Probing single-stranded RNA in the 30S subunit of the E. coli ribosome

J. Gordon Porter
The University of Montana

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PROBING SINGLE-STRANDED RNA IN THE
30S SUBUNIT OF THE E. COLI RIBOSOME

By
J. Gordon Porter
B.A., Lafayette College, 1974

Presented in partial fulfillment of the requirements
for the degree of
Master of Science
UNIVERSITY OF MONTANA
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Approved by:

[Signatures]

Chairman, Board of Examiners

Dean, Graduate School

8-1-83

Date
Radiolabeled DNA molecules complementary to the 16S rRNA of E. coli were synthesized with reverse transcriptase using both random and specific priming techniques. Polyadenylated rRNA was first synthesized using the enzyme poly(A) polymerase (New England Nuclear). Then in the presence of an oligonucleotide primer complementary to the poly(A)-16S RNA junction, a cDNA copy was produced using AMV reverse transcriptase. Alternatively, DNA synthesis was primed at a number of random internal sites on the 16S RNA using primers derived from calf thymus DNA.

Products of the reaction primed specifically at the 3' end of the 16S RNA were shown to consist of discrete transcriptional intermediates ranging in size from 30 to 1500 bases long. DNA intermediates are believed to accumulate during the elongation of cDNA due to secondary structure or methylated bases in the RNA template that act as attenuators of DNA polymerization. In time, however, the polymerase was shown to extend the primers to full length cDNA.

A DNA specie 32 bases long was isolated from these products. Its nucleotide sequence was determined and was shown to be exactly complementary to the 3' 23-nucleotide sequence of the 16S rRNA. This specie was used to probe the structure of the 30S ribosomal subunit, and shown to hybridize specifically to the 3' end of the 16S RNA in the ribosome at 37°C in a specifically defined hybridization buffer (40 mM Tris-HCl (pH 7.4), 500 mM KCl, 5 mM MgCl₂). Ribosomes were shown to maintain their native, folded structure throughout these experiments. This result indicates that the 3' end of the 16S rRNA is exposed on the surface of the intact ribosome. Hybridization experiments performed with cDNA probes complementary to the other regions of the 16S RNA indicate that other regions of single-stranded RNA are also exposed on the surface of the 30S subunit.

These studies suggest that this method is a valuable and powerful technique to aid in the elucidation of the tertiary structure of the rRNA in the ribosome.
ACKNOWLEDGEMENTS

I am grateful to Dr. Walter E. Hill for ideas, advice and especially patience during the course of these studies.

I extend thanks to Dr. Kenneth F. Watson and his research staff, particularly John Ong and Dr. John Olsen for helpful discussions concerning experimental design and reagent preparation. I also wish to acknowledge Bill Tapprich for his help with the colicin experiment.

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<td>adenosine 5'-triphosphate</td>
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<tr>
<td>GTP</td>
<td>guanosine 5'-triphosphate</td>
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<tr>
<td>CTP</td>
<td>cytosine 5'-triphosphate</td>
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<tr>
<td>TTP</td>
<td>thymidine 5'-triphosphate</td>
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<td>Poly(rA)</td>
<td>polyadenylic acid</td>
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<td>Oligo(dT)</td>
<td>oligothymidylic acid</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>CPM</td>
<td>counts per minute</td>
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<tr>
<td>mCi</td>
<td>millicurie</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>Tris</td>
<td>tris(hydroxymethyl) aminomethane</td>
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<td>tRNA</td>
<td>transfer RNA</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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CHAPTER I

INTRODUCTION

Ribosomes are cellular ribonucleoprotein particles responsible for protein biosynthesis. Ribosomes can be found associated with messenger RNA (Gilbert, 1963) in complexes called polysomes (Mangiarrotti and Schlessinger, 1966). Bacterial ribosomes can be isolated either as polysomes (Flessel et al., 1976), or by varying the ionic conditions, as free particles which sediment at 70S (Kholer et al., 1968; Phillips et al., 1969). The 70S ribosome is composed of two unequal subunits, the 50S and 30S particles, each consisting of ribonucleic acid and protein components. In the process of translation, mRNA binds first to the 30S subunit after which the initiation factors and the 50S subunit are attached to form the active 70S complex (Lengyel, 1974). The major functions of the ribosome are believed to take place at the interface between the two subunits and are a result of interactions between the RNA and protein components.

The small ribosomal subunit

The E. coli 30S ribosomal subunit contains one molecule of 16S RNA which has a molecular weight of 499,155
daltons (Brosius et al., 1978; Carbon et al., 1978) and 21 different proteins, ranging in size from 8300 to 65,000 daltons. All of these proteins have had their amino acid sequences determined and a number of them have been characterized by hydrodynamic and X-ray methods (Giri et al., 1983).

Isolated 30S proteins and 16S RNA can be reconstituted to form biologically active 30S subunits (Traub and Nomura, 1969). In addition, the 16S RNA has been shown to bind specifically a number of individual ribosomal proteins (Mizushima and Nomura, 1970; Zimmerman et al., 1972; Hochkeppel and Craven, 1977). Using ribonuclease protection experiments, specific sites on the RNA for the binding of proteins S4, S7, S8, S13 and S20 have been determined. These proteins have been shown to be essential to the reconstitution of the subunit (Muto et al., 1974; Ungewickell et al., 1975; Zimmerman et al., 1975).

The 30S ribosomal subunit of _E. coli_ has a molecular weight of about $1 \times 10^6$ daltons, depending on the method of isolation (Van Holde and Hill, 1974). Subunits which have been exposed to 0.5 M NH$_4$Cl (as those used here) have lost some proteins as well as initiation and elongation factors (Hill et al., 1969; Stanley et al., 1966). Hill et al. (1969) have used X-ray scattering to show that the 30S subunit is best approximately by an ellipsoidal model having dimensions of $55\AA \times 220\AA \times 220\AA$. Other work done with the
electron microscope shows the particle to be more prolate and asymmetric, divided into regions called the base or body, the platform or cleft, and the head. The platform extends from the lower two thirds of the subunit and forms a cleft between it and the upper one-third or head region (Lake, 1980; Lake and Kahan, 1975).

Information concerning the arrangement of the proteins relative to each other and on this model of the subunit has been obtained by crosslinking (Traut et al., 1974; Peretz et al., 1976), immune electron microscopy (Lake, 1976) and neutron scattering (Engelman et al., 1975; Moore et al., 1977; Moore, 1980). Lake et al. (1974) were first to apply immune electron microscopy to the E. coli small ribosomal subunit, and introduced the approach of mapping proteins in three dimensions. Using this method, most of the proteins are located on the upper one third of the subunit, and their interprotein distances agree fairly well with data obtained by neutron scattering (Lake, 1980). Crosslinking studies, where reagents are used that covalently link the epsilon amino groups of adjacent lysine residues also agree with these results (Lake, 1980). No proteins have been located on the lower body of the subunit.

Lake (1980) has established functional domains on the ribosome by correlating the known functions of particular ribosomal proteins and initiation factors, based on reconstitution studies, with their location on the
subunit based on immune electron microscopy. Stöffler et al. (1980) have also identified functional domains on the surface of the *E. coli* ribosome. Three antibiotics, thiostrepton, chloramphenical and puromycin, inhibit protein synthesis by binding specifically to ribosomes and blocking a specific molecular event. By generating antibodies and reacting them to these specific antigens bound on the ribosome, the location of a specific binding and thus function can be visualized through the electron microscope.

**Ribosomal RNA**

An interesting feature of the ribosome is that it is constructed from both protein and nucleic acid components. Although much work has been done on the 30S ribosomal protein constituents, much less is known of the functional role of the ribosomal RNA or of its relative position in the ribosome.

For some time it was thought that ribosomal RNA functioned merely as a scaffold upon which the ribosome is built. More recently a few authors have speculated on the evolutionary origins of ribosomes and have suggested that ribosomal RNA was probably an original component of the particle and thus probably plays a central role in ribosomal function (Woese, 1980; Noller, 1980). This thinking suggests that ribosomal RNA is more than just a structural component, that it probably has a mechanistic role as well.
Enzymatic or mechanical processes controlled or catalyzed by different RNAs have been proposed or demonstrated more frequently over the past few years. Specifically, a molecular ratcheting mechanism has been proposed for tRNA activity in translation (Woese, 1970), an RNA splicing role for small nuclear RNAs (Rogers and Wall, 1980), and most dramatically, a self splicing mechanism for the processing of eukaryotic ribosomal RNA (Kruger et al., 1982). In addition there is now good experimental evidence which suggests that ribosomal RNA participates directly in the functioning of the ribosome in addition to the organization of the particle. Some of the specific functions in which the ribosomal RNA probably participates are: mRNA binding and selection (Shine and Dalgarno, 1974; Steitz and Jakes, 1975; Dunn et al., 1978), tRNA binding (Ofengand et al., 1980), antibiotic sensitivity (Yamada et al., 1978), and subunit association (Herr et al., 1979).

The 16S ribosomal RNA can be isolated from the 30S subunit by phenol extraction (Stanley and Bock, 1965). From sequencing it has been shown to consist of 1541 nucleotides numbered by convention from the 5' end (Brosius et al., 1978; Carbon et al., 1978). The RNA is believed to have a well-defined secondary structure based on several lines of evidence. Noller (1974) has demonstrated specific sites on the 16S RNA that can be modified by kethoxal, a reagent specific for guanine residues in single-strand regions of
RNA (Litt, 1969). The RNA has also been digested with single-strand specific nucleases and resistant fragments of RNA have been isolated (Ehresman et al., 1977). Double-stranded regions of the RNA have been identified by crosslinking with psoralen or photoirridation (Cantor et al., 1980; Zweib et al., 1978; Zweib and Brimacombe, 1980). These data, along with models of maximum predicted thermodynamic stability (Tinoco, 1973) and the existence of analogous structures in other organisms (Woese et al., 1975) have led to comprehensive models for the secondary structure of 16S RNA (Noller and Woese, 1981; Woese et al., 1980; Noller, 1980; Stiegler et al., 1981; Zweib et al., 1981).

According to the model of Noller and Woese (1981), 48% of the linear molecule is present as interstrand helices, hybridized in Watson-Crick type base-pairing. In proposing such a model there is recognized the probability that the RNA undergoes significant folding during the assembly of the subunit (Tam et al., 1981), but it is probable that helical regions are stable during this process.

In the proposed model the RNA is organized into five functional and structural domains (I-V) based on long range base-pairing interactions between positions 50 and 400, 400 and 900, 930 and 1390. Domains I (Bases 24-420) and II (Bases 420-560) are nuclease resistant and contain only two kethoxal reactive sites in active 30S subunits (Noller,
1974). They contain binding sites for proteins S20 and S4 (Zimmerman, 1980) which have been antigenically placed in the body of the 30S subunit (Lake, 1976).

Domain III (Bases 560-890) contains the binding sites for proteins S6, S8, S15, and S18 (Zimmerman, 1974). In addition there are a number of chemically reactive bases which are protected from modification by association of the 30S and 50S subunits, suggesting that this region of the 16S RNA makes contact with the 50S subunit (Chapman and Noller, 1977). It has further been shown that these sites are crucial for the association process (Herr et al., 1979). An alternate helix can be formed within this domain, and it has been suggested that this may function in a conformational switching mechanism important in subunit association (Noller, 1980).

Domain IV (bases 940-1400) binds S7, S9, S10, S13, S19 and is associated with these proteins in nuclease generated subparticles (Zimmerman, 1980). These proteins have been mapped on the head of the 30S subunit (Lake, 1976; Stoffler, 1980). Secondary structure in this region consists of three long range interactions between bases 930-1390, 950-1230 and 985-1215 (Noller, 1980). There are a number of kethoxal reactive sites which show slightly decreased activity in the 70S particle, suggesting that they may lie in the interface region, but not in contact with the 50S subunit (Noller, 1980). In addition the anticodon of
p-site bound tRNA has been affinity labeled to this region of the 16S RNA in the intact subunit, implying that domain IV may be active in the binding of the peptidyl tRNA (Zimmerman, 1980).

Domain V (bases 1400-1541) contains the 3' terminal region of the RNA and consists of two main helical stems. Two dimethylated adenines at positions 1517 and 1518 have been antigenically placed on the platform or ledge of the Lake model using immune electron microscopy (Lake, 1976). In addition, antibodies to low molecular weight ligands attached to the 3' end of the 16S RNA have been visualized in a groove between the head and ledge of the 30S subunit (Shatsky et al., 1979). While also implicated in subunit interaction (Chapman and Noller, 1977; Herr et al., 1979), there is good evidence that the 3' end participates in binding to mRNA during initiation. In 1974, Shine and Dalgarno noticed a significant complementarity between the 3' end of the 16S RNA and the 5' end of mRNAs from various coliphages. They have suggested a role for this region of rRNA in mRNA selection and translation initiation. Since that time significant biochemical and genetic evidence has emerged to support this view (Steitz and Jakes, 1975; Dunn et al., 1978).

The proposed helices in this secondary structure model of the 16S RNA are short, usually less than ten base-pairs. These double-stranded regions are most often
associated with ribosomal proteins in the intact ribosome and so can be placed in general regions of the model of the 30S subunit (Noller, 1980; Zimmerman, 1980; Lake, 1980). An analysis of this type indicates that the 5' and 3' proximal domains exhibit substantial overlap in the upper half of the subunit, while the central domain is situated in the lower half. Experiments crosslinking the 16S RNA 5' and 3' ends with psoralen in situ support these conclusions (Cantor et al., 1980) while the immune electron microscopy data of Mochalova et al. (1982) place the 5' end in the lower half of the subunit. Residues 1-550 of the 16S RNA are very resistant to nuclease digestion and chemical modification while still in the ribosome (Fellner et al., 1970; Rinke et al, 1977; Noller, 1974). The 5' domain is thus believed to be in the structural core of the ribosome, together with the proteins that it binds (S4, S20). The 3' and central RNA domains however are very susceptible to nuclease cleavage and chemical modification in situ and so may lie close to the surface of the ribosome (Zimmerman, 1980; Teterina et al., 1980; Skripkin et al., 1982).

Knowledge of the structure of RNA on the ribosome is only rudimentary compared to the progress with proteins. However, the localization and identification of exposed RNA fragments in ribosomal subunits is of great interest because as indicated above, these RNA regions can be directly involved in the functioning of ribosomes. Cantor (1980) has
pointed out that there can be as many as six different RNAs on the ribosome simultaneously: the intrinsic 16S, 23S, and 5S rRNAs, the bound mRNA, and the tRNAs bound at the peptidyl and aminoacyl sites. The small amount of evidence accumulated so far points to the existence of base-pairing interactions between the different RNAs during the process of translation, yielding the conclusion that single-stranded regions of RNA on the surface of the ribosome may be active as functional centers. Most of the experiments performed that address this point involve some type of chemical modification and so do not give any idea of the length of exposed regions of RNA. It is hoped that new knowledge of the arrangement of 16S RNA in the 30S subunit will illuminate our perception of both the structure and function of ribosomes.

**Reverse transcription of 16S rRNA**

Reverse transcription of 16S rRNA and other ribosomal RNAs has previously been accomplished using techniques where DNA synthesis is primed specifically at the 3' end of the RNA molecule. In the case of 16S RNA, three DNA intermediates 30, 375 and 576 bases long are observed to accumulate during the elongation of cDNA. These are reported to be due to methylated bases in the RNA template which produce pauses or stops (Hagenbuchle et al., 1978; Youvan and Hearst, 1979, 1982). The 16S RNA of *E. coli*
contains 9 methylated bases (Carbon et al., 1979). Two adenines at positions 1517 and 1518 dimethylated in the N₆ position (m₆Am₆A) have been shown to act as an absolute stop to DNA polymerization, presumably due to interference by the methyl groups in Watson and Crick hydrogen bonding. In these experiments a DNA specie approximately 30 bases long was produced (Hagenbuchle, 1978).

Experiments have been performed demonstrating that this block can be circumvented by priming downstream from the dimethylated adenines to produce longer DNA species. In this case guanines at positions 965 and 1206 and methylated in the N₂ position have been shown to attenuate but not stop reverse transcription resulting in DNA intermediates 576 and 335 bases long, respectively (Youvan and Hearst, 1979). X-ray crystallographic evidence indicates that the N₂ methyl group in m²G exists proximal to the imidazole ring in monomer crystals, a position which allows Watson and Crick base-pairing. The evidence does suggest, however, that alkylation of the 2-amino groups leads to alterations in the stacking and conformation of the nucleosides (Ginnel and Parthasarthy, 1978). It has also been shown that poly(m²G) is unable to form a helix with poly(C), presumably due to the altered stacking properties of m²G as compared to G (Ikehara and Hattori, 1977). Thus the attenuation of transcription at the m²G positions may be due not to
interference in hydrogen bonding, but disruption of desired ring conformations and thus base stacking.

Similar work done on mono and dimethylated analogs of adenine and cytosine indicate that rotation of the aminomethyl group is in this case restricted about the C2-N2 bond. The two possible orientations of m6A and m4C lie in the plane of the ring and show a strong preference for syn-positioning of the methyl group (relative to N1 of m6A and N3 of m4C) resulting in preferential interference of the methyl group with Watson and Crick base-pairing (Engle and von Hippel, 1974). Adenines dimethylated at the N2 amino position would of course disrupt hydrogen bonding and presumably transcription in all cases.

Reasons for the occurrence of modified bases in rRNA are unclear but it has been suggested that base alkylation might destabilize certain tertiary structures possible in an unmodified rRNA and preferentially stabilize the biologically active structure (Dimick and Stoltzfus, 1977).

**Proposed Problem**

In these experiments, in order to identify regions of single-stranded RNA that may be exposed on the surface of the 30S ribosomal subunit, radiolabeled cDNAs complementary to the 16S rRNA were used as hybridization probes. The purpose of this study was to investigate and develop methods for making cDNAs complementary to various regions of the 16S
RNA and to identify reaction conditions whereby cDNAs could be hybridized to intact ribosomes without seriously denaturing the folded ribosomal structure. Using RNA-directed DNA polymerase (reverse transcriptase) a number of radiolabeled DNA probes complementary to the 16S RNA were made. The ability of these probes to hybridize to RNA in the intact ribosome was investigated using a single-strand nuclease assay and a gradient hybridization assay.

In particular, the interaction with the 30S subunit of a DNA probe complementary to the 3' end of the 16S RNA was demonstrated to be a specific nucleic acid hybridization. This shows that the 3' end of the 16S RNA is exposed on the surface of the ribosome. It is believed these results lend support to the theory of Shine and Dalgarno concerning the role of the 16S RNA in initiation of protein synthesis. Using cDNAs complementary to 5' and internal regions of the 16S RNA, experiments were performed demonstrating that there are other regions of single-stranded RNA exposed on the ribosome.
CHAPTER II

MATERIALS AND METHODS

Isolation and characterization of ribosomes and ribosomal subunits

*Escherichia coli* strain MRE 600, isolated during the midlogarithmic growth phase and purchased from Grain Processing Company, Muscatine, Iowa, was used throughout this study. Ribosomes were prepared by the method of Hill et al., (1969). Typically, 100 grams of cells were disrupted by grinding with glass beads (0.25-0.30 mm diameter) in a minimill for 1 hr in a high salt buffer containing 10 mM Tris-HCl (pH 7.4), 500 mM NH₄Cl, and 15 mM MgCl₂. The minimill container was surrounded by an ice-salt solution throughout the procedure.

After grinding, glass beads and unbroken cells were removed from the solution by centrifugation at 8000 RPM for 10 minutes in a Beckman JA-17 Rotor. The supernatant was centrifuged at 18,000 RPM for 1 hr in the same rotor (low speed centrifugation). The resulting supernatant was removed and then centrifuged at 50,000 RPM for 3 hrs in a Beckman Ti 60 rotor (high speed centrifugation). The pellet from this high speed centrifugation was resuspended overnight in the same high salt buffer. The low speed
centrifugation was repeated and the resulting supernatant was pelleted again at high speed. The pellet was resuspended in a buffer containing 10 mM Tris-HCl (pH 7.4), 100 mM KCl, and 1.5 mM MgCl\(_2\) (low Magnesium buffer) in order to dissociate the ribosome into its constituent 30S and 50S subunits.

The two subunits were separated by centrifugation at 31,000 RPM for 14 hrs through a 10-30% sucrose gradient containing 10 mM Tris-HCl (pH 7.4), 100 mM KCl, and 1.5 mM MgCl\(_2\) and formed inside a Beckman Ti 15 Zonal rotor (Eikenberry et al., 1970). Fractions of 10 ml were collected in a Gilson fraction collector and analyzed for absorbance at 280 nm. Those fractions corresponding to 30S subunits were pooled, precipitated with 3 volumes of EtOH, resuspended in a small volume (20 ml) of low magnesium buffer and dialyzed overnight against the same buffer. The 30S subunits were then frozen and stored at -70°C.

The ribosome preparations were checked for homogeneity by sedimentation velocity measurements made on a Beckman Model E analytical ultracentrifuge equipped with schleiren optics. In these experiments, ribosomes were diluted to a concentration of 1-2 mg/ml and spun at 48,000 RPM in a Beckman ANE rotor at 4°C. Pictures were taken at 2 minute intervals after the 30S peak cleared the meniscus, using Kodak Metallographic plates. The pictures on the plates were measured using a Nikon 6C microcomparator
equipped with IKL digital micrometers. The sedimentation
coefficient was calculated according to the equation:

\[
S = \frac{\ln \frac{r_1}{r_2}}{\omega^2 (t_1 - t_2)}
\]

\( S \) = sedimentation coefficient
\( r_1, r_2 \) = distance of boundary from the axis of
rotation (cm)
\( t_1, t_2 \) = times when boundaries were at \( r_1 \) and \( r_2 \)
respectively (sec)
\( \omega \) = angular velocity (radians/second)

The sedimentation coefficient is usually expressed in
Svedberg units \((S = 10^{-13} \text{ seconds})\). The results were
corrected to \(20^\circ\text{C}\).

**Isolation and purification of 16S RNA**

The 16S RNA was prepared from 30S subunits by
SDS-phenol extraction according to the method of Stanley and
Bock (1965), modified slightly. Anhydrous phenol was
equilibrated with buffer containing 10 mM Tris-\(\text{HCl}\) (pH 8.8),
100 mM NaCl, 5 mM EDTA. Samples were made 1% in SDS, 20 mM
in EDTA, extracted with 1 volume of phenol three times,
shaken vigorously and centrifuged at 10,000 RPM for 10
minutes each time to separate the phases. All procedures
were performed at room temperature. The aqueous phase was
separated and stored at \(-20^\circ\text{C}\) in one-tenth volume of 4 M
NaCl and three volumes of EtOH. The RNA precipitates were collected by centrifugation at 10,000 RPM in a Sorval HB4 rotor for 60 minutes at 4°C. Trace amounts of phenol and other impurities were removed by washing the pellet twice with a 70% EtOH, 40 mM NaCl solution.

The extracted RNA was purified from degradation products by centrifugation thru 10-30% linear glycerol gradients containing 10 mM Tris-HCl (pH 7.4), 40 mM LiCl, 5 mM EDTA, and 0.2% SDS for 14 hrs at 26,000 RPM in a Beckman SW 27 rotor. Peak fractions containing 16S RNA were pooled and stored at -20°C by the addition of 3 volumes of EtOH and one-tenth volume of 4 M NaCl as described above. In all cases the RNA was subjected to a 65°C heat treatment followed by quick cooling in a saline-ice bath prior to fractionation on glycerol gradients. The purity of 16S RNA samples was monitored by urea-agarose gel electrophoresis using the method of Locker (1979). In some cases, RNA was further purified by centrifugation thru glycerol gradients for 12 hrs at 35,000 RPM in a SW 41 rotor. Peak fractions were again collected, analyzed and stored as above.

Preparation of polyadenylated 16S RNA (Poly(A)^+ 16S RNA)

The 16S RNA was adenylated at its 3' end with poly (A) polymerase (New England Nuclear) according to the method of Sippel (1973). The reaction mixtures (150-450 µl) contained 38-114 µg of 16S RNA; 50 mM Tris-HCl (pH 7.9); 10
mM MgCl$_2$; 2.5 mM MnCl$_2$; 250 mM NaCl; 0.25 mM $[^3]$H rATP (17-60 Ci/mMole) and 3-9 units of poly(A) polymerase. After incubation for 20 minutes at 37°C the reaction was terminated by addition of SDS to 1% and EDTA to 20 mM. The extraction of radioactive nucleic acids from this and all reactions was made using phenol extraction according to the method of Brawerman (1974). To each reaction (20-500 µl) was added one-tenth volume of 10% (w/v) SDS, one tenth volume of 200 mM EDTA and one volume of equilibrated phenol containing 0.1% (w/v) 8-hydroxy quinoline. The mixtures were brought to room temperature, shaken vigorously, centrifuged briefly in the table top centrifuge, and the aqueous phase removed. The phenol phase was reextracted 3-4 time with an equal volume of buffer containing 10 mM Tris-HCl (pH 8.8), 100 mM NaCl and 5 mM EDTA. Labeled RNA products were further purified by gel exclusion chromatography on a G-50 Sephadex column.

Polyadenylated 16S RNA was separated from unreacted RNA using oligo(dT)-cellulose chromatography according to the published methods of Aviv and Leder, (1972) and Bantle et al., (1976). Polyadenylated RNA was bound to the column in 10 mM Tris-HCl (pH 7.4), 0.5 mM NaCl, 5 mM EDTA, 0.2% (w/v) SDS and washed with 10 mls of the same buffer. Bound samples were eluted with 10-15 mls of a buffer containing 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.2% (w/v) SDS.
Intact $[^3\text{H}]$ poly(A)$^+$ 16S RNA was separated from degradation products by centrifugation through 4 ml, 10-30% (v/v) glycerol gradients containing 10 mM Tris-HCl (pH 7.4), 40 mM LiCl, 5 mM EDTA, and 0.2% (w/v) SDS for 4 hrs at 21°C and 54,000 RPM using an SW 60 rotor. Fractions containing $[^3\text{H}]$ poly(A)$^+$ 16S RNA were pooled and stored at -20°C in 75% (v/v) EtOH containing 100 mM NaCl. Purity was further established by polyacrylamide gel electrophoresis and fluorography (Maniatis et al., 1975).

Reverse transcriptase reactions

Reverse transcriptase from avian myeloblastosis virus prepared by the method of Kacian et al., (1971), was supplied by Dr. Kenneth F. Watson.

Poly(rA)$\cdot$oligo(dT)$_{12-18}$ reactions. Reverse transcriptase activity was measured using poly(A)$\cdot$oligo(dT) as template-primer in 25 ul reactions containing 50 mM Tris-HCl (pH 8.2); 40 mM NaCl; 8 mM MgCl$_2$; 10 mM DTT; 0.2 mM $[^3\text{H}]$ TTP (50 cpm/pmole); 6 µg/ml poly(A)$\cdot$oligo(dT); and varying amounts of reverse transcriptase. The template-primer was prepared as described by Olsen, (1982) and was a gift of the laboratory of Dr. Kenneth F. Watson.

Reaction mixtures were incubated at 37°C for 20 minutes and terminated by pipeting onto DE-81 filter papers. Unincorporated $[^3\text{H}]$ nucleotide was removed by washing the filters repeatedly in 5% Na$_2$HPO$_4$ solution (Blatti et al.,
1970). The filters were dried and immersed in a liquid scintillation fluid containing 0.39% (w/v) PPO, 0.008% (w/v) bis MSB, 0.35% (v/v) H₂O and 2.5% NCS tissue solubilizer. The incorporation of radiolabeled isotope into DNA was measured in a Beckman LS-230 liquid scintillation counter.

**Synthesis of cDNA primed with calf thymus DNA fragments (cDNA₅).** DNA complementary to 16S RNA and primed with calf thymus primers was synthesized according to the method of Taylor et al., (1976). Reaction mixtures contained 50 mM Tris-HCl (pH 8.2); 10 mM DTT; 0.2 mM of each of the 3 unlabeled deoxyribonucleotide triphosphates; 0.04 mM of the labeled deoxyribonucleotide triphosphate ([³H]dATP or [³H]dTTP, 17-60 Ci/mMole); 100 g/ml actinomycin D; 880 g/ml calf thymus primers; 80 g/ml 16S RNA and varying amounts of reverse transcriptase. Calf thymus DNA primers prepared by the method of Taylor et al., (1976) were a gift of the laboratory of Dr. Kenneth F. Watson. Reverse transcriptase reactions were incubated at 37°C for 4 hrs and terminated by the addition of SDS to 1% and EDTA to 20 mM. Reaction products were immediately extracted with phenol, hydrolyzed overnight at 37°C in the presence of 300 mM NaOH, neutralized with HCl and purified by G-50 Sephadex chromatography.
Oligo(dT)$_{12-18}$ and dT$_{10}$dAdA-primed DNA synthesis. The synthesis of cDNA-primed from the 3' end of the poly(A) 16S RNA was as described by Hagenbuchle et. al., (1978), with modifications. Reactions contained 50 mM Tris-HCl (pH 8.2); 10 mM MgCl$_2$; 40 mM NaCl; 10 mM DTT; 0.2 mM dATP, dGTP, dCTP and 0.04 mM [$^3$H]dTTP (17-60 Ci/mMole); 100 µg/ml actinomycin D; 50 µg/ml poly(A)$^+$ 16S RNA; 4 µg/ml oligo(dT) or dT$_{10}$dAdA-primer (PL Biochemicals) and saturating amounts of reverse transcriptase. Before the addition of enzyme and actinomycin D to the reaction, primers and template were preincubated for 30 minutes at 37°C. After the addition of enzyme, reactions were incubated at 37°C for 35-180 minutes. The [$^3$H]cDNA products were then subjected to alkaline hydrolysis and purified by extraction with phenol and G-50 Sephadex chromatography. Samples were precipitated by the addition of one-tenth volume of 4M NaCl and three volumes of EtOH.

Purification of a specific hybridization probe—cDNA$_{32}$. Specifically primed cDNA products, synthesized in reactions described above, were fractionated by electrophoresis for 4 hrs at 100 V in cylindrical gels 15 cm long containing 11% polyacrylamide and 98% formamide. (Friedrich et. al., 1977). The gels were sliced and each slice eluted overnight in a shaker bath at 37°C in 0.25 ml of a buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM LiCl,
2 mM EDTA and 0.2% (w/v) SDS. The eluate was assayed for radioactivity and fractions corresponding to DNA 500-1500 bases long and 32 bases long were pooled separately and chromatographed on a G-50 Sephadex column. The excluded volume was concentrated and precipitated by the addition of one-tenth volume of 4 M NaCl and three volumes of absolute EtOH. After storage overnight at -20°C, samples were collected by centrifugation at 10,000 RPM for 1 hr at 1°C in a Sorvall HB4 rotor. The [³H]cDNA products were then washed with either 70% or absolute EtOH and resuspended in appropriate buffers for either hybridization experiments or sequencing.

Gel electrophoresis of nucleic acids.

Analysis of cDNA transcripts and polyadenylated RNA on denaturing polyacrylamide gels was performed according to Maniatis et. al., (1975). Typically, 6% polyacrylamide gels containing 7 M urea, 90 mM Tris-Borate (pH 8.3) and 20 mM EDTA were run in a vertical slab apparatus at 12.5 mA for 2.5 hrs. Radiolabeled samples were resuspended in 95% formamide and 0.25% bromphenol blue, heated at 90°C for 90 seconds, quick cooled in a saline ice bath, and loaded on the gel under reservoir buffer containing 90 mM Tris-borate (pH 8.3) and 20 mM EDTA. DNA sequence analysis was performed on polyacrylamide gels as described in a subsequent section. Gels containing 20% bis-acrylamide; 90
mM Tris-borate (pH 8.3); 20 mM EDTA; and 8.3 M urea were poured, polymerized and cured overnight at room temperature.

Electrophoresis on horizontal alkaline agarose gels was performed as described by McDonnel, (1977). Agarose (1-2%) in distilled water was melted and equilibrated at 50°C, mixed with appropriate amount of reservoir buffer (30 mM NaOH; 2 mM EDTA) and allowed to polymerize at room temperature. Samples were dissolved in reservoir buffer with the addition of 20% glycerol, 0.1% bromphenol blue, and heated at 37°C for 5 minutes prior to loading. Electrophoresis was performed at 150 mA constant current for 4-5 hrs with the slab submerged under buffer.

Urea-agarose gel electrophoresis for analysis of RNA was performed according to the method outlined by Locker, (1979). Agarose was dissolved in 6 M urea, 15 mM iodoacetate, 50 mM Tris-borate (pH 8.3); 1 mM EDTA; boiled briefly, and poured onto a horizontal slab while hot. Gels were cooled to room temperature and subsequently placed at 4°C for several hours to polymerize. Samples were loaded in 90% formamide and electrophoresed for 3-4 hrs at 125 V constant voltage.

Non-denaturing gels were run in cylindrical glass tubes containing 2.85% acrylamide, 5% SDS, 90 mM Tris-borate (pH 8.3) and 20 mM EDTA. Samples were loaded in the same buffer and electrophoresed at 5 mA constant current for 2 hrs.
Nucleic acids were fixed in gels by soaking for one-half hr in 1% (w/v) lanthanum acetate, 1% (v/v) acetic acid. Non-radioactive samples were visualized by staining with either 0.05% methylene blue for 1 hr, followed by destaining with distilled water, or by staining with ethidium bromide (0.5 μg/ml) for one-half hr and viewing with an ultraviolet transilluminator.

**Autoradiography and fluorography.**

Fluorography of agarose and acrylamide gels was performed as described by Chamberlain, (1979). After electrophoresis, nucleic acids were fixed in the gel by soaking in 1% lanthanum acetate/acetic acid as described above, followed by soaking in 5 volumes of 1.5% (w/v) ammonium acetate for 20 minutes. Acrylamide and agarose gels were then soaked in 10 volumes of 1 M sodium salicylate (pH 5-7) for 30 minutes. Gels were placed on wetted Whatman 3MM paper and dried under heat in a gel dryer for 1.5 hrs. The dried gel bound to the 3MM paper was exposed to Kodak XAR-5 film at -70°C in a light tight cassette containing X-ray intensifying screens for an appropriate length of time.

Autoradiography was performed by fixing gels with lanthanum acetate and acetic acid, drying on 3MM paper and exposing to X-ray film as above. Autoradiography of sequencing gels was performed by the wet gel method of Maxam.
and Gilbert, (1980). Immediately after electrophoresis, the gels, still bound to electrophoresis plates, were covered with cellophane and exposed directly to X-ray film as described above.

5' end-labeling of DNA.

Labeling the 5' end of dephosphorylated DNA with $[^{32}\text{P}]$ was accomplished by the method of Maxam and Gilbert (1980). Samples containing 1-50 pmoles of DNA were precipitated with EtOH as described above, washing once or twice with 70% EtOH, 40 mM NaCl and resuspended in a reaction mixture containing 50 mM Tris-HCl (pH 7.6); 10 mM MgCl$_2$; 5 mM DTT; 0.1 mM EDTA; 0.1 mM spermidine and a 5-10 fold molar excess of $\gamma-[^{32}\text{P}]$ ATP (2900 Ci/mMole) and 5 units of T4 polynucleotide kinase.

In the case of DNA with 5' phosphates, the terminal phosphates were first removed in 50 µl reactions containing 10 mM Tris-HCl (pH 8.1) and predetermined amounts of bovine alkaline phosphatase (New England Nuclear). Incubations were for 30 minutes at 37°C. The reaction was stopped by the addition of NaPO$_4$ (pH 6.8) to 5 mM. End-labeling was accomplished by diluting the reaction to 100 µl by the addition of salt solutions to a final concentration of 50 mM Tris-HCl (pH 8.8); 10 mM DTT; 5 mM MgCl$_2$; 1 mM Spermidine, a 5-10 fold molar excess of $\gamma-[^{32}\text{P}]$ ATP (2900 Ci/mMole) and 5 units of T4 polynucleotide kinase (Chaconas and Van de
Sande, 1980). Reactions were then incubated for 35 minutes at 37°C.

Radiolabeled products were extracted with phenol, purified by G-50 Sephadex chromatography, and concentrated with butanol. Excess butanol was removed with ether and residual ether evaporated with nitrogen (Maxam and Gilbert, 1980). The end-labeled samples were then stored at -20°C by the addition of one-tenth volume of 4 M NaCl and three volumes of absolute EtOH.

Nucleotide sequencing of 5'-[^32]P labeled cDNA.

End-labeled cDNA to be sequenced was fractioned on cylindrical polyacrylamide gels as described above. Fractions corresponding to DNA 32 bases long were pooled, purified by G-50 chromatography, precipitated with EtOH, washed once with 70% EtOH, once with 95% EtOH, dried under vacuum, and resuspended in 30 μl of double distilled water.

DNA sequence analysis was performed using the base-specific chemical cleavage method of Maxam and Gilbert (1980). Dimethylsulfate (guanine specific) and hydrazine (pyrimidine specific) reactions were incubated at 20°C for 10 minutes and acid (purine specific) reactions at 20°C for 60 minutes in 1.5 ml siliconized Eppendorf centrifuge tubes. The reactions were stopped and samples were purified either by EtOH precipitation (G, C+T, C) or by lyophilization (A+G). Strand scission reactions were performed with 0.5 M
piperidine in sealed glass capillary tubes at 90°C for 30 minutes, followed by repeated lyophilization. Dried DNA samples were resuspended in 4 μl of 80% (w/v) formamide; 10 mM NaOH; 1 mM EDTA; 0.1% Bromphenol blue, and loaded on 0.5 mm X 12 cm X 30 cm sequencing gels containing 20% (w/v) bis-acrylamide and 8.3 M urea. Electrophoresis was performed at 15 W constant power (900-1200 V) for 3-4 hrs. Autoradiography was as previously described.

Nucleic acid hybridization

Hybridization conditions. Hybridization conditions compatible with ribosome stability were determined by experiment. Reactions contained 40 mM Tris-HCl (pH 7.4); 500 mM KCl; 5 mM MgCl₂; 50-200 μg/ml of 16S RNA or 30S ribosomes; and 0.01-0.05 μg/ml of [³H]cDNA. Incubations were either at 37°C or at 65°C for 1.5 hrs. Hybridization reactions to be assayed by single-strand specific nuclease were diluted into ten volumes of S1 nuclease buffer as described in the following sections. Reactions to be assayed on gradients were chilled on ice after incubation and loaded directly on glycerol gradients.

Ribosomes were determined to have a native conformation in the hybridization buffer by sedimentation velocity measurements made on a Beckman Model E analytical ultracentrifuge. Ribosomes, frozen at -70°C in a low salt buffer, were thawed and diluted into hybridization buffer
(40 mM Tris-HCl (pH 7.4); 500 mM KCl; 5 mM mgCl$_2$) to a final concentration of 1.4 mg/ml. Concentration was measured by absorbance at 260 nm on a Beckman DU-8 spectrophonometer. An extinction coefficient of 14.7 was used. Again, pictures were taken at 2 minute intervals after the 30S peak had cleared the meniscus. Sedimentation coefficients were corrected to 20°C.

**S1 nuclease assay.** The single-strand-specific nuclease prepared from *Aspergillus cryzae* by the method of Vogt (1973) was a gift from the laboratory of Dr. Kenneth F. Watson. Typical reactions contained 30 mM NaAc (pH 4.6); 250 mM NaCl; 1 mM ZnSO$_4$; 5% Glycerol; 10 μg/ml double stranded calf thymus DNA, 10 μg/ml denatured calf thymus DNA, the radio-labeled sample, and predetermined amounts of S1 nuclease. Reactions were incubated for 45 minutes at 37°C. Undigested radioactive DNA was either precipitated with tri-chloro acetic acid and collected on glass fiber filters, or bound to DE-81 paper, dried and counted with a Beckman liquid scintillation counter.

**Gradient hybridization assay.** Hybridization reactions described above were stopped by chilling to 4°C for 5 minutes, layered on a 4 ml 5-20% glycerol gradient containing 40 mM Tris-HCl (pH 7.4); 100-500 mM KCl; 5 mM MgCl$_2$ and centrifuged at 4°C for 3 hrs at 54,000 RPM in a Beckman SW 60 rotor. Fractions were assayed for both
absorbance at 260 nm and radioactivity using an aqueous-compatible scintillation fluid.

**Preparation of colicin E3.**

Colicin E3 was isolated from *E. coli* strain Nol47, which was a generous gift of Dr. Masayasu Nomura, University of Wisconsin, Madison. Colicin was induced from the colicinogenic bacteria by the method described by Konisky and Nomura, (1967). A 4 liter flask containing 1 liter of nutrient broth, (1% Tryptone, 0.5% yeast extract, 0.02M NaCl, 0.005 M CaCl₂, 0.5% glucose, and 0.003 M NaOH to pH 7), was inoculated with one ml of an overnight culture of Nol47 grown in the same media. Cells were grown for 7 hrs on a shaker table at 37°C to a density of 2 x 10⁸ cells/ml. The culture was induced by the addition of mitomycin C (1 μg/ml) and then incubated further for 4.5 hrs. Cells were then collected by centrifugation for 15 minutes at 10,000 RPM in a Sorval GSA rotor, resuspended in 25 mls of 0.05 M guanidine-HCl in 10 mM phosphate buffer (pH 7) and shaken overnight at 37°C. The extract thus obtained was cleared of whole cells by centrifugation. Solid ammonium sulfate was added to the supernatant to 40% saturation and the precipitate removed by centrifugation at 12,000 RPM for 30 minutes. The supernatant was removed, ammonium sulfate added to 60% saturation and the solution chilled on ice for several hrs. The resulting precipitate was collected by
centrifugation, resuspended in 2 mls of 10 mM phosphate buffer (pH 7) and dialyzed overnight at 4°C against a buffer containing 10 mM Tris-Cl (pH 7.8), 50 mM KCl, 10 mM MgAc and 6 mM BME.

Colicin was assayed for activity by serially diluting the preparation and spotting a drop of each dilution on a nutrient agar plate freshly seeded with $10^8$ indicator bacteria (No2147, colicin sensitive bacteria, also a gift of Dr. Nomura) in 5 ml of soft nutrient agar. The highest dilution which gave a clear zone of inhibition of bacterial growth was the concentration used in in vitro reactions with purified ribosomal subunits. In vitro activity was assayed by the inhibition of binding of cDNA$_{34}$ to 30S ribosomes.
CHAPTER III

RESULTS

Preparation and characterization of 30S subunits

Resuspending ribosomes in a low magnesium buffer causes uncoupling of the ribosome into its constituent 30S and 50S subunits (Tissiers and Watson, 1958). Consequently they can be easily separated on a linear 10-30% sucrose gradient (Figure 1). After fractionation and purification, 30S samples were analyzed on the analytical ultracentrifuge. The sample sedimented as one peak at a rate of 30.5S.

Preparation and characterization of 16S rRNA

RNA, when extracted from ribosomes by the method of Stanley and Bock (1966) often contains hidden nicks or interruptions in the covalent structure that may go undetected due to the high degree of secondary structure present in the molecule (Figure 2A). These nicks are attributed to the presence of ribonuclease in the bacterial preparation. It is unlikely that reverse transcriptase can traverse breaks in the structure during transcription. Structural breaks may also yield 3'-OH ends which may cause spurious, uncontrolled initiation of DNA synthesis (Olsen,
Figure 1. Sucrose Gradient Elution Profile from Zonal Sedimentation of 30S and 50S Subunits. Centrifugation was in a 10-30% gradient for 14 hrs at 4°C in low salt buffer (10 mM Tris-HCl [pH 7.4] 100 mM KCl, 1.5 mM Mg) as described in Materials and Methods. Fractions corresponding to the 30S subunit (identified by diagonal lines) were pooled, precipitated, dialyzed overnight in low salt buffer and frozen at -70°C.
Figure 2. Velocity Sedimentation of 16S RNA. 

(A) About 2000 μg of 16S RNA was heated for 2 minutes at 65°C, then quick cooled and centrifuged through a 38 ml 10-30% (v/v) glycerol gradient containing 10 mM Tris-HCl (pH 7.4), 40 mM LiCl, 5 mM EDTA, and 0.2% (w/v) SDS in a SW 27 rotor at 26,000 RPM, 20°C for 22 hrs. Fractions were collected from the bottom of the tube and analyzed for absorbance at 260 nm. Fractions corresponding to the first peak (5-8, 600 μg) were pooled and further purified by centrifugation through a 12 ml, 10-30% glycerol gradient in the same buffer at 35,000 RPM, 20°C, for 12 hrs in a SW 41 rotor (B). Leading fractions (200 μg of RNA) were again pooled and centrifuged through a 4 ml, 10-30% glycerol gradient at 54,000 RPM at 20°C for 3.5 hrs (C).
For these reasons extracted 16S RNA was subjected to further purification.

After extraction, 16S RNA was purified by sedimentation through neutral glycerol gradients as described in Materials and Methods. RNA was subjected to a 60°C heat treatment prior to each centrifugation in order to reduce aggregation and to expose any hidden breaks. As shown in Figure 2C, purified 16S RNA sedimented as one peak after purification.

**Preparation and Characterization of Polyadenylated 16S RNA**

RNA was polyadenylated according to the method of Sippel (1973), as described in Materials and Methods, except for the noted modifications. Poly(A) polymerase (New England Nuclear) in the presence of BSA with tRNA as primer is reported to label only a low percentage of available RNA. The activity of the enzyme does not require a template. Tails are generally very long, between 300 and several thousand bases (Sippel, 1973). It was found in our hands, however, that in 20 minute reactions with 16S RNA where BSA is omitted, over two thirds of the RNA can be labeled consistently. In addition, by monitoring the amount of incorporation relative to the number of moles of RNA labeled, an average tail length of 80 bases can be calculated.
Polyadenylated RNA was separated from unreacted RNA by hybridizing it to oligo(dT)-cellulose in high salt and washing extensively with the same buffer. RNA that did not contain poly(A) was washed off the column at this point. Column fractions were assayed both for absorbance at 260 nm and for radioactivity. About 65% of the RNA was labeled in the reaction and is thus bound to the column. None of the labeled RNA failed to bind to the column, indicating that the poly(A) tails were all long enough to hybridize.

Fractions from the bound volume were pooled, concentrated and centrifuged through a glycerol gradient in order to isolate intact 16S poly(A)$^+$ RNA. It can be seen from the profile in Figure 3 that a small amount of labeled RNA is present as 8-10S breakdown product. This is believed to be due to some nuclease contamination in the enzyme preparation. These species were pooled separately and used as template for synthesis of smaller cDNAs. The larger peak of poly-adenylated RNA migrated consistently just ahead of a 16S RNA marker. The sharpness of the peak in this region indicates that the poly(A) tails were fairly homogeneous. This was corroborated as the purified sample was observed to migrate as a discrete band in polyacrylamide gel electrophoresis (Figure 4).
Figure 3. Velocity Sedimentation of $^3$H poly(A)$^+$ 16S RNA. Poly(A)$^+$ 16S RNA synthesized and purified as described in Materials and Methods was precipitated by the addition of 3 volumes of EtOH and one-tenth volume of 4 M NaCl, collected by centrifugation, resuspended in 150 μl of sample buffer containing 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.05% SDS, heated at 60°C for 2 min. and quick cooled in a saline ice bath. Samples were then centrifuged through a 4 ml, 10-30% (v/v) glycerol gradient containing 10 mM Tris-HCl (pH 7.4), 40 mM LiCl, 5 mM EDTA and 0.2% (w/v) SDS in a SW 60 rotor at 54,000 RPM, at 20°C, for 4 hrs. Fractions were taken from the bottom of the tube and assayed for radioactivity in a Beckman LS scintillation counter. Fractions corresponding to the first peak (16S) and second peak (8-10S) were pooled separately, stored in 3 volumes of EtOH, 100 mM NaCl, -20°C, and used as needed for reverse transcriptase reactions.
Figure 4. Gel Electrophoresis of $[^3H]$ Poly(A)$^+$ 16S RNA. A small sample (50,000 cpm) of $[^3H]$ poly(A)-labeled 16S RNA, purified as described in Materials and Methods was precipitated with EtOH, resuspended in 20 ul of 85% formamide, heated at 90°C for 1 minute, cooled, and electrophoresed at 12.5 mA constant current for 4 hrs on a 4.8% vertical polyacrylamide slab gel containing 8 M urea, 90 mM Tris-borate (pH 8.3), 2 mM EDTA. After electrophoresis, nucleic acids were fixed in the gel by soaking in 1% lanthanum acetate/acetic acid for 20 minutes, followed by soaking in 1 M sodium salicylate pH 5-7 for 30 minutes. Fluorography was performed by subsequently drying the gel on Whatman 3 MM paper and exposing it to X-ray film in a light tight cassette at -70°C for 36 hrs.
16S RNA as a Template for DNA Synthesis

The 16S RNA alone, in the absence of primer, was inactive as a template for DNA synthesis. The reaction is dependent on the presence of hybridizable primers possessing a 3'-OH. Initially, reactions were primed with small fragments of calf thymus DNA as described in Materials and Methods. The complexity of the calf thymus DNA fragments is sufficient so that they have some random sequence complementary with the RNA template, and priming occurs at various specific positions along its length (Taylor et al., 1976). The DNA primers were present in a ten-fold mass excess over RNA in these reactions. This was determined to be optimum by titration.

The DNA products from the reactions (cDNA$_5$;) were analyzed on denaturing polyacrylamide gels. Product size varied continuously and discretely from 70 bases to 900 bases long. Actinomycin D, which specifically inhibits DNA-directed DNA synthesis (McDonnel et al., 1970), was present in all reactions to inhibit plus strand synthesis. DNA products were determined to be complementary to the 16S RNA by hybridization to the template and assaying resistance to single-strand specific nuclease.
**Oligo(dT)-primed DNA synthesis**

Oligo(dT)\textsubscript{12-18} was used to prime DNA synthesis on poly(A)\textsuperscript{+} 16S RNA. Primers were present in a ten-fold molar excess, and were preannealed to the template prior to the addition of reverse transcriptase. Preannealling was found to increase incorporation by about 25\%. The amount of reverse transcriptase used in this reaction was determined by titration. Generally, enzyme was used in saturating amounts. The cDNA products synthesized after 1 hr were analyzed by polacrylamide gel electrophoresis and autoradiography. Two major species were discernable, one about 30 bases long and another ranging from 600 to 1200 bases in length. The reaction products were not discrete.

**dT\textsubscript{10}dAdA-primed DNA synthesis**

In order to obtain cDNAs suitable for sequencing, a primer specific for the poly(A) - 16S RNA junction was used. Primers, obtained from PL Biochemicals, had the structure dT\textsubscript{10}dAdA-3'. The 5' phosphates had already been removed to facilitate end-labeling (Figure 5). Reactions were further characterized by measuring the incorporation of labeled nucleotides at different time points. The rate of incorporation was roughly the same as in the oligo(dT)-primed reaction, with the reaction rate slowing gradually after 1 hr (Figure 6).
Figure 5. Specific Priming of cDNA Synthesis. The arrow indicates the beginning of the native 16S RNA molecule.
Figure 6. Kinetics of Specifically Primed Reverse Transcription of 16S RNA. Each 25 μl reaction contained 50 mM Tris-HCl (pH 8.2); 8 mM MgCl₂; 40 mM NaCl; 10 mM DTT; 0.2 mM each of dATP, dGTP, dCTP and 0.04 mM dTTP (17-60 ci/m mole); 50 μg/ml 16S poly(A)⁺RNA; 4 μg/ml dT₁₀ dAdA-3' primer and reverse transcriptase.
The radioactive products produced after 1 hr were analyzed using 6% polyacrylamide gels and autoradiography. The autoradiograms in Figures 7 and 8 show that the transcription process was uniformly processive, i.e., discrete partial cDNA intermediates exist. In this case the products consisted of a short fragment exactly 32 bases long, and a number of discrete fragments in the range of 200 to 1500 bases as determined from standard markers. Most of the species were discrete, characteristic of specific priming events.

The 32-base fragment (cDNA\(_{32}\)) may be present due to a transcriptional block consisting of a pair of adenines (nucleotides #1517 and 1518) each dimethylated at the nitrogen in position 6 (Nichols and Long, 1966). The methyl groups are on the hydrogen bonding positions in the template and may prevent the reverse transcriptase from proceeding past the site of the last unmodified base (Hagenbuchle et al., 1978). The smaller, less prominent fragment in the autoradiogram is probably partially extended primers. Analysis of the cDNA transcripts produced at various time points is represented by the autoradiograms in Figure 8. The larger transcripts consist of a reproducible array of discrete species. DNA species ranging in size from 200 to 400 bases are produced within 5 minutes. Longer species (500 to 1500 bases) are produced after about 30 minutes.
Figure 7. Analysis of dT$_{10}$dAdA-Primed Poly(A)$^+$ 16S RNA-Directed DNA Products in a Polyacrylamide Gel. [$^{32}$P]DNA products from a 25 µl reverse transcriptase reaction were purified by extraction with phenol and ethanol precipitation, denatured in formamide and electrophoresed at 12.5 mA constant current for 2.5 hrs in a 6% polyacrylamide slab gel containing 7 M urea. The gel was subsequently analyzed by autoradiography. (a,c) 5'-[$^{32}$P] Hae III-digested φX174 DNA. (b) 60 minute reaction products.
Figure 8. Size Analysis of Poly(A)^+ 16S RNA-Directed cDNA Products as a Function of Time. Aliquots (20 µl) were taken from a 100 µl reaction, purified by extraction with phenol and precipitation with ethanol, denatured in formamide and electrophoresed at 12.5 mA constant current for 3h in a 6% polyacrylamide slab gel containing 7 M urea. [^32P]DNA products were visualized by autoradiography. (a) 5 min. (b) 35 min. (c) 90 min. (d) 130 min. (e) 180 min.
Densitometric tracings of this autoradiogram (Figure 9) indicate the percentage of the total that each component contributes at each time point. This information was used to optimize this reaction with regard to production of cDNA\(_{32}\). From Figure 10 it can be seen that cDNA\(_{32}\) as a percentage of the total is maximum within 5-10 minutes. However, by combining this information with the total measured incorporation at each time point (Figure 6), it was determined that the optimum production of cDNA\(_{32}\) occurs after 120 minutes (Figure 11).

Since the dimethylated adenines at positions 1517 and 1518 have been previously reported as a block to reverse transcription (Hagenbuchle et al., 1978), the idea was considered that some specific downstream priming may be occurring in the interior of the molecule, resulting in longer cDNAs. Upon inspection of the rRNA sequence, some portion of the primer complementary sequence 3'-A\(_{10}\)UU-5' was found in a few positions nearer to the 5' end of the molecule. Combined with an excess of primers in the reaction, this could result in priming and synthesis of longer cDNAs. However, the appearance of molecules which correspond in size to full-length cDNA and the concomitant reduction in the total amount of cDNA\(_{32}\) after 120 minutes (Figure 11) seems to suggest that the reaction primes mainly at the poly(A) tail, pauses significantly in the region of the dimethylated adenines, and then reads through the pause.
Figure 9. Densitometer Tracing of Autoradiogram in Figure 8. The area under the peak corresponding to cDNA was calculated and presented as data in Figures 10 and 11.
Figure 10. cDNA\textsubscript{32} Presented as a Percentage of Total cDNA Products at Various Time Points. Data was obtained by analysis of the densitometer tracing in Figure 9.
Figure 11. Kinetics of Synthesis of cDNA

Data were obtained by multiplying percentages presented in Figure 9 by the total amount of [32P] nucleotide incorporated at each time point (Figure 6).
to eventually produce full-length cDNA. This was shown to be true by the results of an additional experiment where isolated $^{32}$P labeled cDNA$_{32}$ was utilized as a primer for reverse transcription of the poly(A)$^+$ 16S RNA template using unlabeled nucleotides. The 32-base fragment was active as a primer and was extended to full-length cDNA (not shown).

The discrete cDNA species which persist at all time points are probably due to regions of RNA secondary structure or chemical modification which temporarily interrupts or slows reverse transcription (Youvan and Hearst, 1979). A major species 345 bases long previously reported as corresponding to a transcriptional pause at a methylated guanine in position 1206 (Youvan and Hearst, 1979) is visible in the autoradiogram in Figure 8. Another reported pause at a second methylated guanine in position 965, corresponding to a transcript 586 bases long is not clearly evident from these data.

Other previously unreported major species approximately 180, 270, 440, 480 and 950 bases long are observed, corresponding to kinetic pauses near positions 1370, 1280, 1110, 1070 and 580 on the 16S RNA. It is possible that these may be due to regions of RNA secondary structure as predicted by the Noller model of 16S rRNA (Noller and Woese, 1980). Numerous, less prominent discrete species are also observed. In this way, the transcription
of 16S ribosomal RNA is similar to the transcription of natural RNA templates.

**Isolation and sequencing of cDNA\textsubscript{32}**.

The 32-base cDNA fragment was easily separated from longer species by fractionation on a cylindrical polyacrylamide gel as described in Materials and Methods. Gels were sliced, DNA eluted from the slices overnight, and the eluate assayed for radioactivity (Figure 12). Fractions corresponding to cDNA\textsubscript{32} and to longer fragments were pooled separately and subsequently used for hybridization experiments. The 5' ends of these products were labeled with $[^{32}\text{P}]$ either at this point or prior to fractionation. To positively identify cDNA\textsubscript{32}, it was subjected to the chemical cleavage reactions described by Maxam and Gilbert (1980). The sequence determined from the autoradiogram in Figure 13 can be read from the end of the primer up to 22 bases into the cDNA fragment. It is, without ambiguity, a perfect complement of the 3' end of the 16S RNA, the sequence of which was originally determined by Brosius et al. (1978). In each lane only the strongest bands are read. In the oligo(dT)-primer region some fainter out of phase bands appear between the thymine cleaved products. These could be end-labeled RNA, a contaminant encountered during sample preparation. In some cases adenine bands appear with less intensity in the guanine lane. This is probably due to
Figure 12. Preparative Polyacrylamide Gel Electrophoresis of cDNA22. [3H] or [32P] DNA product from a 100 nl reverse transcriptase reaction was first purified by alkaline hydrolysis (0.3N NaOH, 37°C, 1 hr), extraction with phenol and precipitation with EtOH. The sample was then denatured in formamide and subjected to electrophoresis in a 15 cm cylindrical gel containing 11% polyacrylamide and 98% formamide at 100 V for 4 hrs. After electrophoresis, the gel was sliced, the DNA eluted overnight, and the eluate assayed for radioactivity. Fractions corresponding to cDNA22 (41-46) and cDNA600-1500 (1-20 not shown) were pooled separately and subjected to further purification as described in Materials and Methods.
Figure 13. Nucleotide Sequence of cDNA2 Isolated from Poly(A) † 16S RNA-Directed DNA Transcripts. 5' [32P] cDNA was subjected to 4 base-specific degradation reactions as described in Materials and Methods. Samples were subsequently purified and subjected to electrophoresis in 20% polyacrylamide gels containing 8.3 M urea for 3.75 hrs at 12.5 W constant power. After electrophoresis the gel was analyzed by autoradiography.
adenine reaction to a lesser extent in the dimethylsulfate reaction.

The sequencing also identifies a specific priming event at the junction of the 16S RNA and the poly(A) tail. Priming actually occurs three bases into the RNA molecule. It should be mentioned that if priming were not specific, sequencing from the 5' end would not be possible. The sequencing also identifies the specific pause in transcription at the position just prior to the dimethylated adenines.

Optimization of ribosome hybridization reactions

Typical RNA-DNA hybridization reactions are performed in 0.5 M NaCl and 1 mM EDTA at 68°C for a period of 2-3 hrs (Leong et al., 1972). Ribosomes cannot exist in these conditions in any viable form, so one of the goals of this research was to develop buffer conditions compatible with both ribosomes and nucleic acid hybridization. Specifically, the approach was to systematically vary the standard hybridization conditions to bring them closer to conditions necessary to maintain ribosome integrity. A given set of conditions was determined to be capable of supporting hybridization by reacting calf thymus-primed [³H] cDNA with the 16S RNA template and assaying for resistance to single-strand specific S1 nuclease.
Potassium is a standard monovalent component used in ribosome studies. Little effect was seen on nucleic acid hybridization when potassium replaced sodium in the reaction mixture. EDTA was replaced by magnesium, in order to maintain ribosomes in a folded 30S conformation (Tissieres and Watson, 1958). Very little effect was seen on hybridization rate in this case. Finally, reactions were performed at 37°C instead of 68°C in order to prevent denaturation of ribosome conformation. Figure 14 represents 16S RNA-DNA hybridization at both temperatures. Whereas hybridization was nearly complete in 2 hrs at 68°C, as much as 8 hrs was required for complete hybridization at 37°C. However, the steep initial curve observed in the 37°C reaction shows that nearly 50% of the DNA is hybridized within 1 hr. This is the incubation time used in hybridization of cDNA to ribosomes. When incubated in 40 mM Tris-HCl (pH 7.4); 500 mM KCl; 5 mM MgCl₂ at 37°C for 1 hr, 30S ribosomes were shown to sediment at a rate of 31.2S when analyzed in the analytical ultracentrifuge. This is interpreted to mean that 30S ribosomes maintain their native, folded conformation when incubated for a short time in this buffer.

Ribosome hybridization experiments with cDNA³².

Hybridization of cDNA to ribosomes was assayed by measuring the comigration of radioactive DNA with ribosomes.
Figure 14. Kinetic Analysis of 16S RNA•DNA Hybridization at 68°C and 37°C. 0.5 ng of $[^3]H$ cDNA was incubated at 68°C and at 37°C in 100 μl reactions containing 40 mM Tris-HCl (pH 7.4), 500 mM KCl, 5 mM MgCl$_2$, and 200 μg/ml 16S RNA. At various times 10 μl aliquots were removed from the reaction, diluted with 100 μl of a chilled mixture containing 30 mM NaOAc (pH 4.6); 250 mM NaCl; 1 mM ZnSO$_4$; 5% glycerol and 20 μg/ml ct DNA, and incubated for 45 minutes at 37°C with saturating amounts of S$_1$ nuclease. Reactions were then assayed for S1 resistant $[^3]H$ cDNA.
in a glycerol gradient. Typically, after a 40 minute incubation the entire reaction was layered on a 4 ml glycerol gradient and centrifuged at 54K for 3 hrs. The results of hybridization of oligo(dT)-primed cDNA product to 30S ribosomes are presented in the profile in Figure 15A. Here it is seen that a small portion of the DNA binds to the ribosome, relative to a control run with nonspecific DNA (Figure 15B).

The cdna^32 represents a specific probe of the 3' end of the 16S RNA, so this was isolated and sequenced as described, and more hybridization experiments performed. It is evident from the profile in Figure 15B that the fragment binds strongly to the 30S subunit. The second, slower sedimenting peak is probably an unfolded form of the subunit, and it reproducibly binds the fragment at an even higher rate. These interactions were determined to be specific relative to controls run with various types of nonspecific DNA in which no binding was observed.

Experiments were subsequently performed to demonstrate that this specific binding is a true nucleic acid hybrid, rather than some specific nucleic acid-protein interaction. As shown in Table I the DNA probe is resistant to single-strand specific nuclease after a 45 minute incubation with ribosomes. Roughly the same amount of DNA is resistant to S1 nuclease as is bound to the ribosome in the gradient assay. Figure 16 represents increasing
Figure 15. Hybridization of [³H] Poly(A)⁺ 16S RNA-Directed DNA Transcripts to 30S E. coli ribosomes. [³H] cDNA (0.5 ng) was incubated in a 100 µl hybridization reaction containing 40 mM Tris-HCl (pH 7.4); 500 mM KCl; 5 mM MgCl₂, 150 µg/ml 30S ribosomes for 40 minutes at 37°C. Reactions were then chilled on ice for 5 minutes and layered on a 4 ml, 5-20% glycerol gradient containing 40 mM Tris-HCl (pH 7.4); 500 mM KCl; 5 mM MgCl₂ and centrifuged at 4°C for 3-4 hrs at 54,000 RPM in a Beckman SW 60 Rotor. Fractions were collected from the bottom of the tube and assayed for both absorbance at 260 nm and radioactivity, using an aqueous compatible scintillation fluid. (A) [³H]cDNA (B) [³H]cDNA₃₂ (C) [³H] viral DNA.
<table>
<thead>
<tr>
<th>Template</th>
<th>% DNA Hybridized/40 minute incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S RNA</td>
<td>45%</td>
</tr>
<tr>
<td>30S Ribosomes</td>
<td>37%</td>
</tr>
<tr>
<td>23S RNA</td>
<td>2%</td>
</tr>
</tbody>
</table>
Figure 16. Kinetics of Hybridization of cDNA$_{32}$ to 16S RNA and 30S Ribosomes. Analysis was as described in Figure 14 except that 16S RNA or 30S ribosomes were used and incubations were at 37°C. Again, reactions were assayed at various time points for S1 resistant [$^3$H]cDNA.
resistance of hybridized cDNA$_{32}$ with time. The curve is compared with one where the cDNA probe is hybridized with 16S RNA. The curves have the same shape with a difference in amplitude. Initially the rates are the same, but the 30S ribosome reaction plateaus earlier. It is possible that the difference between the two curves is due to steric hindrance, or perhaps a shorter accessible region of RNA in the ribosome reaction.

Table II represents hybridization of the cDNA$_{32}$ probe to 30S ribosomes in varying salt conditions. Monovalent salt (potassium) concentration was varied between 100 and 500 mM with other conditions held constant. These data demonstrate an increasing percent of total DNA hybridization as monovalent salt is increased, a phenomenon characteristic of nucleic acid hybridization, and decidedly uncharacteristic of an ionic interaction with protein. It is also true that ribosome conformation may loosen somewhat as the monovalent-divalent ratio is increased, increasing the accessibility of the probe to the correct region of RNA. This was not detectable in the gradient assays performed.

To further demonstrate that the cDNA binding was due to nucleic acid hybridization, experiments were performed where the 16S RNA in the ribosome was cleaved at a specific site 50 nucleotides from its 3' terminus with the enzyme colicin E3. A crude preparation of colicin was used, isolated from colicinogenic E. coli. by the method of
**TABLE II**

HYBRIDIZATION OF $[^3H]cDNA_{[2]}$ to 30S RIBOSOMES IN VARYING SALT CONCENTRATIONS

<table>
<thead>
<tr>
<th>Monovalent Salt Concentration</th>
<th>% DNA Hybridized/40 Minute Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>100mM</td>
<td>19</td>
</tr>
<tr>
<td>200mM</td>
<td>20</td>
</tr>
<tr>
<td>300mM</td>
<td>28</td>
</tr>
<tr>
<td>400mM</td>
<td>27</td>
</tr>
<tr>
<td>500mM</td>
<td>37</td>
</tr>
</tbody>
</table>

Hybridization conditions and reactions were as described in Figure 14 except KCl concentration was varied as indicated in the table. Hybridization was assayed by the gradient method already described and quantitated by summing the counts over each peak.
Konisky and Nomura (1967) as described in Materials and Methods.

In an initial experiment, cDNA_{32} was first hybridized to the 30S ribosome in the standard reaction conditions. The complex was then treated with colicin for 20 minutes at 37°C and centrifuged through a 4 ml glycerol gradient as previously described. As illustrated by the data in Figure 17, the DNA did not migrate with ribosomes and is believed to be hybridized with the released 50-base colicin fragment at the top of the gradient.

In a converse experiment, colicin-treated ribosomes were incubated with cDNA_{32} and then centrifuged through a glycerol gradient. The profile obtained was identical to the one in Figure 17. These data show that colicin treated ribosomes did not bind the cDNA. In a further experiment, ribosomes were first treated with colicin and then fractionated on a glycerol gradient as above. Appropriate fractions were then tested for their ability to hybridize to cDNA_{32}. Fractions near the top of the gradient show a significantly higher ability to protect cDNA_{32} from S1 nuclease than the fractions containing ribosomes. This indicates that the colicin fragment is cleaved and separated from the ribosomes during centrifugation. In all experiments, the mobility of ribosomes in the gradient was seen to be unaffected by colicin treatment. These data indicate that the binding of cDNA_{32} to 30S ribosomes is
Figure 17. Hybridization of cDNA$_{32}$ to Colicin Treated Ribosomes. 0.5 ng of cDNA$_{32}$ was incubated at 37°C for 40 min. in 50 μl reactions containing 40 mM Tris (pH 7.4); 500 mM KCl; 5 mM MgCl$_2$ and 300 μg/ml 30S E. coli ribosomes, followed by the addition of 50 μl of a mixture containing 10 mM Tris-HCl (pH 7.8); 10 mM MgAc; 50 mM KCl; 6 mM mercaptoethanol and an excess of colicin. Reactions were then incubated at 37°C for an additional 20 min. Alternatively, ribosomes were first treated with colicin for 20 minutes followed by addition of [³H] cDNA$_{32}$ and further incubation for 40 minutes at 37°C. Reactions were then chilled on ice for 5 minutes and centrifuged through a 4 ml glycerol gradient as described previously. Fractions were collected from the bottom and assayed for absorbance and radioactivity. Similar results were obtained from both experiments. Controls run with identical conditions without colicin showed active hybridization of cDNA$_{32}$. 
dependent on the presence of the complementary region of 16S RNA.

**Ribosome hybridization experiments with cDNA\textsubscript{5}.**

In order to investigate the possibility that regions of 16S RNA other than the 3' end are exposed on the surface of the ribosome, it was necessary to prepare cDNAs which do not contain sequences complementary to the 3' 24-nucleotides of the 16S RNA. This was accomplished by treating 30S ribosomes with colicin E3, purifying them on a gradient, extracting the RNA and synthesizing cDNA in a calf thymus DNA-primed reaction as described in Materials and Methods. Radiolabeled cDNA products (cDNA\textsubscript{5}) were analyzed by gel electrophoresis and autoradiography and were seen to consist of species ranging in size from 70 to 900 bases long. That no species shorter than 32 bases or as long as 1500 bases were observed supports the contention that these cDNAs were transcribed from a portion of the RNA molecule some distance 5' from the dimethylated adenines in positions 1517 and 1518.

These cDNA species were then cleaved into smaller fragments using a modification of the purine specific partial hydrolysis originally described by Maxam and Gilbert (1980). Samples were incubated for 6 hrs at 20°C in 1 M piperidine formate followed by incubation for 1 hr with hot (90°C) piperidine. Resulting DNA fragments were
subsequently lyophilized, dissolved in water, and used in hybridization reactions exactly as described previously.

The results of a gradient hybridization assay presented in Figure 18A indicate that a significant portion of the cDNA$_5$ molecules were able to bind to the ribosome and thus migrate with it in the gradient relative to a control run with no ribosomes (Figure 18B). The binding is enhanced significantly by cleaving the DNA into smaller fragments as described above. Again, this result indicates a specific cDNA-ribosome interaction.
Figure 18. Hybridizing of $^{3}$HcDNA, Fragments to 30S E. coli. Ribosomes (A). $^{3}$HcDNA, cleaved into smaller fragments by a purine specific Maxam and Gilbert sequencing reaction, was incubated with 30S ribosomes in hybridization reactions and the reactions assayed on a glycerol gradient exactly as described in Figure 25. (B) Control run with $^{3}$HcDNA, alone.
CHAPTER IV

DISCUSSION

In this study I have demonstrated a new approach for identifying and mapping single-stranded regions of RNA on the surface of the ribosome. Using this method, radiolabeled cDNA complementary to the 3' end of the 16S rRNA is shown to be capable of hybridizing specifically to the rRNA in the 30S ribosomal subunit. This indicates that the 3' end of the 16S RNA is exposed on the surface of the subunit. Using other cDNAs, the 30S subunit is also shown to possess other regions of exposed single-stranded RNA. These results demonstrate this method to be a viable and valuable tool for discerning the general topography of rRNA on the ribosome.

Reverse transcription

For the purpose of generating a 3'-specific cDNA probe, the reverse transcription protocol outlined by Hagenbuchle et al., (1978) was used. The 16S RNA was first polyadenylated and then cDNA synthesis was initiated with reverse transcriptase in the presence of a primer specific for the 16S RNA-poly(A) junction. In this study it was found that a number of transcriptional intermediates are
synthesized on the way to production of full-length cDNA, as determined by analysis via polyacrylamide gel electrophoresis and autoradiography. These partial transcripts seem to be kinetic intermediates that accumulate during the elongation of cDNA due to kinetic modulators in the RNA template. Possible template attenuators of the rate of transcription include signals in the primary sequence, base or ribose methylations, and RNA secondary structure (Youvan and Hearst, 1979).

The smallest of these transcripts, cDNA$_{32}$, was sequenced and shown to be complementary to the known sequence of the 3' end of the 16S RNA. This intermediate is believed to be present due to a pair of dimethylated adenines at positions 1517 and 1518 which interfere with Watson and Crick hydrogen bonding between the template and the nascent cDNA. Hagenbuchle et al., (1978) have previously shown these modified bases to constitute an absolute stop to DNA transcription, i.e., only DNA species 32 bases long were produced in every case. The results presented here show that larger cDNA intermediates are produced and they seem to be extensions of cDNA$_{32}$. In addition, isolated cDNA$_{32}$ is active as a primer, catalyzing synthesis of full-length cDNA, when annealed to 16S RNA. These results indicate that m$_2^6$Am$_2^6$A is not a stop, but that in time the enzyme is able to read through and produce longer cDNAs.
It has been suggested that the 3' terminal base pairing requirement is less stringent for a primer than for cDNA elongation. Primers complementary to a region up to and including a modified base have been shown to be quite active, despite the fact that upstream priming results in a stop at this base (Youvan and Hearst, 1981). Hence a structural block in elongation may not constitute a block to the priming event. Thus, DNA synthesis may proceed to the block, stop as the enzyme dissociates from the template, and then reinitiate and continue. The rate of reinitiation would probably be dependent on the concentration of enzyme in the reaction. This would explain the presence of transcripts of different discrete lengths, and the ability of cDNA\textsubscript{32} to act as a primer. It is not entirely clear from my results whether cDNA\textsubscript{32} includes nucleotides complementary to the dimethylated adenines, or if transcription terminates just before this point.

A further explanation of the discrepancy between these results and those of Hagenbuchle et al., (1978) may lie in the possibility that a portion of the population of the 16S RNAs in this strain of \textit{E. coli} lack the dimethylated adenines, thus allowing transcription to proceed through this region. Alternatively, it may be true that limited interruptions in base-pairing between the transcript and the template are simply not an absolute block to polymerization of DNA by reverse transcriptase. Evidence that this is true
comes from experiments by Youvan and Hearst (1979) where methylated guanines which are known to disrupt base-pairing (Ginnel and Parthasarthy, 1977) are shown to only partially attenuate reverse transcription of 16S RNA.

I have also observed numerous cDNA intermediates 200-1500 bases long other than the three previously reported. It is likely that these are due to the other methylated bases on the RNA, or to secondary structure in the template as described for DNA transcription by T4 DNA polymerase (Huang and Hearst, 1980). The enzyme may slow as it enters base-paired regions of the template, however Youvan and Hearst (1979) did not observe this for reverse transcription of 16S RNA.

**Hybridization of cDNA to Ribosomes**

The cDNA\textsubscript{32}, complementary to the 3' end of the 16S RNA, was shown to hybridize specifically to RNA in the intact subunit. This result indicates that the 3' end of the 16S RNA is exposed on the surface of the small ribosomal subunit. This supports the conclusions of Teterina et al., (1982); Skripkin et al., (1982) and Noller (1980) based on ribonuclease digestion and base modification experiments. Other work by Shine and Dalgarno (1974), Steitz and Jakes (1975) and Dunn et al., (1978), has suggested that the 3' end of the 16S RNA acts in the initiation of translation by first binding to the 5' end of a messenger RNA. Nearly all
prokaryotic mRNAs contain a nucleotide sequence complementary to the consensus sequence: CCUCC, present on the 3' end of the 16S RNA. The complementary binding site is usually centered about 9 or 10 bases 5' to the AUG start codon on the mRNA (Steitz, 1980). The binding of mRNA by 3' rRNA during initiation is a dramatic example of an RNA functional center on the surface of the ribosome.

A number of tests were performed to prove that the binding of the cDNA\textsubscript{32} probe was indeed a hybridization to the complementary 3' end of the 16S rRNA. The binding of cDNA\textsubscript{32} to 30S ribosomal subunits was shown to be specific compared to assays performed using non-specific viral DNA (Figure 15). When bound to the ribosome, the cDNA\textsubscript{32} was also shown to be resistant to digestion with a single-stranded specific nuclease, indicating that the interaction was probably a nucleic acid hybrid. At this point there remained the possibility that the cDNA was involved in a specific interaction with ribosomal proteins, which also might protect the DNA from digestion by single strand nuclease. There is a well-defined protein neighborhood in the region of the 3' end consisting of proteins S1, S7, and S2, all of which seem to be involved in initiation (van Duin et al., 1975). However, the fact that the nuclease digestions were performed in relatively high salt and low pH suggests that the interaction was not with protein. This view is further supported by the data in
Table II which show more extensive binding as ionic strength increased, indicating that the interaction was probably not ionic, but was a true hybridization reaction. Finally and most conclusively, binding to the 30S subunit by cDNA\textsubscript{32} was shown to require the presence of the 3' end of the 16S RNA, i.e., ribosomes in which the 3' end had been enzymatically removed failed to bind the 3' cDNA probe.

The kinetics of hybridization of cDNA\textsubscript{32} to 30S ribosomes (as assayed by resistance to S1 nuclease -- Figure 16) appeared very similar to the kinetics of hybridization to isolated 16S RNA under similar conditions. Differences occurred only in the amplitude of the curves, the 16S RNA curves showing a larger amount of DNA protected from nuclease at each time point. A possible explanation of this result may be the existence of a shorter region of accessible 3' RNA in the ribosome. However, this interpretation is contrary to the data of Teterina et al., (1980) and Skripkin et al. (1982) where up to 120 nucleotides of the 3' end of the 16S RNA are shown to be susceptible to RNase, and to the fact that colicin E3 is able to clip the RNA in situ specifically 50 bases in from the 3' end (Bowman et al., 1971).

The 3' end of the 16S RNA in the ribosome is believed to have a native secondary structure involving at least one hairpin (Woese et al., 1980; Noller, 1980; Wickstrom, 1983). Initiation factor 3 (IF3) is believed to
be involved in destabilizing this structure in order to facilitate its binding to mRNA during initiation (Wickstrom, 1983). The 30S subunits used in these experiments have had their initiation factors removed by washing with salt (Stanley et al., 1966). Consequently, the 3' RNA secondary structure in the ribosome is very likely present in these samples and may prevent hybridization of part of the probe. The isolated 16S RNA used here, however, has been denatured by heating several times during purification and so may lack significant amounts of native secondary and tertiary structure, yielding a 3' end more accessible to a hybridization probe. This may account for differences in rate of hybridization in the two cases.

In Figure 15B two forms of the 30S subunit were evident. The more slowly sedimenting form is possibly an unfolded ribosome, denaturation apparently occurring during incubation due to the very high monovalent/divalent salt ratio (100). It is interesting that the unfolded form reproducibly binds the cDNA probe at a higher rate than the faster sedimenting form. This suggests that the unfolding may render the 3' end of the the 16S RNA more accessible to the cDNA probe. That the 30S subunit unfolds in discrete steps in vitro has been well documented (Traub and Nomura, 1969; Held and Nomura, 1973; Blair et al., 1981). In addition it is believed that the ribosome undergoes conformational changes during translation (Noller,
1980). Some authors have suggested that rRNA undergoes coiling and uncoiling during translation and that this is a key element in rRNA function (Weidner, et al., 1977). Wickstrom (1983) has also proposed that the 30S subunit undergoes conformational change during initiation. This begins with a change in the conformation of the 3' end induced by either the binding of IF3, mRNA, or both. The results presented here seem to indicate that the 30S ribosomal subunit is capable of undergoing a discrete conformational change involving the region containing the 3' end of the 16S RNA. A more conservative interpretation of these results is that the more slowly sedimenting form has simply lost ribosomal proteins in the region of the 3' end of the RNA. However, there is no evidence that proteins are lost from the ribosome in this environment.

Experiments performed with cDNA probes complementary to the 5' end and internal regions of the 16S RNA indicate that there are other regions of RNA exposed on the subunit (Figure 18). Much of the binding seen in this profile is due to the unfolded or incomplete 30S particles evident from the broad, trailing peak. The lower body of the 30S subunit is believed to contain the 5' and possibly central domains of the 16S RNA. No proteins have been antigenically located in this region (Noller, 1980; Mochlova et al., 1982; Lake, 1980). The results here may support this contention, though experiments have yet to be performed to identify specific
internal sequences of RNA exposed on the ribosome. When end-labeled restriction fragments of cloned 16S rDNA are obtained, the DNA footprinting method of Patton and Chae (1982) may be quite useful in this regard. Buffer conditions may have to be readjusted to eliminate any unfolding of the ribosome in order to obtain a reproducible result with this method.

Internal RNA sequences lack free 3' or 5' ends and so the formation of an RNA·DNA helix of an extended length may be quite difficult. However, judging from the proposed model of helical structure, a hybrid of 10 bases would require only one complete turn (Watson and Crick, 1953). Moreover, the proposed models of 16S RNA secondary structure exhibit single-stranded regions of 20 bases or less.

Another experimental approach, possibly combined with the one described above, involves the chemical synthesis of short oligonucleotide fragments (10-15 bases) of specific sequence, and performing assays of the type already described for cDNA\textsubscript{32}. Information obtained through these methods will contribute significantly to our understanding of the topography of rRNA on the ribosome.
CHAPTER V

SUMMARY

cDNA molecules complementary to the 3' end and to general internal and 5' regions of the 16S RNA have been synthesized using reverse transcriptase and used as probes of the structure of the 30S ribosomal subunit. The cDNA complementary to the 3' RNA end has been shown to hybridize specifically to this region of RNA in the intact ribosome in a buffer containing 40 mM Tris-HCl (pH 7.4), 500 mM KCl, and 5 mM MgCl₂. This end of the 16S RNA is thus proposed to be single-stranded and exposed on the surface of the ribosome, along with as yet undefined regions of ribosomal RNA.
BIBLIOGRAPHY


