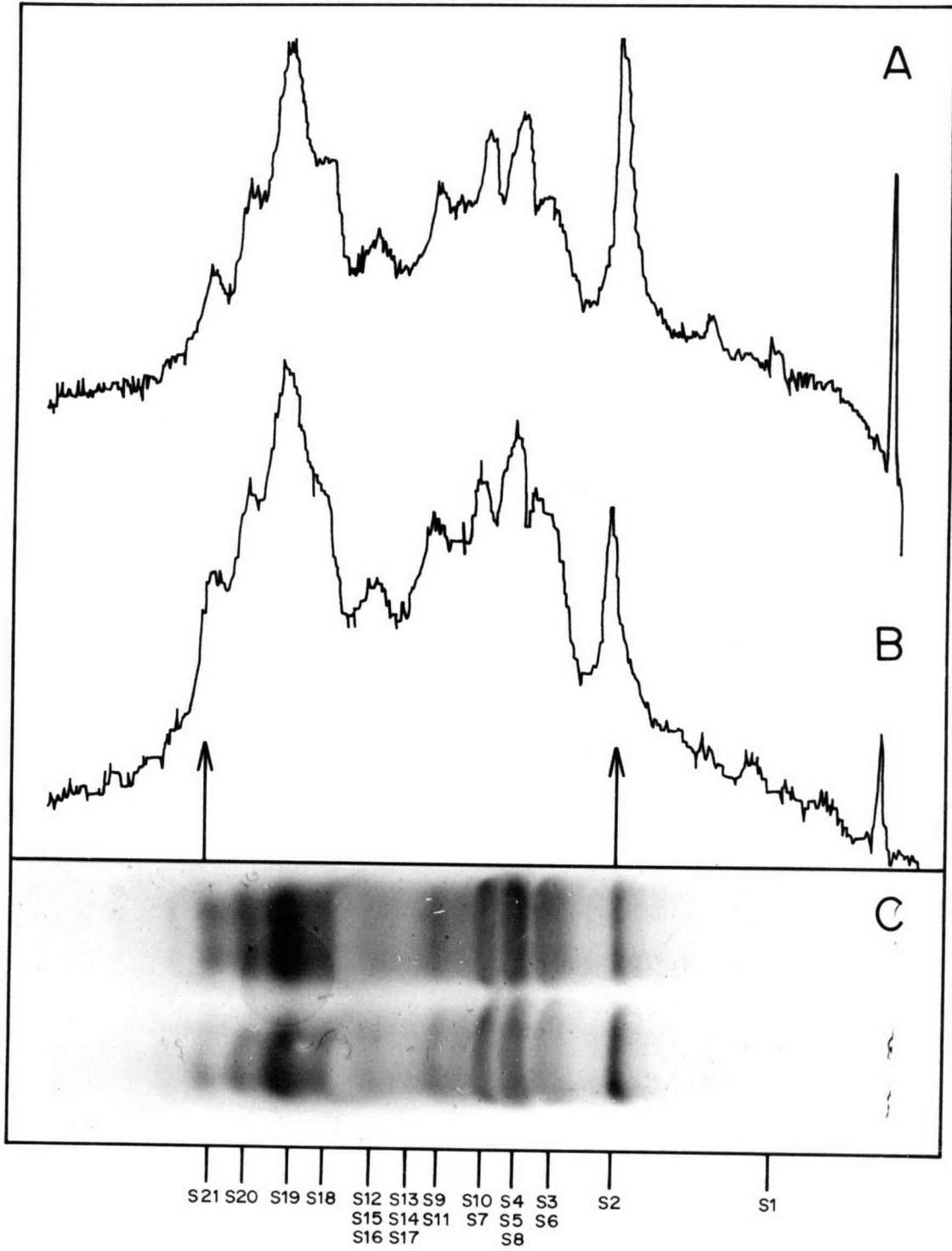   reports (48).

Figure 10

DISCUSSION

The Shine and Dalgarno sequence is a highly conserved sequence unique to prokaryotes. The removal of this sequence from the RNA without impairing in vitro 30S assembly was a major objective. The strategy employed yielded terminal deletions of 16S rRNA and was efficient. However, in the initial attempts to remove the 3'-terminus of 16S rRNA probe was hybridized with the RNA using the conditions described, but only E. coli RNase H was used in the digestions. Under these circumstances the digestion efficiency appeared to be about 50% based upon the amount of [32P]-label removed from the 3'-end of labeled 16S. Likewise the use of only calf-thymus RNase H resulted in about a 50% removal of 3'-terminal sequences. The 16S RNAs which have 8 to 10 bases removed can not be separated from normal molecules which are 1542 nucleotides in length. A 50% background of normal 16S RNA was not satisfactory for straightforward analysis of the characteristics of reconstituted particles. It was therefore decided to use a mixture of RNase Hs to obtain higher cleavage efficiencies. The use of the modified RNAs in the reconstitution system provided the opportunity to test the importance of the 3'-end nucleotides in assembly. A difference in the assembly characteristics of the two base different RNAs would have been detected in terms of a low assembly efficiency. Overall the data from the sucrose gradients indicate that nearly all of the RNA is incor-

porated into particles that co-sediment with 30S marker particles. In addition to this, RNase Tl digest of modified RNA recovered from these particles indicate that both of the RNAs are incorporated into the subunits. The competency of the RNAs in in vitro reconstitution experiments to form particles which sediment at 30S is not compromised by the removal of the ten 3'-terminal nucleotides.

The concern that the 16S rRNAs from which the terminal nucleotides had been removed might not be assembly competent was highlighted by the observation that the modified RNA manifested consistently a lower sedimentation velocity than 16S rRNA. When the modified RNA was compared to marker 16S RNA on sucrose gradients the peak zone for it was typically one to two fractions behind the 16S peak. Since only 8 to 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 its frictional coefficient and would account for the slower sedimentation. Such a change could have resulted in the denaturation of the molecule in a manner that would have impaired it in subunit reconstitutions (50, 51, 52). If the altered sedimentation properties do reflect tertiary structural changes these may well mirror functional characteristics of the initiation process in protein synthesis. Van-Duin et al. have observed that 30S ribosomes which are protein S21 deficient are not

able to properly bind MS2 mRNA during initiation of pro-
tein synthesis (41,42). Addition of S21 to the particles
restored functional initiation. The conclusion was that
the S21 yielded a configurational change that made the
Shine and Dalgarno sequence available for mRNA interac-
tion. The question remained as to whether or not there
was a direct interaction between S21 and the terminal
nucleotides or was there simply a displacement of the
terminal nucleotides. In the former case, the removal of
the terminal-nucleotides might reduce S21 incorporation
into reconstituted particles since a binding site for the
protein had been removed.

Nomura et al. have reported that colecin E3 treated
16S rRNA does not bind S21 when the RNA is used in in
vitro reconstitution experiments (53). Analysis of the
protein composition of particles generated using the
modified 16S revealed no obvious difference between
control and experimental particles with regard to protein
S21. Suggesting that the actual tight binding zone for
this protein is located nearer the colecin E3 cleavage
site at position about 50 nucleotides from the 3'-ter-
minal. Although this does not preclude a weak interaction
the magnitude of the effects of S21 on initiation of pro-
tein synthesis suggest a less than subtle effect. The
notion of displacement seems more attractive given the
observation that removal of this sequence appears to alter
16S conformation. One possibility is that the 3'-end

interacts with other zones within the molecule that form part of the S21 binding region, and the binding of S21 simply disrupts the interaction. This suggestion shrouds the conclusion of Van-Duin and his coworkers that S21 plays a direct role in the process of initiation. Although it is possible that S21 s' function could be just to prevent the 3'-end interaction, it would not be surprising to discover a more direct function or functions for this protein such as those suggested by others (33, 54, 55).

The basic assumption in the preceding discussion has been that the Shine and Dalgarno proposal is indeed correct. It is not inconceivable that it is S21 that is essential in a direct manner for initiation of protein synthesis or that its presence may permit interactions other than those which have been proposed. The observation here that the removal of the Shine and Dalgarno sequence does not impair in vitro reconstitution and that all of the normal ribosomal components are present in the subunit will permit a direct test of the need for this precise sequence in in vitro protein synthesizing systems.

It is worth noting that the approach taken here avoids some of the difficulties encountered when recombinant DNA techniques are used to generate deletions within ribosomal operons (56, 57). It appears that the in vivo response to manipulation, even in zones far from rRNA

processing sites, is impairment of rRNA maturation. The approach described here bypasses this facet of the problem and permits a direct assessment of the utility of terminal sequences in the process of protein synthesis without concern for the disruption of RNase processing sites.

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APPENDICES

APPENDICES

Extraction of 16S rRNA and 30S Ribosomal Proteins of E. coli

4.5 mg of purified 16S rRNA was recovered from 8.5 gm of E. coli cells. The 16S rRNA were separated from other rRNAs in the cells by zone ultracentrifugation (figure 11). This RNA was used as a substrate for deletions and re-constitution experiments. Ribosomal proteins were extracted from 30S subunits which were also prepared by zone ultracentrifugation (figure 12). A modified acetic acid extraction procedure was used to prepare protein from 30S particles. This method yields about 95% of the ribosomal protein with little RNA contamination. Protein concentration was determined by Bio-Rad protein assay (figure 13).

Figure 11.   Sedimentation profile or total rRNAs in $\underline{E.}$
$\underline{coli}$ on a sucrose gradient in TSM buffer
$\overline{(10}$ mM Tris-HCl, 10 mM MgCl$_2$, 0.3 mM succinic
acid, pH 8). Sedimentation is from right to
left, shows the region of 23S rRNA, 16S rRNA
and 5S rRNA. The total rRNAs was extracted
as described in material and method. The
separation was performed with a Sorvall
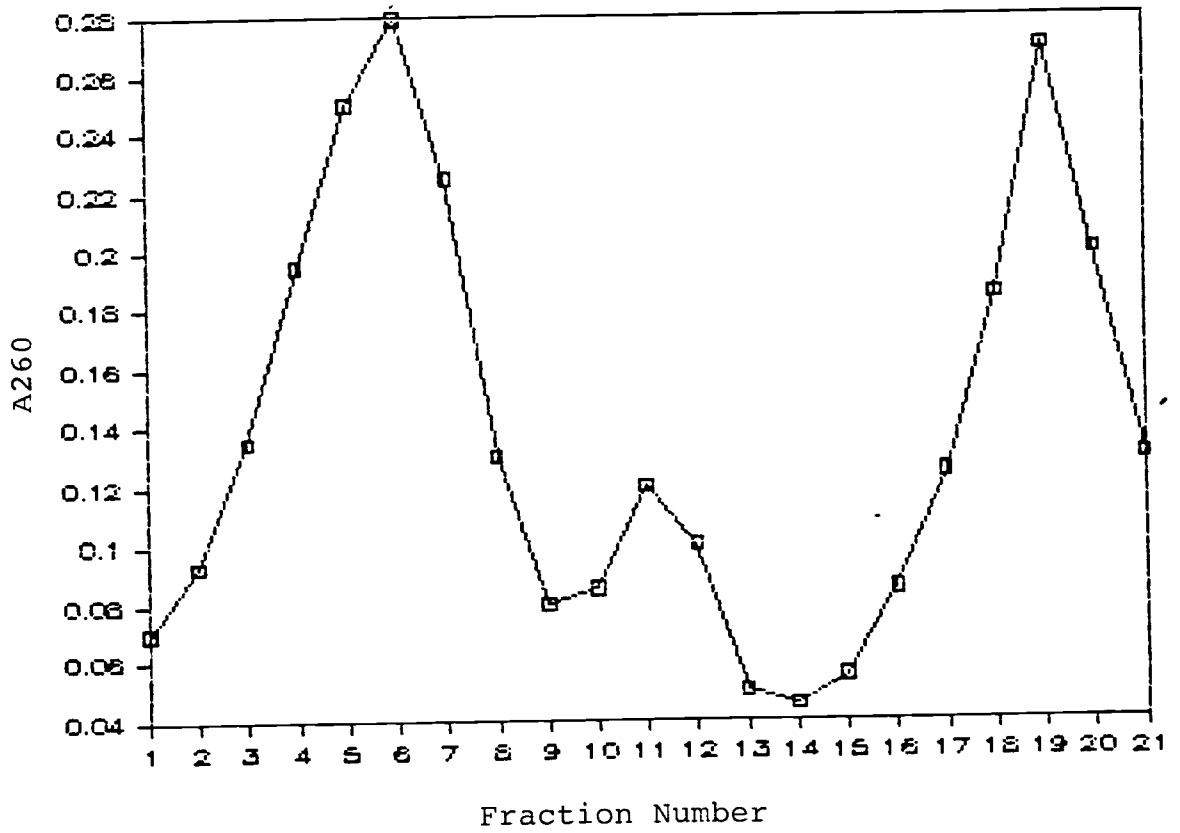AH627 rotor at 24,000 rpm, 4°C for 21 hours.

Figure 11

Figure 12.   Sedimentation profile of 70S ribosome sub-
units isolation in 15-30% sucrose gradient in
gradient buffer (10 mM Tris-Base, 50 mM KCl,
0.3 mM KCl, 6.8 mM B-mercepto ethanol, pH
7.6).   70S ribosomes were extracted by the
method described by Nomura et al. (1968)(38).
Sedimentation is from right to left, shows the
region of 50S subunit, 30S subunit and tRNA.
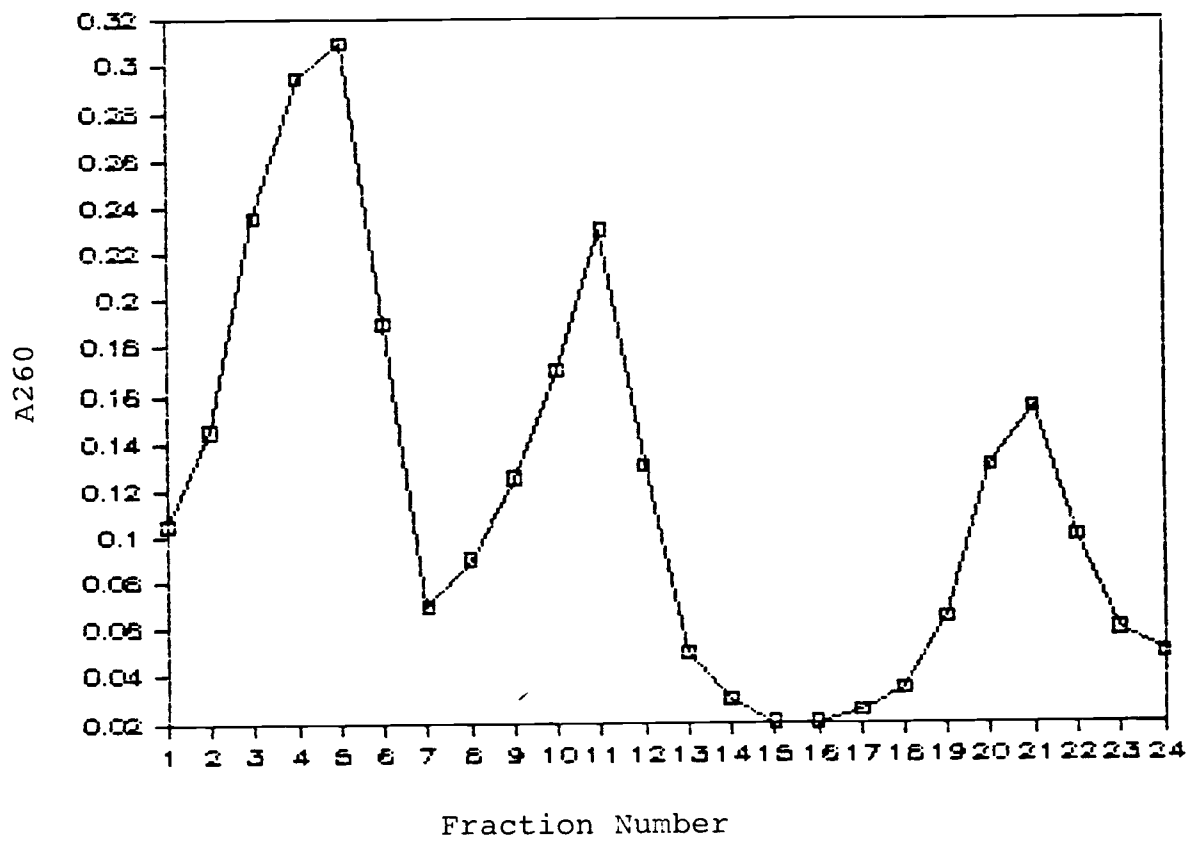The separation was performed with a Sorvall
AH627 at 24,000 rpm, 4°C for 21 hours.

Figure 12

Figure 13.   Standard curve for the Bio-Rad Protein Assay
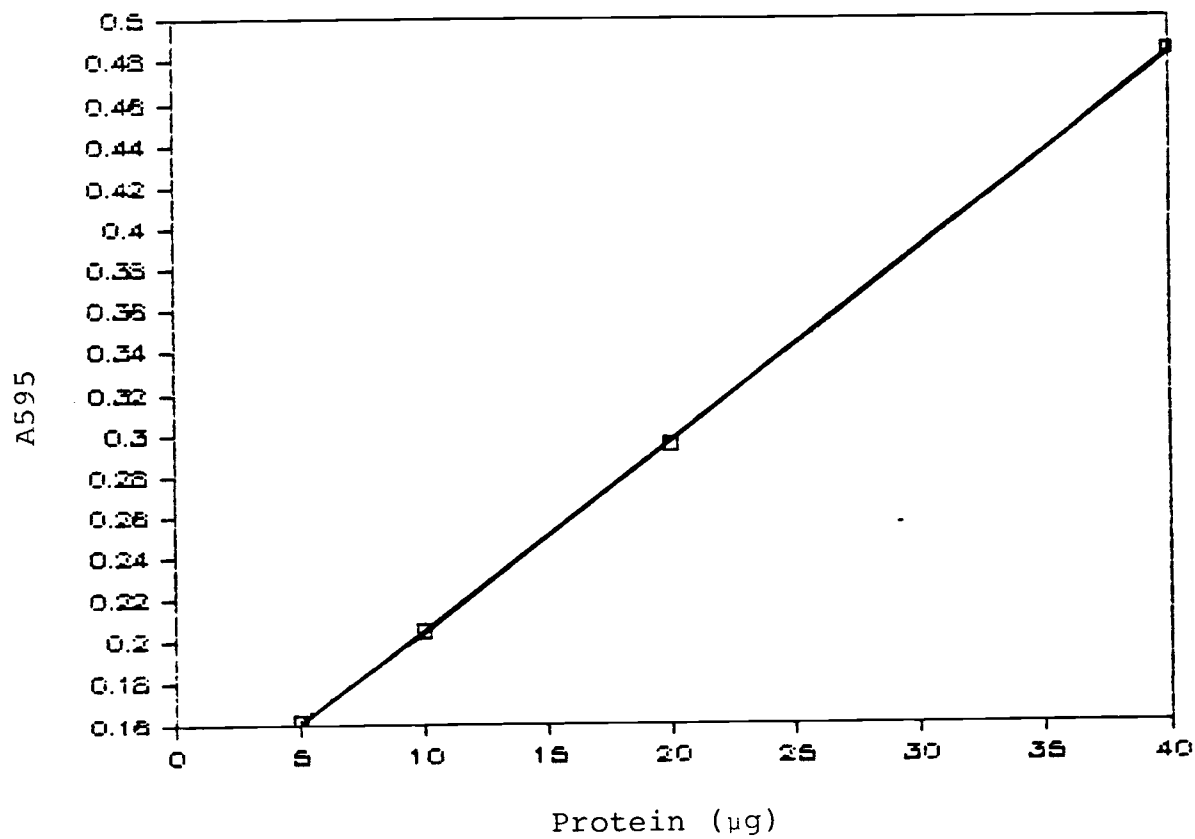   using Bio-Rad Protein Standard.

Figure 13

Figure 14. Autoradiograph of 5' end ^{32}P-labeled 10 base
length of DNA probe (pTAAGGAGGTG). Lanes
1 and 3 purified 5' end ^{32}P-labeled probe.
2. Total DNA probe 5' end ^{32}P-labeled before
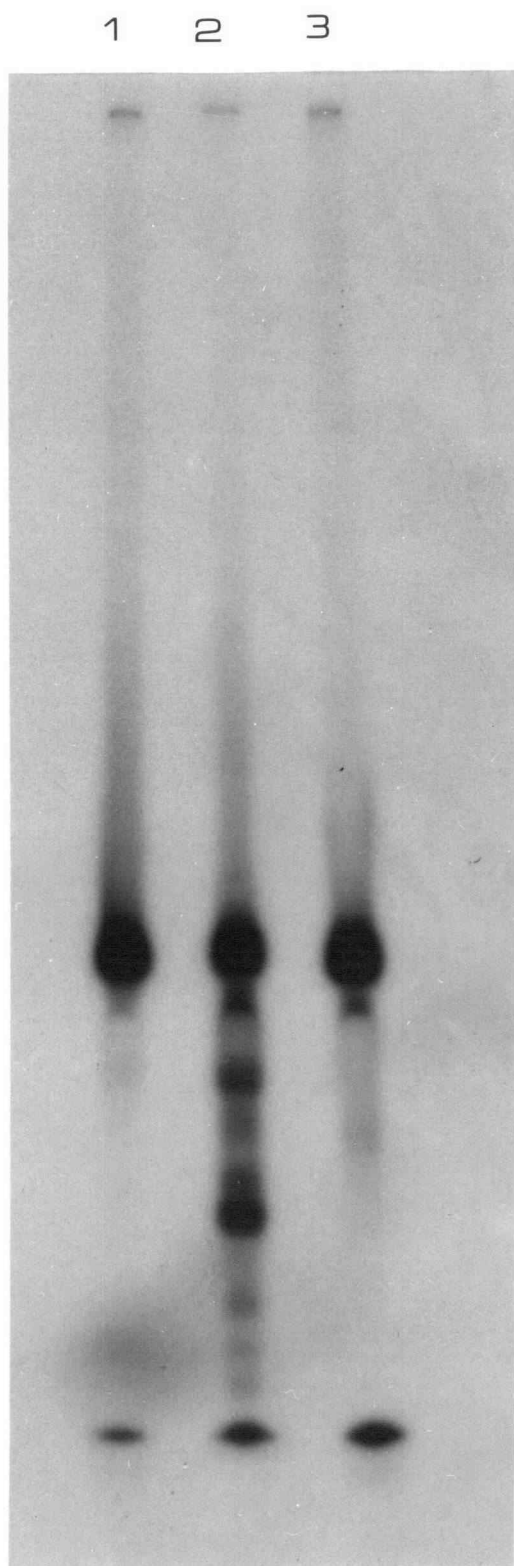purified through HPLC.

Figure 14

Figure 15.   Laser densitometer scan of autoradiograph
              of 16S rRNA in a polyacrylamide gel (the
              gel was a split gel of 10% acrylamide in
              the lower portion and 2.8% acrylamide on
              the upper half). Gels were run at room
              temperature for 2.5 hours at 5 MA. The
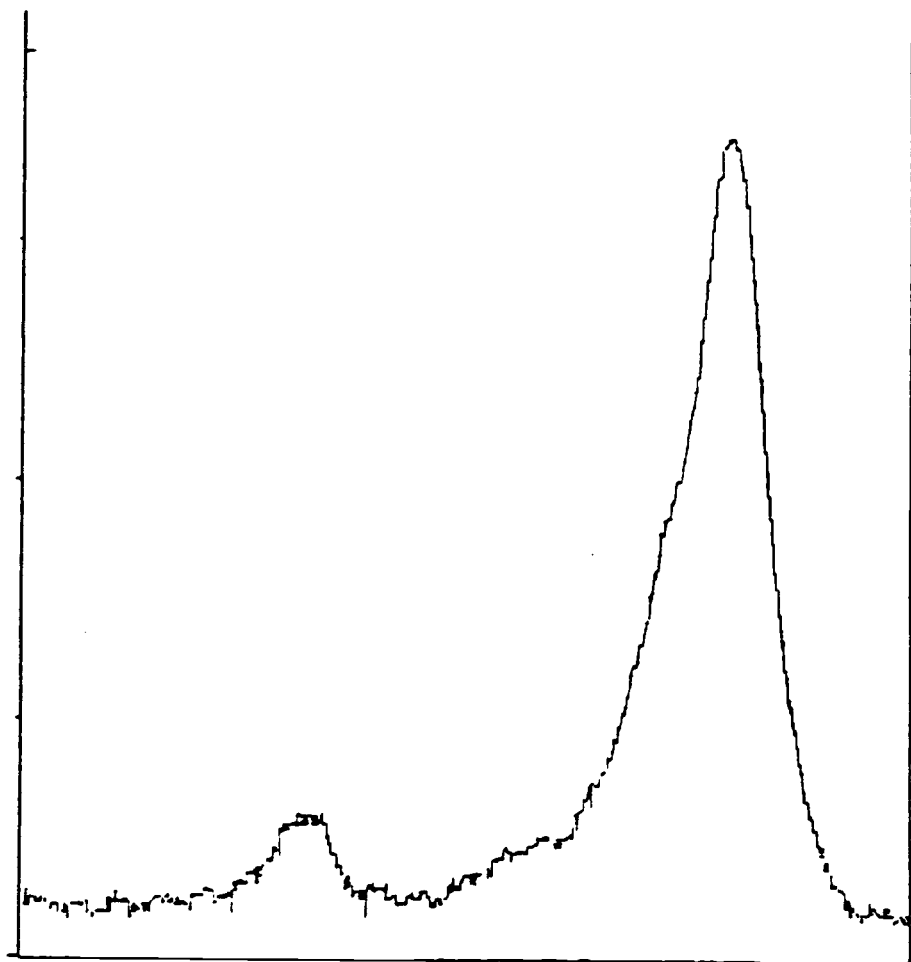   direction of migration is from right to
              left.

Figure 15

Figure 16.   Laser densitometer scan of autoradiograph
            of 16S rRNA from control deletion reaction.
            Separation was effected by polyacrylamide
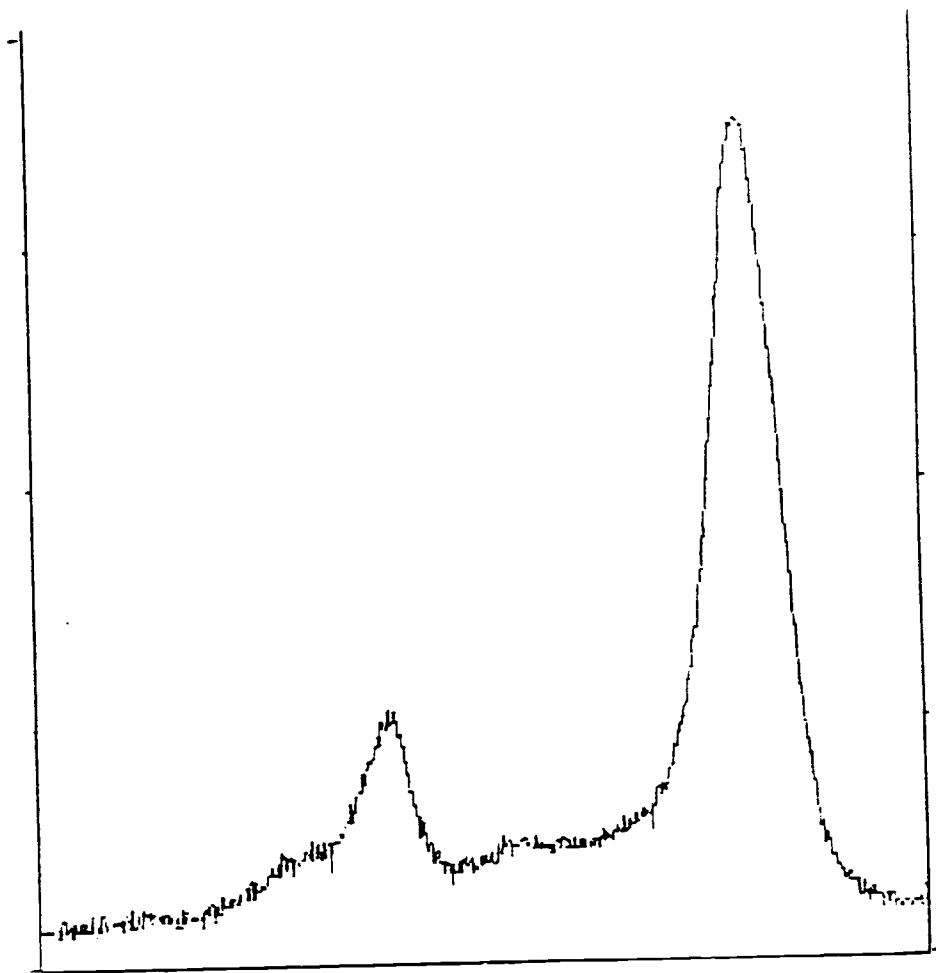   gel electrophoresis as described in
            figure 15.

Figure 16

Figure 17.   Sedimentation profile of 16S rRNA on sucrose
              gradient in 1x TSM buffer.   Sedimentation
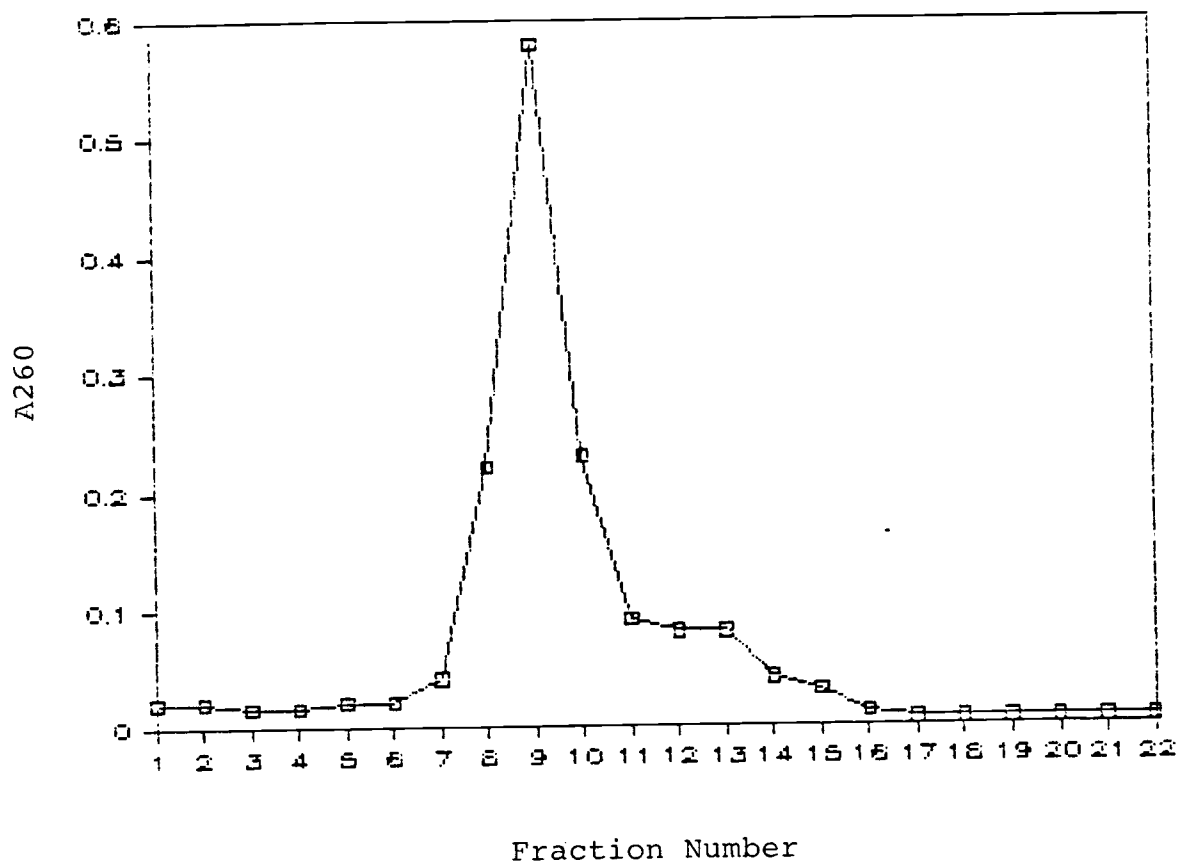   was performed as described in figure 5.

Fraction Number

Figure 17

Figure 18.   Sedimentation profile of 30S particles recon-
             stituted with normal 3' end labeled 16S rRNA.
             Sedimentation is from left to right and was
             performed as described in figure 5.   Fraction
             9 through 13 contained the reconstituted 30S
             particles and incorporated $(5'-^{32}P)pCp$ remains
             in fractions 1 through 5.
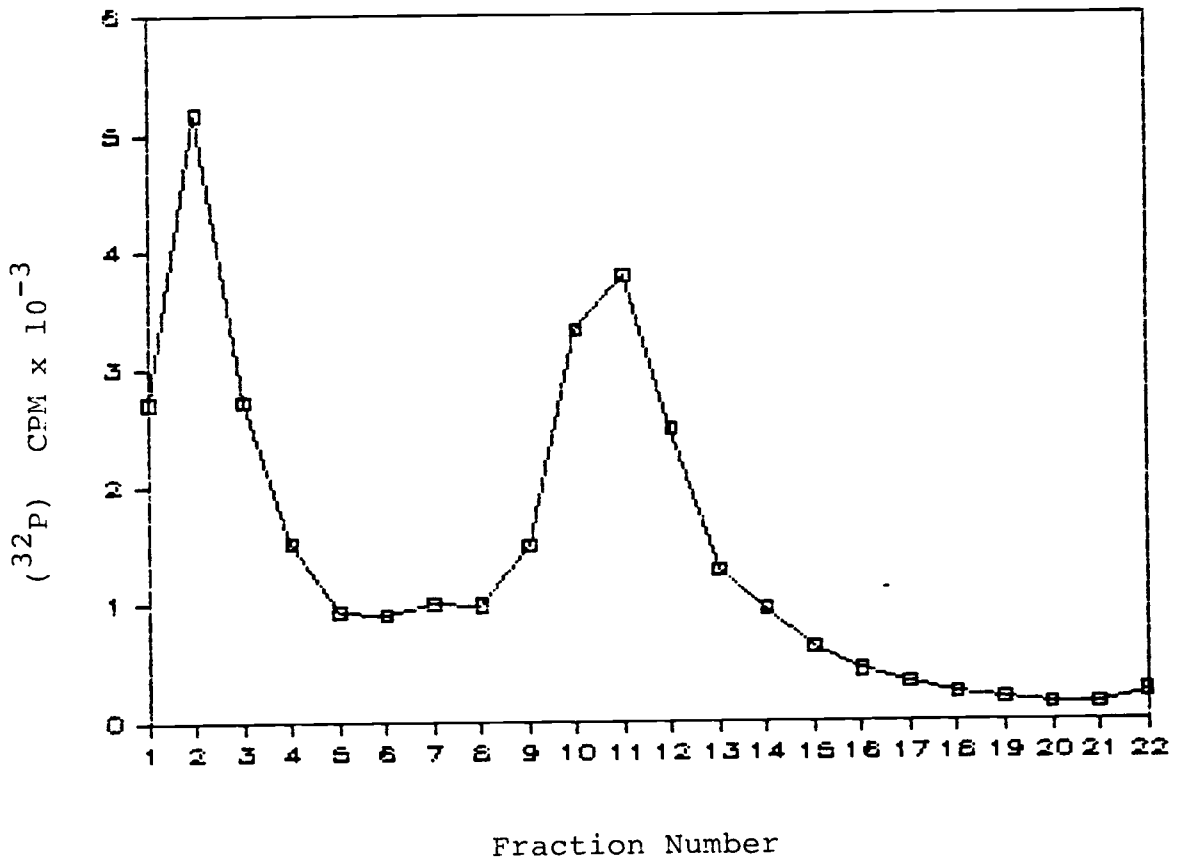
Fraction Number

Figure 18

Figure 19.   Sedimentation profile of 30S ribosomal sub-
             unit.   30S subunit recovered as from figure
             12 was rerun on sucrose gradient.   Sedimen-
             tation is from right to left and was
             performed as described in figure 5.   Frac-
             tions 8 through 13 contained 30S subunit
             region and some 50S contamination in
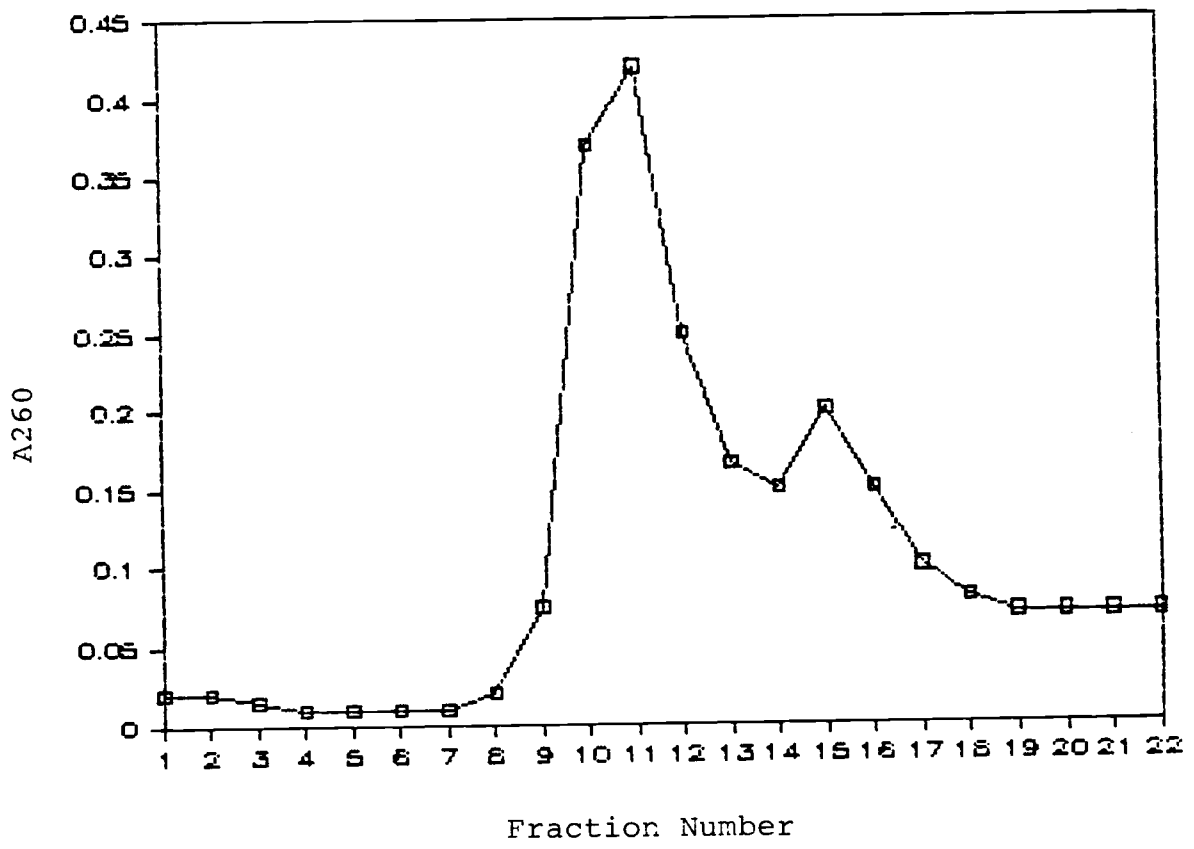   fractions 14 through 16.

Figure 19