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Title: Generation and in vitro Assembly Evaluation of a Site-Specific Deletion in Escherichia coli Small Subunit Ribosomal RNA

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Henry W. Schaup

Ribosomes are intricate macromolecular complexes which are a major element of the protein biosynthetic machinery in all life forms. In Escherichia coli they contain about 50 distinct proteins and 3 ribosomal RNAs. The small 30S ribosomal subunit in E. coli incorporates 21 proteins and a 16S rRNA. The 16S rRNA associated with this subunit was the focus of the investigations described in this dissertation. In order to explore the functional properties of this rRNA an in vitro procedure was developed to generate site-specific internal deletions in the RNA. The C-1400 region of the 16S rRNA was selected for manipulation because the sequence in this zone of the molecule has been shown to be intrinsically universal in all sequenced small subunit rRNAs. Through the use of synthetic DNA, RNase H, and RNA ligase, a four-nucleotide deletion between positions 1400 and 1405 was constructed. The manipulated RNA was tested for competency in in vitro ribosome reconstitution experiments and yielded particles which manifest a sedimentation coefficient comparable to normal 30S

subunits. Therefore, this portion of the conserved sequence did not emerge to be a ribosome assembly imperative and must fulfill an essential function during translation.

Generation and in vitro Assembly Evaluation of a Site-Specific Deletion in Escherichia coli Small Subunit Ribosomal RNA

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Chairman of Department of Biochemistry and Biophysics

Redacted for Privacy

Dean of Graduate School

Date thesis is presented May 4, 1987

Typed by Barbara Hanson for Young Sook Yoo

DEDICATION

This thesis is dedicated to my wonderful parents, the rest of my family, to my son, Sang Hyun, and especially to my beloved husband, Choong Hee Nam, for his warm understanding, support, and encouragement.

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I. LITERATURE REVIEW

1. Functional Aspects of Escherichia coli Ribosomal Proteins

Studies on ribosomes have concentrated on isolation, characterization of the numerous ribosomal components, elucidation of ribosomal architecture, and investigation of ribosomal functions in protein biosynthesis. The molecular composition of bacterial ribosomes is well known and most ribosomal components are well characterized in terms of physical and primary structural characteristics (1-4). It is difficult to assign specific functions to individual ribosomal components since its functional properties appear to be determined by cooperative interactions among RNA and protein components (5-7). Nevertheless, a number of the ribosomal proteins have been reported to be associated with specific functions in the process of protein synthesis.

E. coli mutants with altered ribosomal proteins have been isolated. Most of these strains are antibiotic resistant or temperature sensitive (8-11). These mutants have been used as tools in identifying ribosomal functions associated with specific ribosomal proteins. For example, a single amino acid substitution in protein S12 produces a change from streptomycin sensitivity to resistance (12,13). Mutant strains dependent on streptomycin for growth also have a modified S12 protein (14). Streptomycin interferes with the

process of initiation of protein synthesis and also disrupts the fidelity of the translation process. It, therefore, has been suggested that S12 plays some role in the initiation process and maintenance of translational fidelity. Spectinomycin-resistant mutants exhibit an alteration in protein S5 (15). Mutations in the gene coding for protein S4 as well as in S5 can suppress the dependence upon streptomycin thereby in streptomycin-dependent mutations influencing misreading of the message during translation (16-19). The action of spectinomycin resembles in several respects that of streptomycin, but has less drastic effects. Thus, the fidelity of translation seems to be influenced by the interactions of proteins S4, S5, and S12.

Mutants which are resistant to kasugamycin contain an altered form of protein S2 as shown by two-dimensional gel electrophoresis (20, 21). Of greater interest are the kasugamycin-resistant mutants in which 16S rRNA is altered (22,23). Kasugamycin inhibits the binding of fMet-tRNAf into the 30S initiation complex and thus appears to be a specific inhibitor of initiation of translation process, as shown by N. Tanaka and colleagues (20,21).

Ribosome binding to the messenger RNA depends not only on the initiation factors, but also on the ribosomal proteins S1 and S21. Ribosomal protein S21 is the smallest and most basic protein of the 30S ribosomal subunit of *E. coli*. The estimate of its stoichiometry is 0.7-0.8 copy per 70S ribosome. Van Duin and Wijnands (24) examined the function of protein S21 in protein synthesis by two independent approaches: inactivation of S21 *in situ* with antibodies against S21, and the use of 30S subunits reconstituted in the absence of S21. They

showed that the effects of S21 on 16S rRNA structure are closely paralleled by functional change in ribosome. For instance, subunits which are treated with antibody or lacking the protein S21 can only initiate on synthetic message, but not on MS2 phage RNA. Partial RNase T1 digestion of MS2 RNA relieved the S21 requirement, indicating that the defect is confined to the binding of 30S particles to MS2 RNA at the initiation stage, and also S21 has been shown to be an important protein for binding of Qβ bacteriophage RNA to 30S subunits. These results indicate that the binding of natural templates differs from that of random messages. Backendorf et al. (25) observed that the 30S subunits which are missing protein S21 cannot bind oligonucleotides complementary to the 3'-terminus of 16S rRNA. This finding could explain why S21 is not required for translation of synthetic templates, but is indispensable for phage translation. The function of S21 as described here resembles that of protein S1.

The ribosomal protein S1 has been shown to be involved in the initiation of protein synthesis on natural messenger RNA (26-29). Physical properties, structure of protein S1, and the proposed mode of action of protein S1 have been reviewed by Subramanian (30). Protein S1 is known to be identical to one subunit of the bacteriophage Qβ replicase enzyme, and to interference factor iα (31-34).

2. Functional Aspects of Escherichia coli Ribosomal RNA

Although only a few functional roles for ribosomal RNA have been identified, the idea that the rRNAs not only provide scaffolding for ribosomal protein binding in the assembly of ribosomes, but also

possess important functional roles in translation is generally accepted. Kasugamycin-resistant mutants in which 16S rRNA is altered strongly suggest a functional role for rRNA. Kasugamycin blocks the initiation step in translation. Resistant mutants of the type ksgA lack a methylating enzyme which places four methyl groups on two adjacent adenosines located 23 nucleotides from the 3'-terminus of 16S rRNA (21, 22). This methylase enzyme generates the sequence G-m_2^6A-m_2^6A-C, which is highly conserved in both prokaryotic and eukaryotic small subunit rRNA. Another kasugamycin-resistant mutant involves protein S2 (35). Particles which carry the modified S2 do not bind appreciable amounts of the antibiotic. These two mutations, one involving RNA modification and the other protein modification, provide a clear demonstration of the cooperative nature of the particles. That is, no single function can easily be assigned to a single protein.

The functional importance of the 3'-end of 16S rRNA is also indicated by the mechanism of colicin E3 inactivation of 30S particle. Colicin E3 causes a specific single cleavage of the phosphodiester bond about 50 nucleotides ("E3 fragment") in from the 3'-end of the 16S rRNA (36,37). The cleavage completely abolishes initiation of protein synthesis.

Although the mechanism of inhibition is not clear, the inhibition probably involves an uncoupling of important 16S rRNA-mRNA interactions such as that suggested by Shine and Dalgarno (38). These investigators suggested that a sequence near the 3'-terminus of E. coli 16S rRNA (bases 1532-1542) participates directly in the initiation of protein biosynthesis by forming several Watson-Crick

base pairs with complementary sequence in the leader region of the mRNA centered approximately 10 nucleotides upstream of AUG or GUG initiator codon. Indeed, all initiator regions from bacteriophage or E. coli mRNA analyzed so far have a complementary polypurine stretch of 3 to 8 nucleotides located about 10 bases 5' to the initiator codon. From 3 to 7 contiguous bases within this region of each mRNA can potentially pair with some portion of the polypyrimidine sequence in the 3'-end of the 16S rRNA (39). A dissociable oligonucleotide complex has been isolated, consisting of the 3'-terminus of the 16S rRNA and an RNA fragment from a protein initiator region of R17 phage RNA; the two fragments contained a seven-base complementary sequence, the 3'-terminus of the 16S rRNA being released from the 30S subunit as a 49-nucleotide fragment, under very mild conditions of colicin E3 cleavage (40,41). The Shine and Dalgarno proposal that the 3'-end of 16S rRNA plays a fundamental role in the initiation of protein synthesis is consistent with several different observations. First, crosslinking and chemical modification experiments suggest that the 3'-end of 16S rRNA provides the binding sites for initiation factors and certain ribosomal proteins which are implicated in initiation (42). Second, kasugamycin sensitivity and colicin E3 inactivation show that their site of action lies in the 3'-end of 16S rRNA. Third, random copolymers rich in A and G are the best competitive inhibitors of initiation on natural mRNAs, implying the importance of polypurines in ribosome binding to initiator regions (43). Finally, mutations affecting the mRNA of the 0.3 protein of phage T7, have been examined (44); this mutation is a G-to-A transition located 11 bases to the 5' side of initiator codon (44). This transition mutation eliminates a

five-base pairing with a sequence near the 3' end of 16S rRNA, thus causing the site of ribosome binding to shift about 15 nucleotides to the 3' side, centered on an internal AUG codon. This new site has only a poor potential interaction with 16S rRNA. In a revertant derived from a suppressor mutation, a rate of 0.3 protein synthesis and a behavior of ribosome binding is restored to essentially wild-type level. These results provide strong support for the idea that a base-pairing interaction between 16S rRNA and mRNA is involved in specific initiation of protein synthesis of E. coli, and indicate that this interaction is fundamental in selecting the ribosomal binding site in mRNA.

There are several portions of prokaryotic small subunit ribosomal RNAs which are essentially universal, such as the sequence which occurs around position 1400 in E. coli 16S rRNA (45). Cytidine-1400 is in the center of a 17-mer that has been almost totally conserved among the small subunit ribosomal RNAs of all species so far examined, including yeast. N-Acetylvalyl-$tRNA_{val}$ was reported by Prince et al. to be photochemically crosslinked to 16S rRNA in ribosomes primed with poly (U_3G) messenger RNA (46). The tRNA anticodon wobble position was shown to be primarily crosslinked to C-1400 within 16S rRNA by cyclobutane dimer formation. The tRNA is believed to be bound in the ribosomal P site in the nonenzymatic system used on the basis of its reactivity with puromycine (47). This highly conserved single-stranded region of the 16S rRNA was also suggested to be important in maintaining translational fidelity (48).

Chemical modification of 16S, 23S, and 5S ribosomal RNA with kethoxal, which modifies guanines in single-stranded regions, has been

used to identify possible functional roles for ribosomal RNA. The number of kethoxal-reactive sites in ribosomal RNA has been shown to be fewer in 70S ribosomes when compared with dissociated 30S and 50S subunits (49-51). Brow and Noller observed that some kethoxal-reactive sites which are not protected or only partially protected in 70S ribosomes show clear protection in particles associated with polysomes (52). In 16S rRNA, positions 530, 966, 1338, and 1517 showed protection in polysomes; all these sites are located within highly conserved primary and secondary structural zones which include several methylated nucleotides.

Mutants resistant to antibiotics which inhibit initiation, tRNA binding, translocation, and peptide bond formation have been reported for the 50S subunit. The molecular alterations generating resistance are localized to specific proteins, with the exception of thiostrepton resistance, which in one case involves the modification of ribosomal RNA. A methylase enzyme in the thiostrepton-producing organism Streptomyces azureus renders ribosomes completely resistant to thiostrepton (53). This is achieved by a single methylation event, the product of which is 2'-O-methyl-adenosine located 1067 residues from the 5' end of E. coli 23S rRNA (54). The primary binding site for the antibiotic is on 23S rRNA although binding is greatly enhanced in the additional presence of protein L11. And also, Stöffler et al. (7) showed that the thiostrepton binding site is in close proximity to the antigenic site of protein L11 by using immuno-electron microscopy.

The conserved sequence in the TψC loop of tRNA is complementary to a sequence in 5S rRNA (55,56). There have been suggestions that

base pairing between 5S rRNA and tRNA could be involved in stabilizing amino-acyl-tRNA in an A site (57,58). This base pairing interaction, along with the Shine and Dalgarno sequence base pairing with mRNA, indicates that the complementary interactions between regions of RNA are important not only in the secondary and tertiary structure of the individual rRNA molecules, but are also becoming increasingly implicated in various steps of the protein-synthetic process.

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II. *IN VITRO* GENERATION AND ANALYSIS OF A SITE-SPECIFIC DELETION IN 16S RIBOSOMAL RNA FROM *ESCHERICHIA COLI*

Y. S. Yoo and H. W. Schaup*

Department of Biochemistry and Biophysics

Oregon State University

Corvallis, Oregon 97331

ABSTRACT

The ribosome is the single most important component in the protein biosynthetic apparatus. Few clear functional roles for ribosomal RNAs have been reported. In order to elucidate the functional properties of rRNA, *in vitro* procedures were used to generate site-specific internal deletions in the highly conserved C-1400 region of 16S rRNA of *Escherichia coli*. A combination of DNA probes, RNase H, and RNA ligase were used to produce a four-nucleotide deletion between positions 1401 and 1404. The manipulated RNA was functional to the extent that particles were reconstituted which appeared to manifest a sedimentation coefficient comparable to normal 30S subunits.

INTRODUCTION

Ribosomes play an essential role in the biosynthesis of proteins in all life forms. Bacterial ribosomes are intricate macromolecular complexes composed of about 50 distinct proteins and three ribosomal RNAs (Wittmann, 1982). The small ribosomal subunit of the eubacteria Escherichia coli is composed of 21 proteins and a 16S rRNA. Here we report the development of approaches which seek to identify functional properties associated with the small subunit rRNA.

The primary structure of 16S rRNA from E. coli is known and a secondary structure model has been proposed (Woese et al., 1983). Currently ribosomal RNAs are thought not only to provide ribosomal protein binding sites, furnishing a foundation for the assembly of ribosomes, but they also are believed to bear important functional roles in translation (Shine and Dalgarno, 1974; Prince et al., 1982). Unfortunately, with the exception of a highly conserved short 3'-terminal sequence centered about position 1536 in eubacterial 16S rRNAs (Shine and Dalgarno, 1975; Steitz and Jakes, 1975), clear functional roles for rRNAs have not been delineated. This portion of the sequence appears to be important for initiation of protein synthesis and maintenance of translational fidelity (Steitz, 1979; Abdul-Latif and Schaup, 1987). There are other portions of small subunit rRNA which are essentially universal (Pace et al., 1986; Helser et al., 1972; Prince et al., 1982; Woese et al., 1983). That is, they have been found in homologous positions in all small subunit rRNAs examined. One such sequence centers around position 1400 in E.

coli 16S rRNA (Woese et al., 1983). We selected this zone for locus-specific mutagenesis experiments.

The advent of recombinant DNA technology brought with it numerous in vitro methods for site-specific mutagenesis (Landick, 1982). Unfortunately, there are circumstances when these procedures are of limited use. Analysis of 16S rRNA function is an example, because rRNA is likely to be multifunctional, being required for both ribosome assembly and subsequent activity in protein synthesis. Difficulty arises when an alteration in the structural gene impacts on a consensus tract required for proper nucleotide modification or nucleolytic post-transcriptional treatment. An alteration causing a defect in processing would allow assessment of that functional facet of the molecule, but it would not permit analysis of other putative functions which require proper ribosome biogenesis. The inherent assumption made here is that assembly-required sequences are subsequently vital for unique steps in the protein synthetic event. This dual use of transcripts is almost certainly a universal feature of all of the nontranslated RNAs employed in protein synthesis. Comparable tiers of complexity are also likely to occur in RNA viruses which are both economically and biomedically important.

We wished to assess the working aspects of 16S rRNA which are not associated with post-transcriptional alterations of the molecule. Since in vitro mutagenesis tenders the most opportunistic method for doing this, a technology to take advantage of fully processed RNAs was required. Here we present an approach designed to permit manipulation of specific segments of mature E. coli 16S rRNA. The procedures are general and applicable to any RNA. Through the use of DNA probes,

RNase H, and T4 RNA ligase, we have been able to construct altered RNAs with several nucleotides excised. Insertions are also possible.

MATERIALS AND METHODS

Mid-log phase E. coli D-10 cells were the source of ribosomal components. The cells were disrupted with a French pressure cell at 15,000 psi. Ribosomal RNAs were isolated from phenol-extracted cell lysates by zone ultracentrifugation in 15-30% sucrose gradients made in TSM buffer, which contained 10 mM Tris-HCl (pH 8.0), 3 mM succinic acid, 10 mM $MgCl_2$, and 50 µl/l β-mercaptoethanol (Schaup et al., 1970). Uniformly labeled 16S rRNA was isolated from cells grown in medium containing [^{32}P] orthophosphate (Cowgill de Narvaez and Schaup, 1979). 30S ribosome subunits were isolated by zone ultracentrifugation and 30S ribosomal proteins were extracted with acetic acid as described by others (Hardy et al., 1969). Protein concentration was determined using the Bradford procedure (Bradford, 1976). [5'-^{32}P]pCp (Cytidine 3',5'-bis (phosphate), tetra (triethylammonium salt, [5'-^{32}P]) was purchased from NEN or synthesized as described previously (England et al., 1980). The following products were obtained commercially: calf intestinal alkaline phosphatase (E.C. 3.1.3.1) (NEN), T4 polynucleotide kinase (E.C. 1.7.1.78) and E. coli Ribonuclease H (E.C. 3.1.26.4) (BRL), T4 RNA ligase (E.C. 6.5.1.3) (P-L Biochemicals or BRL), RNase T1 (E.C. 3.1.4.8) (Sankyo), RNA sequencing system (BRL), E. coli tRNA (Calbiochem), [γ-^{32}P]ATP (NEN or ICN Radiobiochemicals), ultrapure acrylamide and N,N'-methylene bisacrylamide (BRL), electrophoresis grade urea (Schwarz/Mann), Sephadex G-50 (Pharmacia), SEP-PAK C18 cartridge (Millipore), and Matrex gel PBA-30 (Amicon Corporation).

Synthesis of DNA Oligomer

DNA oligomers were synthesized on an Applied BioSystems DNA Synthesizer Model 380A using activated nucleoside phosphoramidites (Beaucage and Caruthers, 1981; Matteucci and Caruthers, 1981). The completed DNA oligomers were purified by reverse-phase ion pairing high pressure liquid chromatography (Haupt and Pingoud, 1983), or by extraction from polyacrylamide gels as described earlier with some minor modifications (Sanchez-Pescador and Urdea, 1984).

5' and 3'-End Labeling

The 5'-terminal phosphates of RNAs were removed by using calf intestinal alkaline phosphatase. RNA was incubated for 30 minutes at 37°C with 1 unit of enzyme per 2.77 A_{260} units of RNA in phosphatase buffer containing 100 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, and 0.1 mM $ZnCl_2$. When required, the dephosphorylated RNA and DNA oligomers were 5'-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase as described earlier (England et al., 1980). RNA was 3'-end-labeled by using [5'-^{32}P]pCP and T4 RNA ligase (Meyhack et al., 1978). The labeled RNA was then mixed with two volumes of Peattie tracking dye and next incubated at 90°C for a half minute (Peattie, 1979). The RNA was then purified by polyacrylamide gel electrophoresis and eluted from excised gel fragments by the method of Cory et al. (1972). Samples to be removed from gels were located by either autoradiography, UV shadowing, or with the aid of an AMBIS beta scanner (Automated Microbiology Systems, Inc., 3939 Ruffin Road, San

Diego, CA 92123).

RNA Sequence Analysis

Nucleic acids to be sequenced were either 3'- or 5'-end-labeled and analyzed by the partial enzymatic cleavage methods (Donis-Keller, 1979).

In vitro Reconstitution of 30S Ribosomes

Reconstitution of 30S ribosomal subunits were done as previously described (Traub and Nomura, 1969; Amils *et al.*, 1978).

RESULTS

Deletion Construction

The steps required for generating deletions within the universally conserved region positioned about residue 1400 in E. coli 16S rRNA are summarized in Figure II-1. Each stage in the process was monitored by denaturing polyacrylamide gel electrophoresis of the products of each reaction. In phase one, the 5'-terminal phosphate was removed from the purified 16S rRNA by using calf intestinal alkaline phosphatase. Dephosphorylation of the 16S rRNA was required to prevent circularization of the rRNA during a subsequent ligation reaction. The phosphatase was removed by phenol extraction and the rRNA was separated from spurious degradation products by using a denaturing polyacrylamide gel. In stage two, synthetic DNA oligomers were hybridized to the phosphatased 16S rRNA. The DNA probes were nine oligonucleotides long and complementary to nucleotides from position 1396 to 1404, and 1399 to 1404, respectively. They are identified as probe 1396 and probe 1399. The alignment of the probes with respect to 16S rRNA are shown in Table II-1.

Figure II-2 shows an autoradiograph of purified DNA oligomers used in this work. Hybridizations were performed under conditions similar to those described by Donis-Keller (1979). Preparative hybridization reactions (40-50 µl) contained approximately 100 µg of 16S rRNA, 10 µg of DNA oligomer and an excess of E. coli tRNA (5-10x mass excess to 16S rRNA) in 50 mM Tris-HCl (pH 8.3), 25 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol and 0.03 µg/µl bovine serum albumin. The

Figure II-1. Schematic diagram showing the strategy for construction of internal deletions in 16S rRNA using DNA "sliding" probes as described in the text.

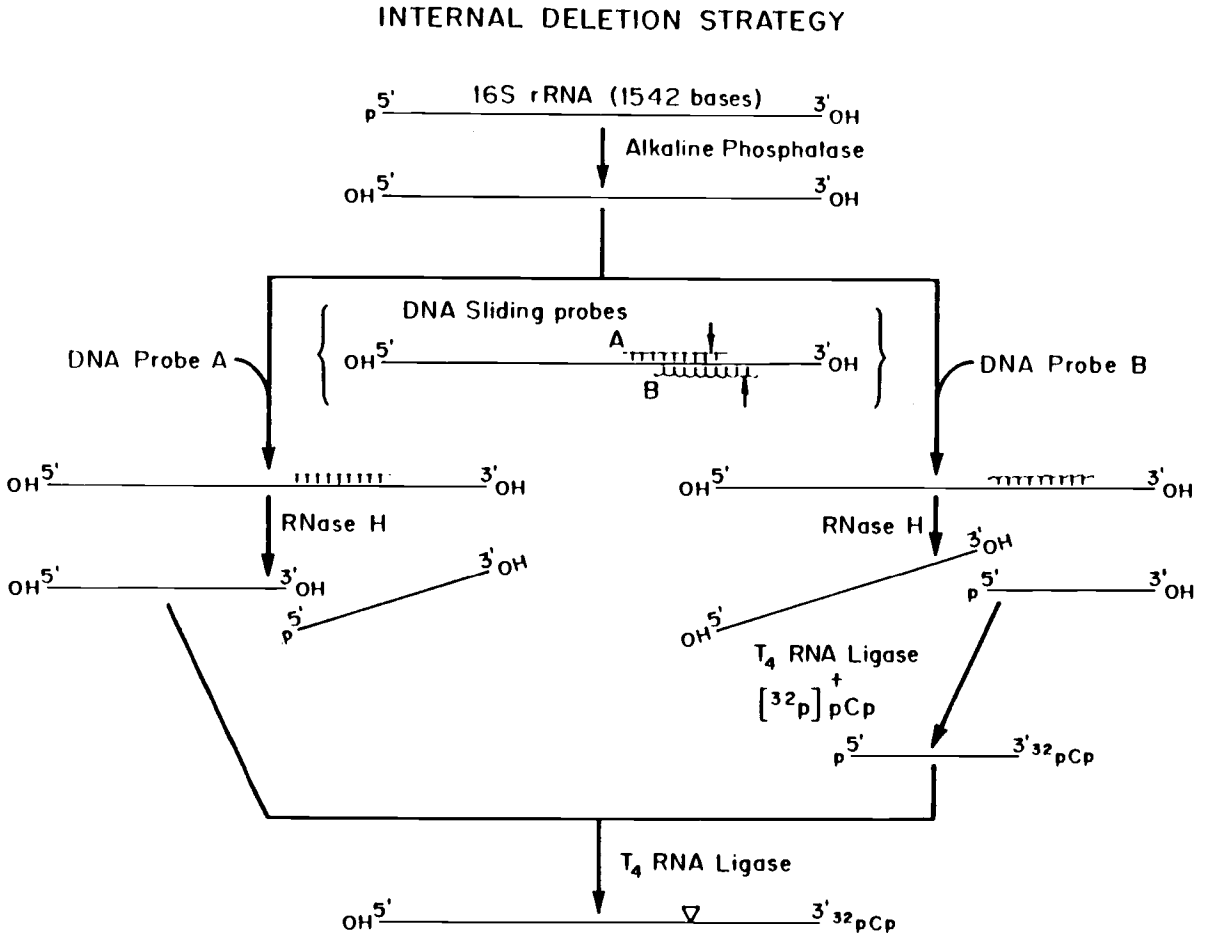
INTERNAL DELETION STRATEGY
16S rRNA (1542 bases)
Alkaline Phosphatase
DNA Sliding probes
DNA Probe A
DNA Probe B
RNase H
RNase H
T4 RNA Ligase
T4 RNA Ligase

Figure II-1

Table II-1: Probe Alignment

DNA 1396	T-G-T-G-G-C-G-G-G
DNA 1399	G-G-C-G-G-G-C-A-G
16S rRNA*	G-U-A-C-A-C-A-C-C-G-C-C-C-G-U-C-A

*16S is shown 5' to 3' beginning at position 1392.

Figure II-2. Autoradiograph of 20% polyacrylamide gel showing 5'-end [^{32}P]-labeled DNA probe used in this work. Lanes 1 and 3: purified DNA as described in Materials and Methods. Lane 2: crude DNA before HPLC purification. The direction of migration is from top to bottom.

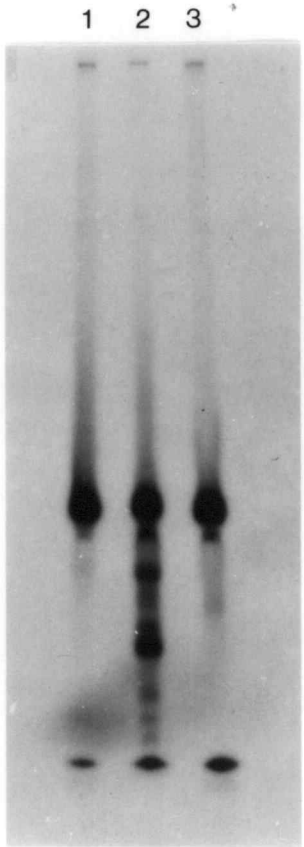

Figure II-2

tRNA was added to diminish nonspecific loss of rRNA in subsequent handling procedures. The reaction was preincubated at 50°C for 20 minutes and then for 30 minutes at 40°C to allow annealing of the DNA oligomer.

In stage three, E. coli RNase H (0.7 units/µg of RNA substrate) was added and the digestion allowed to proceed for 50 minutes at 40°C. In preliminary experiments at 32°C, cleavage of the 16S rRNA with the DNA probe 1396 was observed at sites other than the target zone. This was due to partial hybridization with regions within the 16S rRNA which partially repeat the conserved sequence. A computer search of 16S rRNA revealed partial repeats of four or more continuous bases beginning at positions 51_D, 174_D, 269_D, 313_D, 327, 400_D, 501_D, 718, 734_D, 808_D, 878_D, 931_D, 970_D, 1207_D, 1227_D, 1394, 1408_D, 1533. The longest repeat was six bases in length and the positions marked with a subscript D are located in proposed double-stranded regions of the molecule. The undesirable cleavages were eliminated by increasing the stringency of the hybridization and performing the digestions at 40°C. Titration experiments to determine the optimal amount of RNase H required for efficient cleavage of the hybrid molecule showed that at least 0.7 units of RNase H per 1 µg of substrate 16S rRNA are needed.

Analytical digestion experiments were first performed under the suboptimal conditions using uniformly [^{32}P]-labeled 16S rRNA and the purified DNA probe to assess RNase H specificity for the RNA-DNA hybrid region and also the purity of the enzyme. The suboptimal conditions were achieved by adding less than a stoichiometric amount of DNA relative to RNA and were intended to permit visualization of intact 16S rRNA in the same reaction in which cleavage occurred. The

Figure II-3. Autoradiograph of a polyacrylamide gel showing targeted RNase H digestion of uniformly labeled 16S rRNA. The rRNA was uniformly labeled using [^{32}P]-orthophosphate as described in Materials and Methods. Lane 1: 16S rRNA incubated with RNase H but no DNA present. Lane 2: 16S rRNA cleavages by RNase H in the presence of the DNA less than stoichiometric amounts of RNA. Lane 3: 16S rRNA marker. The denaturing gel was formed by layering a 2.8% gel over a preformed 10% gel and its dimensions were 15x10 cm (0.75 mm thick).

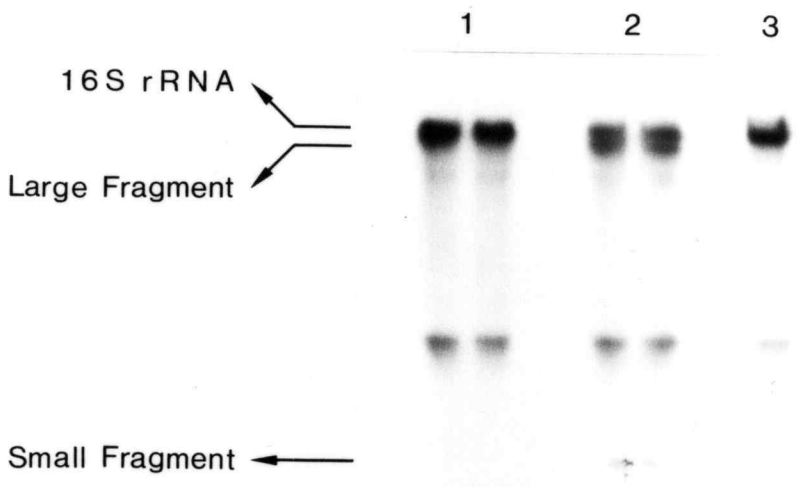

Figure II-3

use of uniformly labeled RNA permitted monitoring of the condition of the entire molecule. An autoradiograph displaying the results of such an experiment is shown in Figure II-3 (lane 2). The autoradiograph was deliberately over-exposed to reveal nonspecific cleavages. Two bands, as expected, were observed in the 16S rRNA zone of the gel. The upper band represents uncleaved 16S rRNA and the lower one was generated by the RNase H attack. This large digestion fragment was about 140 nucleotides shorter than intact 16S rRNA. The common band in all of the lanes found near the middle portion of the gel is located at the interface between the 2.8% and 10% gel. This material represents less than 15% of the total sample. The faint spot in the 10% part of the polyacrylamide gel represents the 3'-side small fragment derived from the digestion. This cleavage occurred only in the presence of both the DNA probe and RNase H; there was no specific cleavage when the 16S rRNA was incubated with RNase H alone (Figure II-3, lane 1). Analysis of this cleavage reaction under the optimal cleavage conditions using 3'-end [^{32}p]pCp-labeled 16S rRNA is shown in Figure II-4. Since the label is only on the 3'-terminus of the molecule, we expect only to see the small fragment generated by the attack of RNase H. The distribution of the label between the high and low molecular weight zones within the gel was a measure of the digestion efficiency; typically the cleavages were nearly 100% efficient. When the same samples were analyzed by zone ultracentrifugation under nondenaturing conditions, the cleavages appeared to be only 30-40% efficient (Figure II-5). This suggests noncovalent interactions between the 3'-terminal fragment and other portions of the molecule.

Figure II-4. Autoradiograph of a polyacrylamide gel as in figure II-3 but using 3'-end [^{32}P]pCp labeled 16S rRNA. Lanes 1-3: 16S rRNA DNA targeted digestion by RNase H. Lane 4: polyacrylamide gel-purified small fragment. Lanes 5 and 6: 16S rRNA.

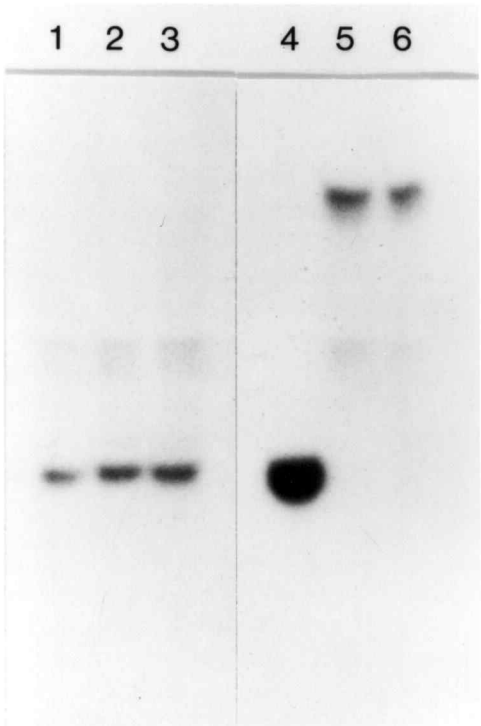

Figure II-4

Figure II-5. Sedimentation profile of DNA-targeted RNase H-digested 16S rRNA. The 16S rRNA was 3'-end [^{32}P]pCp labeled. 1. 16S rRNA. 2. A sample of RNase H digested 16S rRNA from the same preparation analyzed by polyacrylamide gels shown in figure II-4 conditions. Sedimentation was from left to right in a 15% to 30% sucrose gradient made in TSM. The centrifugation was accomplished with a Sorvall AH 650 rotor at 49,000 rpm for 3 hours.

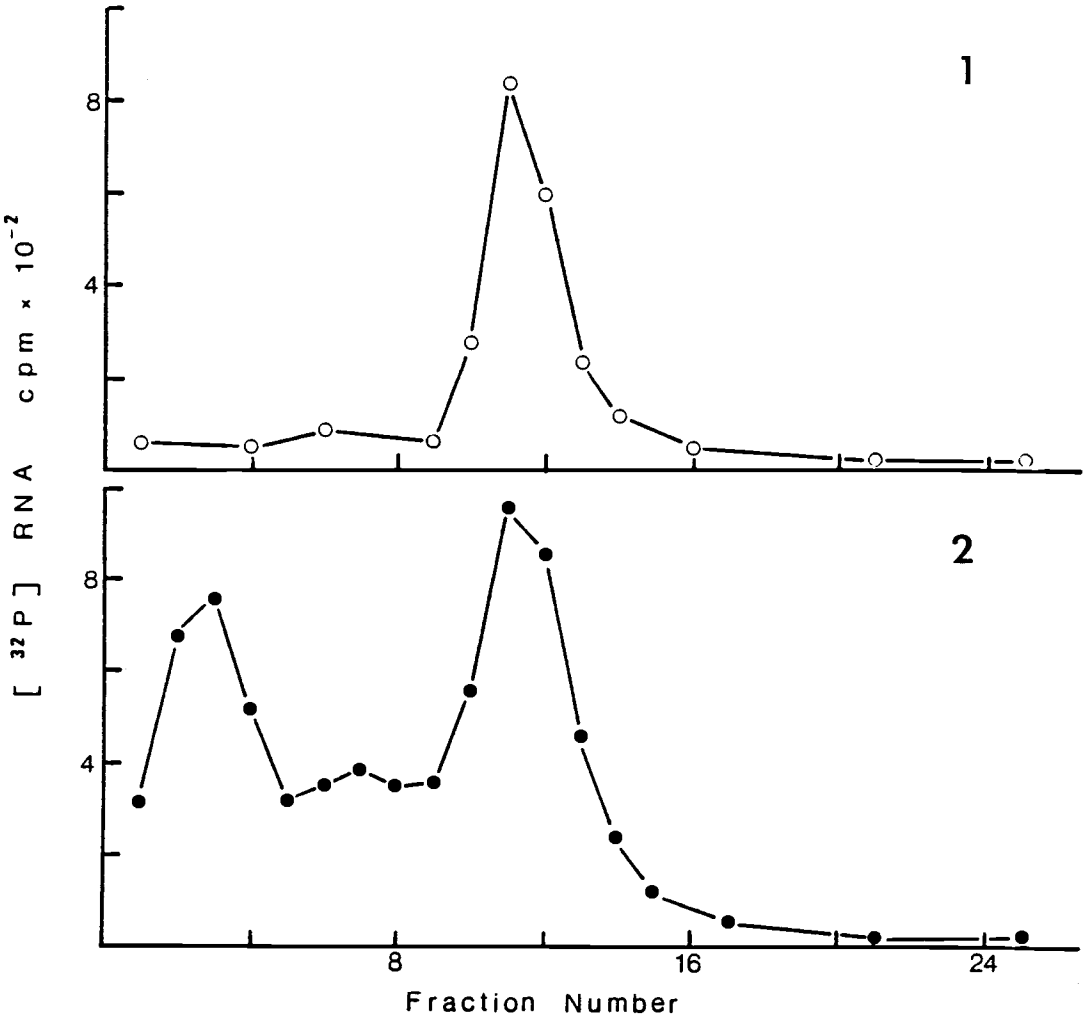

Figure II-5

T4 RNA ligase performs an ATP-dependent ligation reaction. The enzyme requires a free 3'-hydroxyl group to function as an acceptor and a free 5'-phosphate group as a donor molecule (Uhlenbeck and Gumport, 1982). The pCp is used to label the RNA in this experiment and functions as a 3'-end blocking agent on the small fragment generated by the RNase H digestion of the 16S rRNA. Treatment of the 16S rRNA with alkaline phosphatase leaves the larger fragment's 5'-end without a phosphate, making it an unsuitable donor in T4 RNA ligase reactions. Therefore, a mixture of these two fragments in the presence of T4 RNA ligase could not yield ligation of two large or small fragments together; neither could the smaller fragment become attached to the 5' end of the large fragment.

Fragment Cleavage Sites

The products of the digestions in the presence of the individual DNA probes were purified by polyacrylamide gel electrophoresis as described in Materials and Methods. This was done to determine the cleavage sites of RNase H in these reactions. Since the RNA sequencing technique requires either a 5'-end or 3'-end label, the RNA was not pCp labeled when sequence analysis was required. RNA sequence analysis was performed by partial enzymatic digestion. Figure II-6 shows the partial sequencing analysis of the 3' end of the large fragment generated when probe 1396 is used to target the RNase H attack. Examination of the 3' end of the large fragment showed that the cleavage site in the presence of probe 1396 was between C1400 and G1401.

Figure II-6. Autoradiograph of enzymatic RNA sequencing polyacrylamide gel. The enzymes used were RNase CL3, RNase U2, RNase Phy M, and RNase T1. The respective cleavage specificities are as follows: C, A, A+U, and G. The RNAs were 3'-end [^{32}P]pCp-labeled. Set 1 shows 16S rRNA and set 2 shows the large fragment generated using DNA probe 1396. The gel was a 15% polyacrylamide - 7 M Urea gel 25x26 cm (0.5 mm thick).

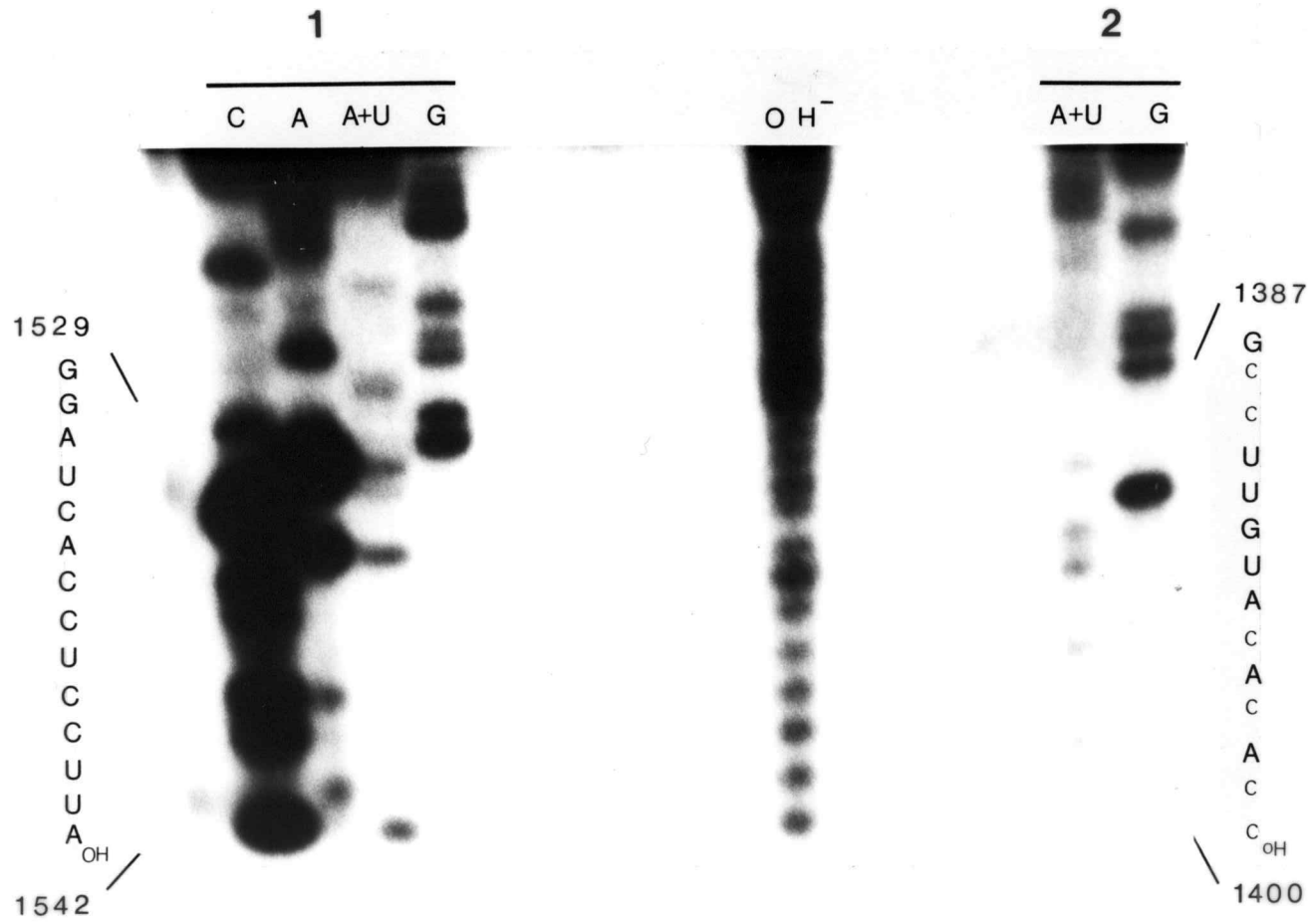
1
C A A+U G
OH⁻
2
A+U G
1529
G
G
A
U
C
A
C
C
U
C
C
U
U
A
OH
1542
1387
G
C
C
U
U
G
U
A
C
A
C
A
C
C
OH
1400

Figure II-6

Figure II-7. Autoradiograph of partial digestion pattern of 16S rRNA and two intact small fragments generated using DNA probe 1396 (lane 1) and probe 1399 (lane 2). RNA was 3'-end [^{32}P]pCp labeled. The enzymatic cleavage sites are shown for 16S rRNA above the lanes and the position within the sequence is indicated by the numbers in the margin. Electrophoretic analysis was performed as described in Figure II-6.

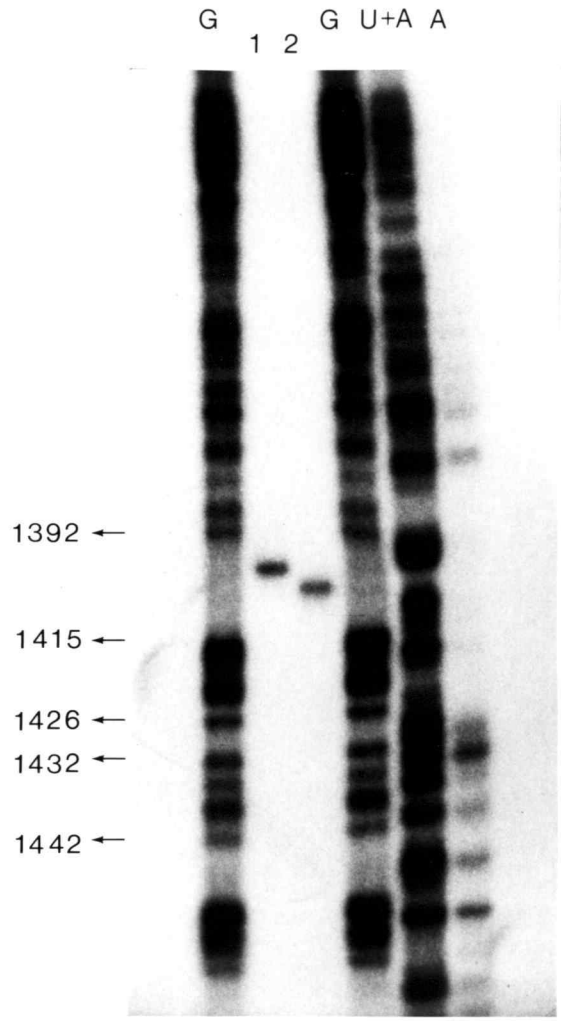

Figure II-7

The two small fragments generated using selected DNA probes were compared with partial digestion patterns of the 16S rRNA to confirm the homogeneity of the preparations and cleavage sites (Figure II-7). When probe 1396 was used, the site was, again as expected, between C1400 and G1401. When probe 1399 was used, it was between C1404 and G1405.

Fragment Ligation

The RNase H digestion products (large fragment with 5'- and 3'-hydroxyl generated by probe 1396, small fragment with 5'-phosphate and 3'-pCp generated by probe 1399) were respectively gel purified by polyacrylamide gel electrophoresis. The fragments were then rejoined to form a 16S rRNA-like molecule using T4 RNA ligase. It is important to note at this point that the cleavage products obtained from RNase H digestions have a free 3'-end OH and a 5'-end phosphate. Therefore, the small fragment has a suitable donor and the large fragment has an acceptor. Because of the treatment of the 16S rRNA used to generate these fragments (phosphatasing and pCp blocking), there was only one possibility to ligate and only one polarity. This means that the 16S rRNA had to be restored properly, but in this case with a specific set of nucleotides missing. Analysis of this rejoining reaction by gel electrophoresis (Figure II-8) and the data of laser scanning densitometer (data not shown) showed that greater than 15% of the 3' side small fragment (lower band) was converted to a slower moving band which migrated close to intact 16S rRNA.

To prepare the altered RNA for further work after ligation, the

Figure II-8. Autoradiograph of a polyacrylamide gel showing the products of rejoining reaction of a 3'-end [^{32}P]pCp labeled small fragment generated by probe 1399 to the large 5'-end 16S rRNA fragment generated by probe 1396. Gel electrophoresis was performed as described in Figure II-3.

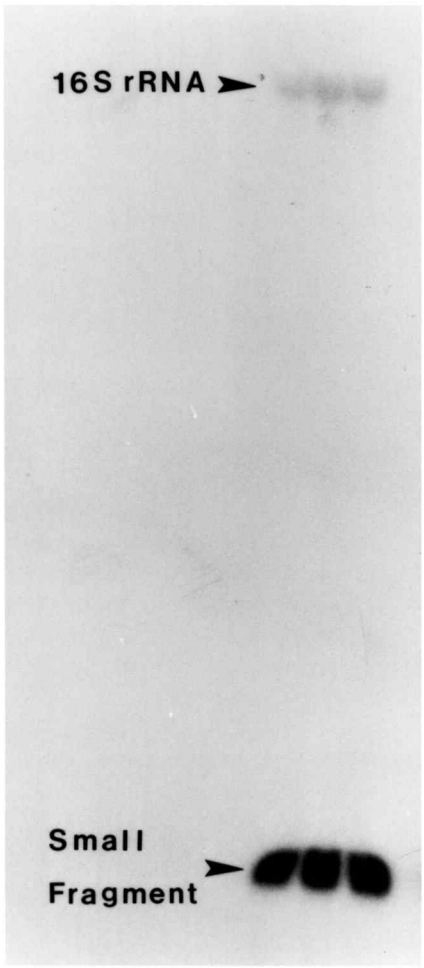

Figure II-8

reaction mixture was passed through a phenyl boronate agarose column (PBA-30). Phenyl boronate agarose columns separate polyribonucleotides on the basis of the presence of 2',3'-terminal cis-diols (Pace and Pace, 1980). Normally RNAs have these groups available on their 3' ends. Under specific conditions, these columns can be used for separations involving molecules of the size of 16S rRNA (Pace and Pace, 1980). Ligated molecules should not be bound to the columns because they have been 3'-end blocked. However, unreacted large fragment should be bound. This procedure ensures that the gel-purified material is substantially free of the closely migrating large fragment generated by the initial RNase H attack (Figure II-3).

RNA Construct Sequence Conformation

The reconstructed RNA molecule should have four nucleotides removed. To confirm the sequence of this deletion quickly and easily without using appreciable amounts of the product, a nine-base DNA oligomer complementary to the new region in the construct was synthesized. The new DNA nanomer (5' TGACGGTGT 3') and the DNA probe 1396 (5' GGGCGGTGT 3') were 5'-end-labeled using polynucleotide kinase and [γ-^{32}P]ATP. The [^{32}P]-labeled DNA oligomers were respectively hybridized to the rejoined RNA molecule and then subjected to nuclease S1 attack. The hybridization conditions were as described for experiments where RNase H was used to generate fragments. Nuclease S1 will not attack double-stranded nucleic acids under these conditions (Berk, 1981). Therefore, if the anticipated ligations had occurred, one would expect probe 1396 (5' GGGCGGTGT 3') to be destroyed because

Figure II-9. Autoradiograph showing S1 nuclease mapping to confirm deletion in reconstructed molecules. DNA probes were 5'-end [^{32}P]-labeled as described in Materials and Methods. Lane 1: DNA probe nanomer (5' TGAC^GGTGT 3'; ^ indicates the site of 4-nucleotide deletion) hybridized to altered 16S rRNA and then subjected to S1 nuclease digestion. Lane 2: DNA probe 1396 (5' GGGCGGTGT 3') hybridized to altered 16S and subjected to S1 nuclease digestion. Lane 3: DNA nanomer (5' TGACGGTGT 3') subjected to S1 nuclease digestion in the absence of any rRNA. Lane 4: DNA nanomer (5' TGACGGTGT 3'). The digestion and hybridization conditions were as described in materials and methods. The specific activity of the DNA was $1x10^7$ cpm/μg, and 0.02 μg of RNA were present in the reaction mixtures.

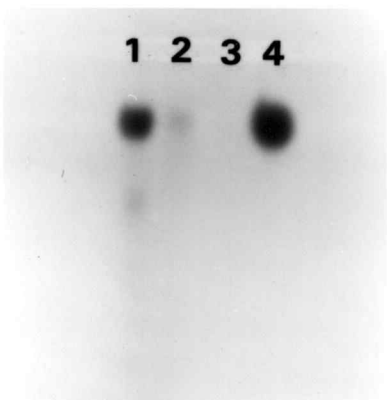

Figure II-9

its 5'-end will not have a complement with which to hybridize. The nanomer (5' TGACGGTGT 3') should be fully complementary with the altered RNA and thus protected. Analysis of these digestion reactions on 20% polyacrylamide gels (Figure II-9, lane 1) support the conclusion that the recovered product was the desired RNA construct. As anticipated, DNA probe 1396 complementary to normal 16S rRNA was not protected when hybridized to the new 16S rRNA (lane 2, Figure II-9). We take this to confirm the construction in which the sequence $G^{4}_{m}CmCC$ has been deleted (positions 1401 to 1404).

An in vitro Reconstitution Experiment

The modified 16S rRNA construct was then used in a simple *in vitro* reconstitution experiment to determine competency for forming 30S particles. The [^{32}P]pCp-labeled RNA molecule which has been modified was mixed with total 30S ribosomal proteins in the presence of nonlabeled normal 16S rRNA as internal marker to compare location of the reconstituted particles on sucrose gradient ultracentrifugation (Figure II-10). Results of these *in vitro* reconstitutions indicate that the four missing bases do not impair reconstitution to the extent that particles which sediment at 30S can be recovered. Although 30S particles appeared to be formed, one cannot conclude that a satisfactory assembly occurred until the protein composition of the particles is analyzed quantitatively.

Figure II-10. Sedimentation profile of particles reconstituted with altered 16S rRNA. The solid line shows distribution of 3'-end [^{32}P]pCp labeled modified 16S rRNA and the dotted line shows the profile for normal 16S rRNA which was included in the reconstitution to provide an internal standard. The arrow indicated the location of 16S rRNA. Sedimentation was from left to right and the separation was achieved using a Sorvall TV850 rotor. The run time was 2 hours at 45,000 rpm in a 15% to 30% sucrose gradient made in TSM.

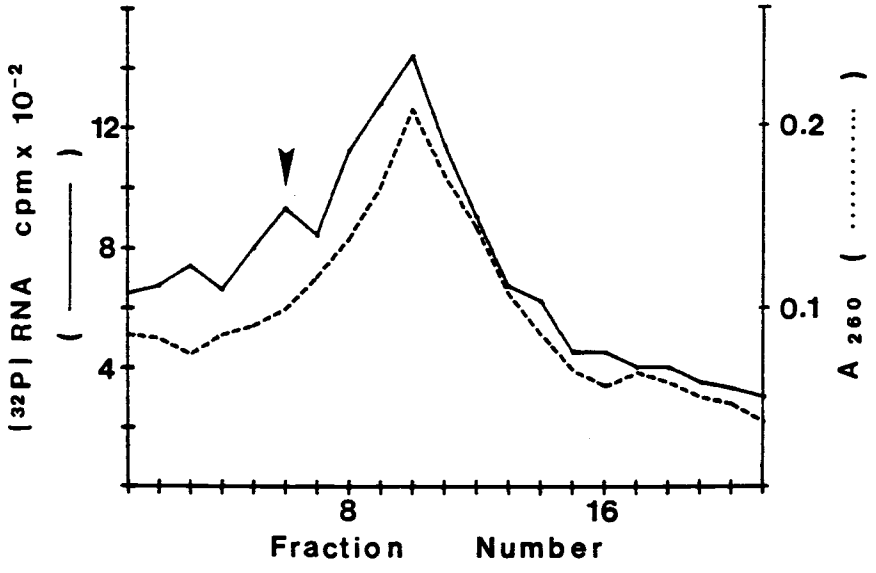

Figure II-10

DISCUSSION

All of the previous work with RNA ligase has been done using small donors of one nucleotide, such as pCp, to about 30 nucleotides in length (Uhlenbeck, 1983). A comprehensive investigation of factors required for efficient ligation of large molecules has not been reported. The efficiency of the ligations required to complete the 16S rRNA reconstruction embody the most challenging facet of the procedure. If the work with small donors is taken as a guide, then one would expect the yields at this phase in the process to depend upon the terminal nucleotide donor and acceptor. Others have shown that pCp is the best and pGp is the worst donor, and homopolymers or oligomers containing A residues are the most suitable acceptors, whereas those with U are the poorest (McLaughlin et al., 1985). In this work, the donor is a G, and the acceptor is a C residue. The choices made here were based upon an interest in using this construct in future investigations. The secondary structure of the molecule to be ligated must also be considered. The ligase appears to require, for both donor and acceptor, at least a two- to three-nucleotide single-strand extension beyond a double-stranded conformation (Bruce and Uhlenbeck, 1982). Blunt ligations are reported not to work well (Bruce and Uhlenbeck, 1978).

We have found that it is extremely important to prepare samples of high purity when attempting constructions of the type described here. This obviously insures proper preparation of the desired product, but it is also a prerequisite for good yields. Very small amounts of nucleotide or oligonucleotide contaminants are the dominant

substrates in RNA ligase reactions. For example, 16S rRNA isolated from sucrose gradients is a poor substrate for [^{32}P]-pCp labeling. When analyzed on polyacrylamide gels by autoradiography, most of the [^{32}P]-pCp is incorporated into low molecular weight oligonucleotides. Yet, when the same RNA is located on these gels by staining methods, very little material is found in these zones. When 16S rRNA is purified from denaturing polyacrylamide gels, it is an excellent substrate for the labeling reaction.

An additional requirement must be met when the described technique is to be used. The target site must be single-stranded or otherwise made so in order to obtain efficient hybridization of the DNA probes used to target the RNase H cleavages. This is because the hybridization with the DNA probe will follow pseudo second-order kinetics, but the folding of an RNA upon itself to reform or form double-stranded regions will approximate first-order kinetic conditions. This would exclude or out-compete the probe for the target track. We have prepared probes for zones within 16S rRNA which have been proposed to be double stranded and found that the targets are not cleavable under the conditions used here.

Although the work described here concerns the construction of a four-nucleotide deletion, it is clearly possible to construct larger or smaller deletions through appropriate probe design. The size and nature of the target locus will present limitations if the single-stranded track is short because of the need to have the donor and acceptor end extend several bases beyond the double-stranded regions. It is also necessary to consider the size of the probe in order to maintain high specificity for the entire site. We believe

nine-base probes are at the practical limit when using a molecule of the size of 16S rRNA for manipulations. It should also be possible to construct insertions by adding a nucleotide or so to the 3' terminus of the acceptor molecule with the aid of RNA ligase. This would introduce additional steps which in turn would be certain to lower overall yields. However, to produce a tandem repeat of a sequence which was initially targeted for deletion, choose the reciprocal products of the initial RNase H digestions and ligate these together. This insertion path would reduce the number of steps required to obtain the product and improve yields. It also offers, at least for 16S rRNA, an interesting molecule for further investigations.

When the 16S rRNA with four bases ($G^{4}_{m}CmCC$) deleted was used in in vitro reconstitution experiments, 30S particles were observed. A complete analysis of the protein composition of these particles will be necessary to characterize them fully. However, it is anticipated that all of the proteins normally associated with the particles will be present because this portion of the 16S rRNA does not appear to be associated with 30S subunit proteins (Lake, 1985; Oakes et al., 1986; Brimacombe and Stiege, 1985). Nevertheless, this optimism must be tempered by the observation that the RNase H cleavages do not release the 3'-terminal fragment from the larger 16S rRNA 5'-end product unless the molecules are first denatured. We interpret this to mean that portions of the smaller fragment are interacting with areas in the 5'-end segment. It is conceivable that removal of these four nucleotides could alter the 16S rRNA folding pattern in a manner that impairs the binding of one or more ribosomal proteins. Experimental evidence exists which suggests that this portion of the rRNA may

interact with tRNA in the peptidyl site of the ribosome (Prince *et al.*, 1982). *In vitro* protein synthesis with these particles will most likely provide the insight required to understand the extraordinary conservation observed for this portion of the 16S rRNA.

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III. ENHANCEMENT OF T4 RNA LIGASE LARGE MOLECULE LIGATIONS

Y. S. Yoo and H. W. Schaup*

Department of Biochemistry and Biophysics

Oregon State University

Corvallis, Oregon 97331

ABSTRACT

T4 RNA ligase has not been used to ligate oligoribonucleotides which surpass 100 nucleotide residues in length because previously established conditions are exceptionally ineffectual when large donor and acceptor molecules are employed.

In this investigation, various factors including polyethylene glycol, hexamine cobalt chloride, the ratio of donor versus acceptor, NAD^+, and a non-ionic detergent (NP-40) have been examined in an effort to improve the efficiency of T4 RNA ligase intermolecular ligations of large molecules. Small subunit ribosomal RNA from Escherichia coli and a 3'-terminal 138-nucleotide fragment derived from it were used as test acceptor and donor in ligation reactions. Polyethylene glycol at 1% and a molar excess of acceptor over a donor molecules produced the greatest enhancement in ligation efficiency.

INTRODUCTION

T4 RNA ligase catalyzes ATP-dependent formation of a phosphodiester bond between terminal 5'-phosphate (the donor) and 3'-hydroxyl (the acceptor) of oligo- or polyribonucleotides (1-3). Because of the catalytic diversity of the enzyme, the use of T4 RNA ligase has greatly augmented our ability not only to synthesize oligoribonucleotides (4), but also to manipulate RNA sequences. T4 RNA ligase has been used to modify 5S rRNA, the anticodon of tRNA (5,6) and to perform specific labeling of 3'-termini of a variety of RNAs (7).

The yields of the intermolecular reactions vary generally depending upon the size of the substrate, the presence of terminal secondary structure and the nucleotide composition of the termini. Since T4 RNA ligase displays a preference for small RNA, and the largest reported molecule modified at an internal position was in the size range of tRNA (6), enhancing the ligation efficiency for the manipulation of a large RNA molecule such as 16S or 23S ribosomal RNA would considerably expand the utility of the enzyme.

Recent investigations have shown that volume exclusion using high concentrations of polyethylene glycol (8,9) or hexamine cobalt chloride (10) can enhance the rate of blunt end joining of DNA fragments by T4 DNA ligase. We have examined the effects of these compounds on T4 RNA ligase-mediated ligations in addition to the following factors: the ratio of donor versus acceptor, NAD^+ as an energy source, and a non-ionic detergent (NP-40). Small subunit 16S rRNA isolated from *E. coli* and a 3'-terminal 16S rRNA fragment of 138

nucleotides in length were used respectively as the acceptor and donor in these experiments. Both polyethylene glycol and a molar excess of acceptor were found to enhance ligation efficiency.

MATERIALS AND METHODS

Materials

Calf intestinal alkaline phosphatase (E.C. 3.1.3.1) was purchased from New England Nuclear. T4 RNA ligase (E.C. 6.5.1.3) and *E. coli* RNase H (E.C. 3.1.26.4) were secured from Bethesda Research Laboratories. [5'-^{32}P]pCp (cytidine 3',5'-bis(phosphate), tetra(triethylammonium) salt, [5'-^{32}P]-) was a product of New England Nuclear. Chemicals for polyacrylamide gel electrophoresis were acquired from Bethesda Research Laboratories, and urea (electrophoresis grade) was obtained from Schwarz/Mann. Polyethylene glycol 6,000 (PEG) was procured from Sigma, and the stock solution of 40% (w/v) was made in deionized water. Hexamine cobalt chloride ($(NH_3)_6CoCl_3$) was purchased from Aldrich Chemical, and Nonidet P-40 (NP-40) was obtained from Particle Data Laboratories. All other chemicals used were reagent grade.

Preparation of RNA substrates

16S ribosomal RNA from mid-log phase *E. coli* D-10 cells was isolated by zone ultracentrifugation on 15-30% sucrose gradients made in TSM buffer containing 10 mM Tris-HCl (pH 8.0), 3 mM succinic acid, 10 mM $MgCl_2$, and 50 µl β-mercaptoethanol per liter (11). Phenol extracts of cell lysates were used as a source of cellular RNA (12). The 5'-phosphate of 16S rRNA was removed by using calf intestinal alkaline phosphatase. The RNA was incubated for 30 minutes at 37°C

with 1 unit of enzyme per 2.77 A_{260} units of RNA in phosphatase buffer containing 100 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, and 0.1 mM $ZnCl_2$. The dephosphorylated 16S rRNA served as a donor molecule in the present study and was also used for the preparation of the acceptor molecule. Site-specific cleavage of 16S rRNA was performed using a DNA oligomer and RNase H to generate a 3'-terminal 16S rRNA fragment to be used as an acceptor molecule. A nine-nucleotide-residue DNA complementary to the 3'-domain of 16S rRNA beginning at position 1399 was synthesized on an Applied Biosystems' DNA synthesizer Model 380A. The DNA probe was then hybridized to the 16S rRNA, and RNase H digestion were performed under conditions analogous to those described by Donis-Keller (13).

Intermolecular joining reactions were accomplished with unlabeled 16S rRNA as an acceptor molecule and 3'-end [^{32}P]pCp-labeled small fragment as a donor molecule. The general reaction conditions were 50 mM Hepes (pH 7.5), 20 mM $MgCl_2$, 3.3 mM dithiothreitol, 15 ng/µl bovine serum albumin, 10% (v/v) dimethyl sulfoxide, with 4 µg of T4 RNA ligase per reaction as previously described (14). After an overnight incubation at 4°C, the reactions were assayed either by zone ultracentrifugation or gel electrophoresis.

3'-terminal labeling

The small fragment was 3'-end-labeled using [5'-^{32}P]pCp and T4 RNA ligase (7). 6.5 pmoles of small fragment (1 µg equivalent to 16S rRNA) was incubated at 4°C overnight with 4 µg of RNA ligase in a 20-30-µl reaction mixture containing 10-20 µCi [5'-^{32}P]pCp, 36 pmoles

of ATP (0.02 μg), 50 mM Hepes (pH 7.5), 20 mM $MgCl_2$, 3.3 mM dithiothreitol, 15 ng/μl bovine serum albumin, and 10% (v/v) dimethyl sulfoxide. Two volumes of tracking dye were added to the reaction (15), the resulting mixture was heated at 90°C for one-half minute and subjected to separation by polyacrylamide gel electrophoresis.

Gel electrophoresis

Electrophoresis of RNA was performed on denaturing polyacrylamide gel using a slab gel apparatus as described previously (16). Gels with [^{32}P]-labeled RNA were autoradiographed by using Kodak Omat-R X-ray film. Unlabeled RNA was located by UV shadowing, cut out with a razor blade, and then the RNA was eluted by the method of Cory et al. (17). For ligation experiment analysis, autoradiographs were scanned by using a Biomed Instruments soft laser scanning densitometer interfaced with an Apple IIe computer.

RESULTS AND DISCUSSION

Acceptor to Donor Ratio

Usually 3'-terminal [^{32}P]pCp labeling reactions are performed using a substantial excess of donor molecule (7). The joining reaction for the modification at an internal position of tRNA (6) has been done with an acceptor excess of about 2-3 fold. However, there have been no reports describing a systematic comparison of the effect of varying the molar ratio of the substrate molecules in either 3'-terminal labeling or intermolecular joining reactions. A comparison of the effect of a molar excess of acceptor or donor molecule in the ligation reaction is shown in Figure III-1. Ligation experiments using a ten-fold excess of acceptor molecule (Figure III-1,1) showed about an order of magnitude increase in ligation efficiency. Various ratios of acceptor to donor molecule up to a 400:1 ratio were analyzed, but further increases in ligation effectiveness were not observed.

Polyethylene Glycol Effects

PEG was added to the reaction mixtures to investigate its effect on ligation efficiency of larger donors and acceptors. Figure III-2 shows the results of a polyacrylamide gel analysis of a titration experiment in which increasing amounts of PEG were added to ligation reactions. Optimal ligation was observed when the reactions were made to 1% PEG. Less than 1% PEG had no effect on the reaction

Figure III-1. Sedimentation analysis of intermolecular ligation. Nonradioactive 16S rRNA and [^{32}P]pCp 3'-end labeled small fragment were incubated with T4 RNA ligase under the conditions described in Materials and Methods. 1. 2 pmoles of 16S rRNA (acceptor) and 0.2 pmoles of small fragment (donor) were present in the ligation reaction. 2. 0.2 pmoles of 16S rRNA and 2 pmoles of small fragment in the ligation reaction. Sedimentation was from left to right and the separation were performed with a Sorvall AH 650 rotor at 49,000 rpm for 3 hours.

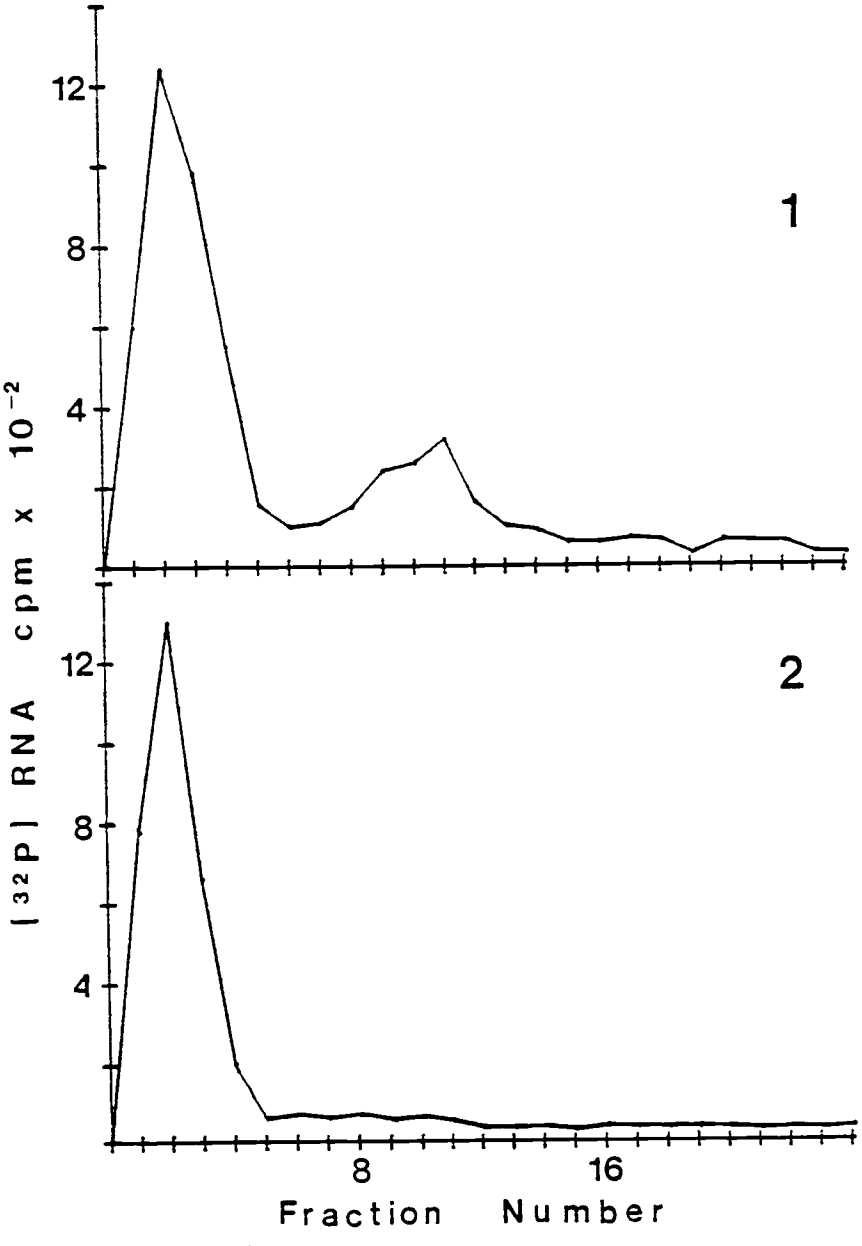

Figure III-1

Figure III-2. Autoradiograph of a polyacrylamide gel displaying the effect of increasing polyethylene glycol concentration on T4 RNA ligase-catalyzed ligations. Ligation conditions were the same as those in Figure III-1 (1) and the small fragment was [^{32}P]-pCp labeled. Nonradioactive 16S rRNA served as the acceptor. Lane 1: control reaction without T4 RNA ligase. Lane 2: 0% PEG and lanes 3 through 8 are reactions containing 1, 4, 10, 16, 22, and 30% PEG in the ligation mixture. The common band in all of the lanes found it the lower portion of the gel is located at the interface between the 2.8% and 10% gel. This material represents less than 15% of the total sample. The polyacrylamide gel was a 2.8% and 10% split denaturing gel as described in materials and methods.

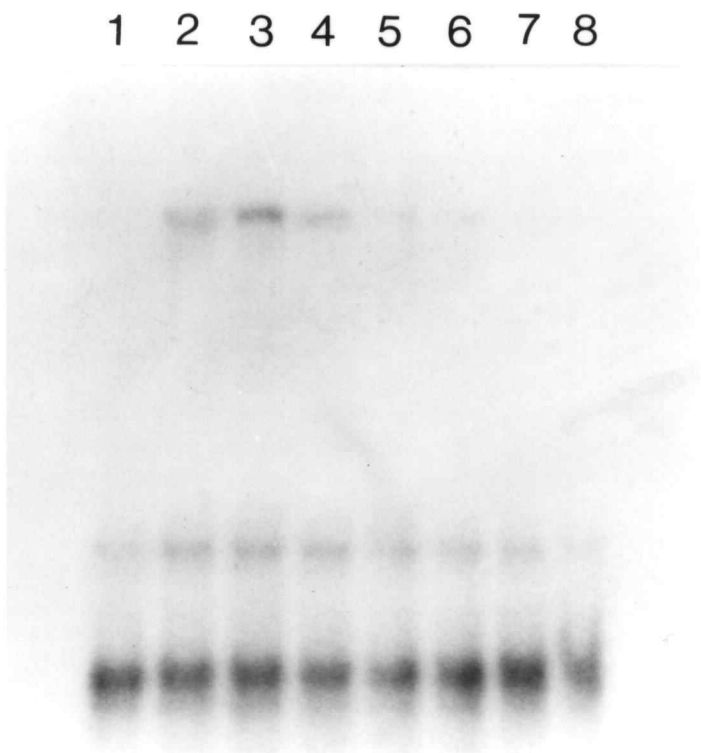

Figure III-2

and increasing it produced a reduction in the joining reaction. Similar effects have also been reported for DNA ligation of blunt ends (18).

Hexamine cobalt chloride, NAD^+, and NP-40

Hexamine cobalt chloride has been reported to increase the efficiency of T4 DNA ligase (E.C. 6.5.1.1) reactions (10). Therefore, we examined the effect of hexamine cobalt chloride ($(NH_3)_6CoCl_3$) on the RNA ligation system. 16S rRNA was used as the acceptor and 5'-end [^{32}P]-labeled small fragment as a donor. The small fragment had 5'-phosphate and, unlike the fragment used in other experiments, it had a free 3'-hydroxyl. Therefore, the small fragment could self-ligate. This was done to provide an internal control with a set of smaller molecules. The HCC was tested over a range from 0.1 mM to 5 mM. No enhancement of ligation was observed and the reaction was inhibited when the HCC exceeded 2.5 mM.

Prokaryotic DNA ligase appears to utilize NAD^+ as an energy source instead of ATP, which is used by eukaryotic and bacteriophage DNA ligase (19). We therefore attempted to use NAD^+ instead of ATP in the ligation reaction. NAD^+ worked as an energy source in the reactions, but without enhancement of the ligation.

Non-ionic detergents have been used to improve enzymatic reactions (20). When NP-40 was added to the ligation reaction at 0.1% and 0.5%, there was no observable improvement of the ligation efficiency.

In conclusion, of the factors investigated, a large molar excess of acceptor over a donor molecule gave the greatest improvement in ligation efficiency. Polyethylene glycol in the reaction also increased the efficiency when present at a concentration of 1%.

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APPENDICES

Figure V-1. Autoradiograph of TLC coated PEI-Cellulose plate showing preparation of [5'-^{32}P]pCp from [r-^{32}P]ATP. [r-^{32}P]ATP was incubated with 1 µl of 10x pCp salts containing 250 mM CHES-KOH (pH 9.5), 10 mM 3'-CMP, 50 mM $MgCl_2$, 30 mM DTT, and 250 mg/ml BSA at 37 C for 90 minutes in the presence of T4 kinase. Lane 1: [r-^{32}P]ATP as a control. Lane 2: [5'-^{32}P]pCp.

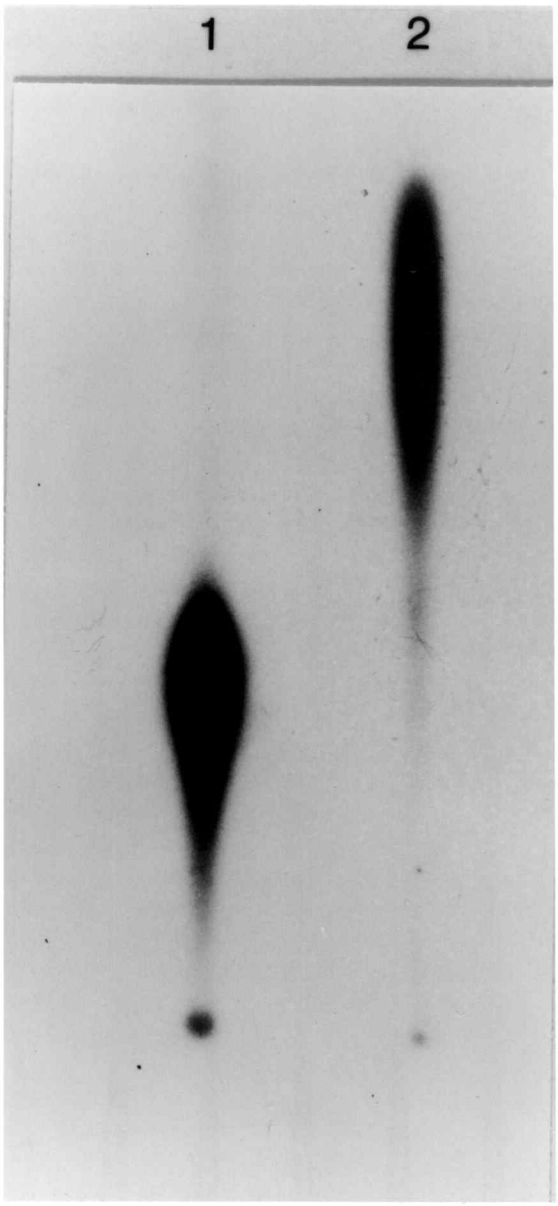

Figure V-1

Figure V-2. Autoradiograph of TLC coated PEI-Cellulose plate showing the activity of phosphatase enzyme. [5'-^{32}P]pCp was treated with calf intestinal alkaline phosphatase in phosphatase buffer containing 100 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, and 0.1 mM $ZnCl_2$. Lane 1: [5'-^{32}P]pCp as a control. Lane 2: [^{32}P] phosphate group was removed from [5'-^{32}P]pCp by the phosphatase enzyme.

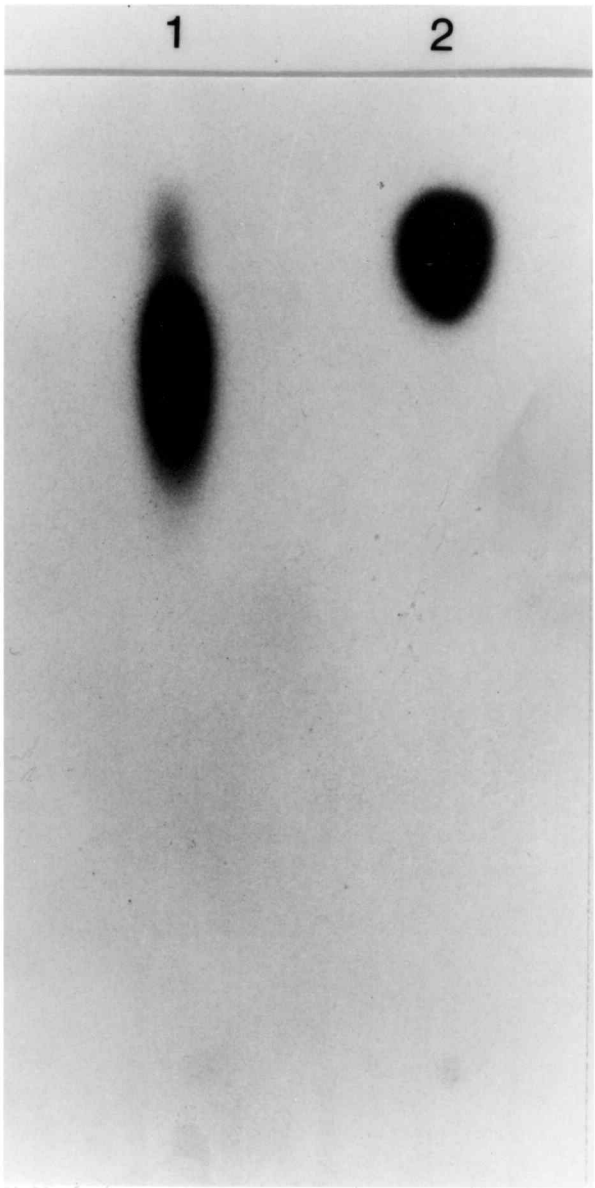

Figure V-2

Figure V-3. Autoradiograph of gel electrophoresis for 3'-end [^{32}P]pCp labeled 16S rRNA purification. 3'-end [^{32}P]pCp labeling of the RNA was performed as described in Materials and Methods of section II.

Figure V-3

Figure V-4. Sedimentation profile of uniformly [^{32}P]-labeled total rRNAs from E. coli on a sucrose gradient in TSM buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 0.3 mM succinic acid, pH 8.0). Sedimentation was from right to left, and separation was performed with a Sorvall AH 627 rotor at 24,000 rpm for 21 hours.

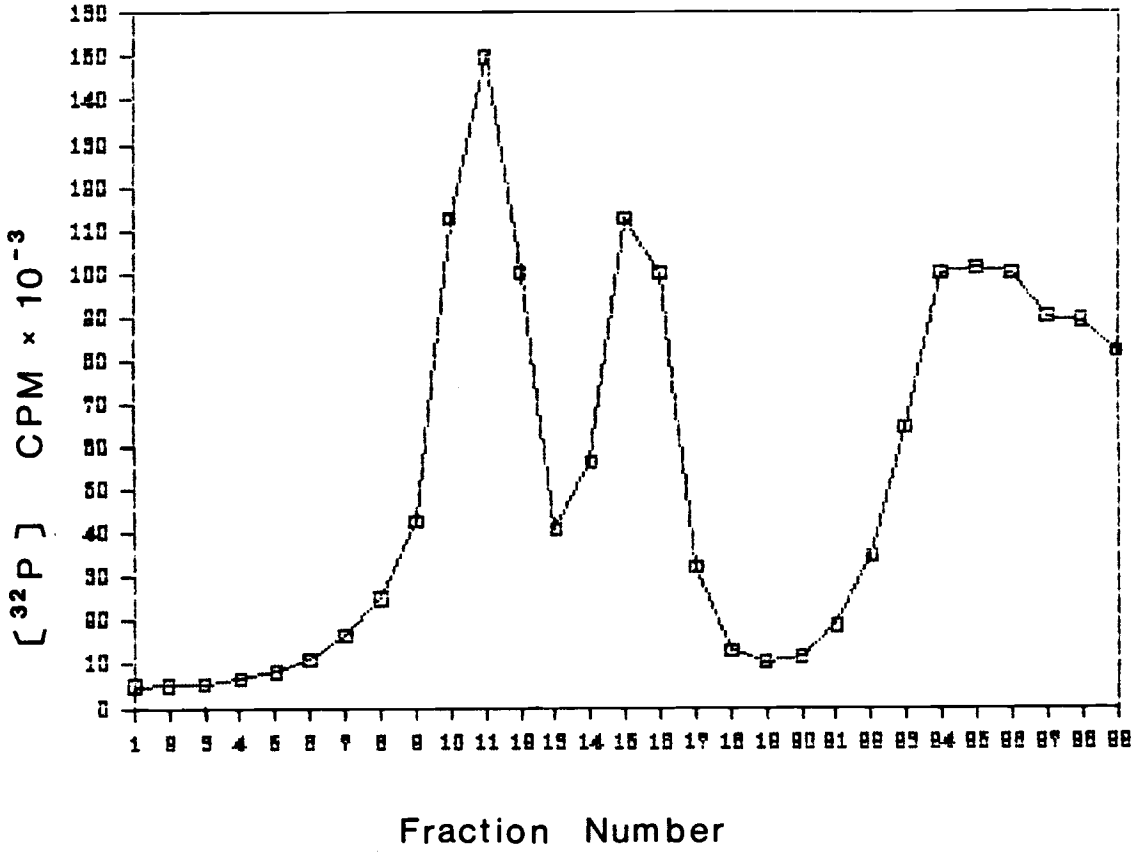

Figure V-4