Studies on the interactions of low molecular weight ribosomal RNAs and ribosomal proteins

Janice Ann Kolberg
Iowa State University

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STUDIES ON THE INTERACTIONS OF LOW MOLECULAR WEIGHT RIBOSOMAL RNAs AND RIBOSOMAL PROTEINS

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Studies on the interactions of low molecular weight ribosomal RNAs and ribosomal proteins

by

Janice Ann Kolberg

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Iowa State University
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ABBREVIATIONS

*B. stearothermophilus*: *Bacillus stearothermophilus*

*E. coli*: *Escherichia coli*

**EDTA**: ethylenediaminetetraacetic acid

*H. cutirubrum*: *Halobacterium cutirubrum*

**MES**: 2-(N-morpholino)ethanesulfonate

**PEG**: polyethyleneglycol

**Poly (U)**: poly (uridylic acid)

**tRNA**: ribosomal RNA

**SDS**: sodium dodecyl sulfate

**tRNA**: transfer RNA

**TEMED**: N,N,N',N'-tetramethylethylenediamine

**Tris**: tris(hydroxymethyl)aminomethane

**Ψ**: pseudouridine

BUFFERS:

Buffer A: 0.1M sodium acetate, pH 5, 1% SDS

Buffer B: 0.1M sodium acetate, pH 5, 0.02M EDTA, 1% SDS

Binding Buffer: 0.005M potassium phosphate, pH 7.4 or 0.02M Tris-HCl, pH 7.4, 0.02M MgCl₂, 0.006M 2-mercaptoethanol or 0.001M dithiothreitol and 0.2M KCl to 0.45M KCl.

Dissociation buffer: 0.005M potassium phosphate, pH 7.4 or 0.02M Tris-HCl, pH 7.4, 0.005M EDTA and 0.006M 2-mercaptoethanol or 0.001M dithiothreitol.
HKB: 0.02M Tris-HCl, pH 7.4, 0.5M KCl, 0.005M MgCl₂

TM2: 10mM Tris-HCl, pH 7.4, 10mM magnesium acetate

TMA2: 10mM Tris-HCl, pH 7.4, 10mM MgCl₂, 60mM NH₄Cl, 6mM 2-mercaptoethanol

TMA4: 10mM Tris-HCl, pH 7.4, 0.1mM MgCl₂, 60mM NH₄Cl, 6mM 2-mercaptoethanol

TMN: 50mM Tris-acetate, pH 7.0, 50mM NH₄Cl, 12mM MgCl₂, 1mM dithiothreitol
INTRODUCTION

Structure of Ribosomes

Since the 1960s when it was realized that ribosomes are complex particles consisting of many individual proteins and several RNA molecules, researchers have tried to determine both the structure and function of the individual components. The early work concentrated on the study of \textit{E. coli} ribosomes and only recently has much work been directed at the study of eukaryotic ribosomes. The structure and possible function of many of the individual components of the \textit{E. coli} ribosome have been determined. While the structure of some components of eukaryotic ribosomes has been determined, there is very little information on the function of the individual components.

\textit{E. coli} ribosomes have a sedimentation value of about 70S and can be dissociated into two subunits with sedimentation values of 30S and 50S. The small subunit has one 16S RNA (1542 nucleotides) and 21 proteins (S1 to S21). The larger subunit has two RNAs; 5S (120 nucleotides) and 23S (2904 nucleotides) and 34 proteins (L1 to L34). All proteins are present in single copies except for protein L7/L12 which is present in four copies per 50S subunit (Noller and Woese, 1981). Protein L12 is identical to L7 except for acetylation of the N-terminal amino acid. Further, S20 is identical to L26 (Wittmann, 1982) and protein L8 is not a unique protein, but a complex of proteins L10 and L7/L12 (Pettersson et al., 1976). There are, therefore, 53 unique proteins in the \textit{E. coli} ribosome. The primary sequence of all 53 proteins (Wittmann, 1982) and all three ribosomal RNAs.
(Brownlee et al., 1968; Brosius et al., 1978; Brosius et al., 1980) has been determined.

Eukaryotic ribosomes have sedimentation values of near 80S and can be dissociated into two subunits with sedimentation values of 40S and 60S. The smaller subunit consists of one 18S RNA (0.7x10^6 daltons) and about 30 proteins, depending on the species (Bielka, 1982). In contrast to E. coli ribosomes, the large subunit consists of three RNAs; 5S RNA (121 nucleotides, 3.9x10^4 daltons), 5.8S RNA (158-162 nucleotides, 5.1x10^4 daltons) and 25S-28S RNA (1.3-1.7x10^6 daltons), and about 40 different proteins (Bielka, 1982).

The general shape of the 50S subunit and 30S subunit of E. coli ribosomes has been determined by electron microscopy and a consensus model has recently been presented (Prince et al., 1983). The shape of the 50S subunit resembles a "crown." From the main body, three protuberances extend. There is a central protuberance and a stalk on one side which contains L7/L12 with a shorter appendage on the other side of the central protuberance (Review Wittmann, 1983). The shape of the 30S subunit is also asymmetric. There is a "head" region comprising about one third and a "base" region comprising about two thirds of the particle. Between these two regions is a thin projection or "platform" tilted towards the subunit interface (Prince et al., 1983). Little information is available on the shape of eukaryotic ribosomes.

Sequences and Secondary Structural Models for Ribosomal RNAs

The primary sequence of high molecular weight ribosomal RNAs from several bacterial species, mitochondria of both plants and animals and
several eukaryotic organisms has been determined (Brimacombe et al., 1983). When the primary sequences of small subunit rRNA or large subunit rRNA from different organisms or size classes are compared, it is clear that a number of regions have been highly conserved. There is extensive homology between the 3' end of *E. coli* 16S rRNA and the 3' end of 18S rRNA from *S. cerevisiae* (Rubstov et al., 1980). Extensive homology also exists between all eukaryotic 18S rRNAs for which sequence data is available (Rubstov et al., 1980). However, all eukaryotic 18S rRNAs studied thus far lack the CUCC sequence which is found near the 3' end of *E. coli* 16S rRNA and is postulated to form a complex with mRNA (Shine and Dalgarno, 1974). There is also sequence homology between *E. coli* 23S rRNA and *Xenopus laevis* 28S rRNA (Walker, 1981). The homology is especially high between the 5' end of *Xenopus laevis* 28S rRNA and positions 158-275 (from 5' end) of *E. coli* 23S rRNA (Walker, 1981). Homologous regions have been identified at the 3' end of 18S RNA and the 5' end of *E. coli* 23 RNA suggesting a functional analogy between 18S rRNA of eukaryotic ribosomes and the 5' end of prokaryotic 23S rRNA (Nazar, 1980).

Models for the secondary structure of *E. coli* 16S and *E. coli* 23S RNA have been proposed. These models are based on different sets of experimental data, yet the overall features of the models are quite similar. In all models, there is a high percentage of base paired regions (review Brimacombe et al., 1983). The differences between the various models are mainly differences in the detailed base-pairing in
individual helical segments (Brimacombe et al., 1983). These models for the secondary structures of *E. coli* 16S and 23S rRNA have been used as the basis for proposing secondary structures for both mitochondrial rRNAs and eukaryotic rRNAs. To accommodate the smaller mitochondrial rRNAs, secondary loops are removed or whole domains are missing. For the larger eukaryotic rRNAs, extra helical regions are added. With these accommodations there seems to be a high degree of conservation of secondary structural features, although the primary sequences differ (Wittmann, 1982).

Numerous prokaryotic 5S RNAs, eukaryotic 5S RNAs and eukaryotic 5.8S RNAs have been sequenced (Erdmann, 1979). There are some sequence differences among various prokaryotic 5S RNAs, but certain regions show a very high degree of homology (Erdmann, 1976). A similar secondary structure has been proposed for all prokaryotic 5S RNAs (Fox and Woese, 1975). The general features of this model have been supported by a number of different experimental approaches (reviewed Wittmann, 1982). The essential features of this model involve four base-paired regions: 1) "molecular stalk," 1-10/110-120, 2) "weak tuned helix," 18-23/60-65, 3) "common arm base," 31-34/48-51 and 4) prokaryotic loop," 82-86/90-94 (Figure 1) (Wittmann, 1982).

Comparison of the sequences of several eukaryotic 5S RNAs shows them to be more highly conserved than the sequences of prokaryotic 5S RNAs (Bielka, 1982). A model for the secondary structure of eukaryotic 5S RNAs has been proposed (Garrett et al., 1981) and it is
Figure 1 (continued)
very similar to the secondary structure proposed for *E. coli* 5S RNA except for the presence of an extra helical region (Figure 1).

The nucleotide sequences of 5.8S RNAs from several mammalian sources are almost identical (Erdmann, 1979) and there is about 75% sequence homology between mammalian and yeast 5.8S RNA (Bielka, 1982). Both prokaryotic 5S RNA and eukaryotic 5S RNA contain about 120 nucleotides and no modified bases (Bielka, 1982). This is in contrast to 5.8S RNA which contains 158-162 nucleotides and several modified bases (Bielka, 1982). Several models for the secondary structure of eukaryotic 5.8S RNAs have been proposed. Two recent models include a model by Nazar et al. (1975) and a model proposed by Luoma and Marshall (1978) based on laser Raman spectroscopy (Figure 1). Both models include a high percentage of base paired regions and base pairing of the 3′ and 5′ ends similar to models for secondary structure of 5S RNAs. However, the primary sequence of wheat embryo 5.8S RNA does not fit either model (MacKay et al., 1980). It appears that none of the current models adequately describe the secondary structure for all eukaryotic 5.8S RNAs.

Computer comparison of the primary sequences of prokaryotic 5S, eukaryotic 5S and eukaryotic 5.8S RNAs led to the conclusion that the sequences of these RNAs are no more related than random sequences (Cedergren and Sankoff, 1976). Despite these sequence differences there still appears to be a conservation of secondary structural features in these low molecular weight rRNAs (Brimacombe et al., 1983).

There is a functional homology between the various prokaryotic 5S
RNAs. All prokaryotic 5S RNAs tested could be incorporated into functionally active Bacillus stearothermophilus 50S subunits (Erdmann et al., 1980). However, neither eukaryotic 5S RNAs or eukaryotic 5.8S RNA were able to be incorporated into active 50S subunits.

Structural Homologies of Ribosomal Proteins

Structural homologies between ribosomal proteins of different organisms have also been studied. Using purified antibodies to E. coli ribosomal proteins, immunological cross reactivity with the ribosomal proteins of other species have been tested. There is broad cross reactivity with ribosomal proteins from other Enterobacteriaceae and less cross reactivity with ribosomal proteins from other classes of bacteria (Brimacombe et al., 1978). Little cross reactivity was observed between E. coli ribosomal proteins and ribosomal proteins from yeast or rat liver (Wittman-Liebold, 1980).

Migration of proteins in two-dimensional electrophoresis gels has also been used to assess homology of ribosomal proteins from different organisms. Similarities in electrophoretic mobilities exist among ribosomal proteins from various bacterial species and among ribosomal proteins isolated from a number of vertebrates, but no detectable similarities exist between bacterial ribosomal proteins and eukaryotic ribosomal proteins (Wittmann-Liebold, 1980). There are also significant differences between electropherograms of yeast and mammalian ribosomal proteins. (Bollen et al., 1981). There are similarities in
the two-dimensional electropherograms of a number of different mammalian ribosomal proteins and this is the basis for a standard nomenclature of mammalian ribosomal proteins (McConkey et al., 1979). Despite the similarity in the two-dimensional gel patterns, there is little immunological cross reactivity between ribosomal proteins from different vertebrates (Bielka, 1982). For example, antisera specific for chicken or rat liver ribosomal proteins recognizes only about 20% of common determinants (Fischer et al., 1978).

Comparison of the amino acid sequences of ribosomal proteins is the most direct method for making conclusions about homology between proteins. Primary sequences have been determined for all 53 \emph{E. coli} ribosomal proteins (Wittmann, 1983) and these sequences have been used to search for homology with ribosomal proteins from other species (Wittmann-Liebold, 1980). Significant sequence homology exists between ribosomal proteins from different bacterial families (Wittmann-Liebold, 1980).

The sequences of two basic yeast ribosomal proteins L41 and L43 have been determined (Itoh et al., 1980). Comparison with the sequences of rat liver ribosomal proteins shows some sequence homology between yeast L43 and rat liver L37 and yeast L42 and rat liver L37a (Wittmann-Liebold, 1980). Some sequence homology also exists between rat liver L37 and residues 21-91 of \emph{E. coli} ribosomal protein L16 (Wool, 1980).

The sequences of a number of acidic ribosomal proteins have been
determined and there are similarities between the sequences of the acidic ribosomal proteins \textit{E. coli} proteins L7/L12, yeast proteins L44/L45 and rat liver proteins L40/L41 (Matheson et al., 1980b). There is also some immunological cross reactivity among these acidic proteins (Wool, 1979). A functional analogy also has been shown between yeast proteins L44/L45 and \textit{E. coli} proteins L7/L12 (Sanchez-Madrid et al., 1981). Many experiments have demonstrated the involvement of \textit{E. coli} proteins L7/L12 in EF-G dependent GTP hydrolysis (Matheson et al. 1980b).

Yeast ribosomes missing acidic ribosomal proteins L44/L45 can be reconstituted with \textit{E. coli} proteins L7/L12 and this reconstituted subunit is able to form an EF-2 GDP complex which exhibits some GTPase activity (Sanchez-Madrid et al., 1981).

Arrangement of Ribosomal Proteins and Ribosomal RNAs Within Ribosomes

For a full understanding of the molecular events that occur on the ribosome during protein biosynthesis, the arrangement and interaction of proteins and RNAs within the ribosome must be understood. Using a number of different approaches, there has been significant progress in the understanding of the \textit{E. coli} ribosome. One of the most fruitful approaches has been immune electron microscopy. Antibodies against individual ribosomal proteins have been prepared. The attachment site of these antibodies on the surface of the ribosome have been visualized by electron microscopy and the location of the proteins thus determined. The location of many 30S proteins and 50S proteins have been determined using this method (Wittmann, 1983). Two principal groups have used this method and the latest results reported
are in general agreement (Lake and Strycharz, 1981; Luhrmann et al., 1981; Prince et al., 1983). Proteins L5, L18 and L25 have been localized on the central protuberance (Wittmann, 1983) and protein L17 has been localized near the bottom of the 50S subunit (Lake and Strycharz, 1981). The location of 5S RNA within the ribosome has also been determined by a similar method (Stoffler-Meilicke et al. 1981). The 3' end of the RNA was oxidized with periodate and then a fluorescein derivative was covalently attached. Antibodies to the fluorescein derivative localized the 3' end of the 5S RNA within the central protuberance of the 50S subunit near proteins L5, L18 and L25 (Stoffler-Meilicke et al., 1981). In a similar manner, the 3' end of the 23S RNA has been located in the lower portion of the 50S subunit below the stalk which contains proteins L7/L12 (Stoffler-Meilicke et al., 1981).

Protein crosslinking studies have generally supported the results of the arrangement of proteins determined by immune electron microscopy (review Traut et al., 1980). Proteins have been crosslinked by a variety of bifunctional reagents, with the most recent work done using 2-iminothiolane. Of particular interest are crosslinks involving 5S RNA binding proteins L5, L18 and L25. Protein L5 has been identified in crosslinks with proteins L2, L3, L7/L12, L11, L17, L23, L25, and L31. Protein L18 has been crosslinked to L32 (Kenny and Traut, 1979).

Another method for identifying interactions between ribosomal proteins within the ribosome is the isolation of protein complexes.
During the isolation of individual proteins, a number of complexes between two or more proteins have been identified (Wystup et al., 1979). These complexes are stable in 6M urea and can be isolated by ion exchange chromatography. Ten such complexes have been isolated. Proteins found in these complexes have usually been found close together in the ribosome by other experimental approaches such as protein crosslinking or immune electron microscopy. A complex containing proteins L2 and L17 has been isolated. Protein L2 has also been found in a complex with L15 and L17 has been found in a complex with L15 and L27 (Wystup et al., 1979). These results seem to indicate that L2 and L17 are located near each other in the ribosome.

Immune electron microscopy has also been used to localize functional domains within the ribosome using antibodies against mRNA, protein factors or antibiotics which have specific binding sites on the ribosome. In a recent report, antibodies to puromycin were used to localize the peptidyltransferase center (Luhrmann et al., 1981). The peptidyltransferase center was located to one side of the central protuberance, opposite protein L7/L12. Proteins L1, L2, L23 and L27 have been localized in the same region indicating possible involvement in peptidyltransferase activity.

Affinity labeling has also been used extensively to determine functional sites within the ribosome (review Cooperman, 1980). Most of these studies have involved the use of photoaffinity labels attached to tRNA or antibiotics. Results of these experiments must be carefully analyzed to insure the labeling is specific, since proteins which are highly nucleophilic might be labeled in a nonspecific reac-
Yet valuable information about functional domains has been obtained using affinity analogues. N-bromoacetyl puromycin was used to label the peptidyltransferase center and proteins L1, L2, L23 and L27 were most heavily labeled (Lührmann et al., 1981). This result is consistent with the localization of these proteins by immune electron microscopy.

Interaction of Low Molecular Weight Ribosomal RNAs and Ribosomal Proteins

The specific interactions of ribosomal proteins with *E. coli* 5S RNA have been studied by a number of investigators using a variety of experimental approaches and a detailed understanding of these interactions is emerging. But detailed information on the interaction of low molecular weight ribosomals RNAs (5S and 5.8S) and ribosomal proteins from eukaryotic organisms is lacking.

An *E. coli* 5S RNA-protein complex can be prepared from 50S subunits by mild ribonuclease treatment and subsequent separation in sucrose gradients containing EDTA (Chen-Schmeisser and Garrett, 1977). The proteins present in this complex were identified as L5, L18 and L25 with small amounts of L1 also present. 5S RNA associated with several proteins can also be released from ribosomes by treatment with a high concentration of NH₄Cl (Gormly et al., 1971). For reattachment of this complex to reconstitute the 50S subunit, a protein fraction containing proteins L5, L18 and L25 is required (Yu and Wittmann, 1973). Similar RNA-protein complexes are formed on incuba-
tion of 5S RNA with total 50S ribosomal proteins in a buffer containing 0.32M KCl and 0.02 M MgCl₂ (Wrede and Erdmann, 1977). The complex is isolated from sucrose gradients and proteins in this complex have been identified as L5, L18 and L25 with lesser amounts of proteins L1, L10, L7/L12, L27 and L30 present. Using ribosomes which had not been exposed to high salt, an additional protein, L31', was identified in a complex with 5S RNA (Fanning and Traut, 1981a).

The interaction of *E. coli* 5S RNA with ribosomal proteins has also been studied in ribosome reconstitution experiments. Functional *E. coli* 50S subunits can be reconstituted from their constituent RNA and protein components using a two step incubation procedure first described by Dohme and Nierhaus (1976a). The first step involves incubation of 23S RNA, 5S RNA and total 50S ribosomal proteins at 4°C in a buffer containing 4 mM MgCl₂. This is followed by a second incubation at 50°C, in a buffer containing 20mM MgCl₂. *E. coli* 50S ribosomal subunits reconstituted by this procedure in the absence of 5S RNA have a reduced activity as measured by a number of different functional assays, and contain reduced amounts of proteins L5, L16, L18 and L25 (Dohme and Nierhaus, 1976b). A nucleoprotein complex can be isolated on sucrose gradients after incubation of 5S RNA and total 50S ribosomal proteins at 0°C in buffer containing 4 mM MgCl₂. A number of proteins are found in this complex with proteins L2, L5, L18 and L25 present in the greatest amounts and lesser quantities of proteins L7/L12, L13, L15, L16, L17, L19, L21 and L28 also present. However,
incubation of \textit{E. coli} 5S RNA and 50S ribosomal proteins at 44\(^\circ\) in 4 mM MgCl\(_2\) yields a nucleoprotein complex which contains only appreciable amounts of proteins L2, L5, L18 and L25. And when the 5S RNA and proteins are first incubated at 44\(^\circ\)C in 4 mM MgCl\(_2\) followed by incubation at 50\(^\circ\)C in buffer containing 20 mM MgCl\(_2\) a complex is isolated which contains only proteins L2, L18 and L25 (Dohme and Nierhaus, 1976b). The results of these experiments show that the proteins L2, L5, L18 and L25 interact strongly with \textit{E. coli} 5S RNA but that a number of other proteins bind weakly to 5S RNA in an interaction which depends on ionic conditions and temperature.

Experiments have been designed to determine the location of protein binding sites on \textit{E. coli} 5S RNA. Early experiments involved mild ribonuclease digestion of the \textit{E. coli} 5S RNA protein complex containing proteins L5, L18 and L25. The RNA fragments were isolated on polyacrylamide gels and the binding sites for proteins L18 and L25 included nucleotides 69-120, with possible involvement of nucleotides 1-11 (Gray et al., 1973). The binding sites for the individual proteins were determined by Zimmermann and Erdmann (1978). The primary binding site for L5 was located at positions 18-57, for L18 at 58-100 and for L25 at 101-116. Another group of investigators determined that the binding site for protein L25 includes nucleotides 69-87 and 90-110 (Douthwaite et al., 1979). And finally using RNA fragments and purified proteins L18 and L25 to form complexes, the binding site for L18 was determined to involve the loop region around nucleotide 40 and
the binding site for L25 included nucleotides 79-97 (Speek and Lind, 1982). The results of these experiments are not in total agreement and the discrepancies might be due to differences in the methods of RNA or protein preparation or to differences in ionic conditions (Wittmann, 1982). But the results of the experiments all indicate that the binding site for protein L25 is located near the 3' end of the *E. coli* 5S RNA molecule.

Experiments are being done to determine the sites on the proteins which interact with the RNA. A recent study looked at the effect of iodination of L18 on its ability to bind to 5S RNA (Fanning and Traut, 1981b). A four fold molar excess of iodine virtually abolishes the binding capacity of L18 for 5S RNA indicating that the protein binding site probably involves tyrosine residues.

Some chemical and physical effects of the interaction of proteins L18, L25 and L5 with *E. coli* 5S RNA have been determined. The binding constants for individual proteins varied from $2.3 \times 10^6$ M$^{-1}$ to $2.3 \times 10^8$ M$^{-1}$ (Spierer et al., 1978). The binding constant was highest for protein L18 ($2.3 \times 10^8$ M$^{-1}$) and lowest for L5 ($2.3 \times 10^6$ M$^{-1}$) with that for L25 being intermediate ($1.5 \times 10^7$ M$^{-1}$). The affinity of L5 for 5S RNA is increased by the presence of L18 in the complex. Optimal binding of all proteins occurred at pH 7.5 to 9 and at a Mg$^{2+}$ concentration of 10-20 mM. The optimal K$^+$ concentration varied from one protein to another and was 0.30-0.40M for L5, 0.10-0.20M for L18 and 0.20-0.30M for L25 (Spierer and Zimmermann, 1978). The effect of
protein binding on the structure of 5S RNA was studied using circular dichroism. Binding of L5 produces no detectable change in the secondary structure of the 5S RNA molecule (Spierer et al., 1978). In contrast, L18 causes a shift in the configuration of one of the double stranded regions of the RNA, possibly in the region 82 to 94 (Spierer et al., 1978a). Results of other circular dichroism experiments show that L25 may cause a small change in the secondary structure of 5S RNA (Bear et al., 1977), although this was not observed in the studies by Spierer et al. (1978a). The results of these experiments show that L18 has a strong interaction with E. coli 5S RNA, which has a profound effect on the secondary structure of the RNA.

A complex of E. coli 5S RNA and B. stearothermophilus ribosomal proteins L5 and L22 has been characterized (Zimmermann and Erdmann, 1978). The regions of the RNA protected from ribonuclease digestion due to the presence of B. stearothermophilus ribosomal proteins L5 and L22 were also protected by E. coli ribosomal proteins L5, L18 and L25, suggesting that the B. stearothermophilus proteins bind to sites on the RNA which are similar to binding sites for the E. coli proteins. A complex can also be reconstituted from B. stearothermophilus 5S RNA and E. coli proteins L18 and L25 (Horne and Erdmann, 1972) again indicating a structural homology between the 5S RNA and the 5S RNA binding proteins of these prokaryotic ribosomes.

Other complexes containing 5S RNA and ribosomal proteins have been isolated. A 5S RNA-protein complex is released from the ribosomes of
the extreme halophile *Halobacterium cutirubrum* when the 50S ribosomal subunit is extracted with buffer containing low concentrations of K⁺ and Mg²⁺ (Smith et al., 1978). The complex contains 5S RNA and two proteins designated HL13 and HL19. These proteins have been purified and preliminary sequence data indicates some sequence homology between protein HL13 and *E. coli* protein L18 and HL19 and *E. coli* protein L5 (Willick et al., 1979). Physical measurements also support the homology between HL13 and L18. The binding constant for HL13 binding to *H. cutirubrum* 5S RNA is $10^8$ M⁻¹, similar to the binding constant for L18 to *E. coli* 5S RNA. Circular dichroism measurements indicate that the binding of HL13 results in significant changes in the secondary structure of its 5S RNA, an effect similar to that induced by L18 binding to *E. coli* 5S RNA (Nazar et al., 1979a). The binding of protein HL19 to *H. cutirubrum* 5S RNA has no effect on the secondary structure, as determined by circular dichroism (Nazar et al., 1979a). When the *H. cutirubrum* 5S RNA protein complex was digested with pancreatic ribonuclease, the protected regions were located mainly near the 3'-end of the RNA molecule (Matheson et al., 1980a). A similar region of the *E. coli* 5S RNA molecule was protected when the *E. coli* 5S RNA-protein complex containing proteins L18 and L25 was digested with pancreatic ribonuclease (Gray et al., 1973). A structural homology between the 5S RNA-protein complexes isolated from *H. cutirubrum* and *E. coli* is suggested by these results.

Nucleoprotein complexes containing 5S RNA have been isolated from eukaryotic organisms including rat liver, rabbit reticulocyte, and yeast ribosomes. When 60S ribosomal subunits from rat livers or rabbit reti-
culocytes are treated with EDTA, a nucleoprotein is released which contains one protein and 5S RNA (Blobel, 1971). The protein in the 5S RNA-protein complex from rat liver ribosomes has been identified as L5 (Terao et al., 1975; 1980). Identification of L5 as part of a native RNA-protein complex is complicated by the observation that EDTA treatment can cause disruption of normal binding sites (Newton et al., 1975). However, irradiation of ribosomal subunits with ultraviolet light prior to isolation of the 5S RNA complex, resulted in the crosslinking of protein L5 to 5S RNA indicating that protein L5 is closely associated with 5S RNA in the native rat liver ribosome (Terao et al., 1980).

A 5S RNA-protein complex can also be isolated from *Saccharomyces cerevisiae* by treatment of 60S subunits with EDTA (Nazar, 1979). This complex contains one protein, identified at YL3, which is one of four acidic proteins found in the yeast 60S ribosomal subunit. This protein has not been identified using the standard nomenclature for yeast proteins (Bollen et al., 1981). Partial sequence data indicate sequence homology between the N-terminus of YL3 and two prokaryotic 5S RNA binding proteins L18 (*E. coli*) and HL19 (*H. cutirubrum*) (Nazar et al., 1979). There is also some sequence homology between *E. coli* ribosomal protein L5 and a region in the C-terminal portion of YL3 (Matheson et al., 1980a). The protein binding site on the yeast 5S RNA was found to be at the 3'-end, similar to the location of the protein binding sites found in the nucleoprotein complexes of *E. coli* 5S RNA and *H. cutirubrum* 5S RNA (Gray et al., 1973; Matheson et al., 1980a). There
appears to be a structural homology between the prokaryotic 5S RNA-protein complexes and the 5S RNA-protein complex from the eukaryote Saccharomyces cerevisiae.

While treatment of yeast 60S ribosomes with EDTA (1mg/mL) released a 5S RNA-protein complex, 5.8S RNA was not released (Nazar, 1978). The 5.8S RNA together with 5S RNA could be released from these ribosomes by brief heat treatment at 60°C or incubation in 50% formamide (Nazar, 1978). Ribosomal proteins were not released by the brief heat treatment, indicating that the interactions that occur between ribosomal proteins and 5.8S RNA within the yeast ribosome are quite different from those of 5S RNA.

Synthesis of Ribosomal RNAs

There are similarities in the synthesis of low molecular weight ribosomal RNAs from prokaryotic and eukaryotic organisms. E. coli 5S RNA is synthesized as part of a large 30S ribosomal RNA precursor molecule which contains the sequences for all three ribosomal RNAs. The order of sequences within the 30S RNA molecule is 5'-16S-spacer-23S-spacer-5S-3' (Abelson, 1979). The molecule is processed in a number of enzymatic events to yield the mature ribosomal RNAs, 5S RNA, 16S RNA and 23S RNA.

In eukaryotic organisms, there is also a single high molecular weight ribosomal RNA precursor which contains sequences for 17-18S RNA, 5.8S RNA and 25-28S RNA, but there is a separate transcription unit for 5S RNA. The genes for the rRNAs in S. cerevisiae have the following sequence [5S-spacer-[spacer 18S-5.8S-25S-spacer]-spacer-5S] (Nath and Bollen, 1977; Bell et al. 1977). In yeast, the primary transcript is a
37S RNA molecule which is processed to yield mature 18S, 25S and 5.8S RNAs (Nikolaev et al., 1979). Yeast 5S RNA is a separate primary transcription product, transcribed by RNA polymerase III (Trapman and Planta, 1975).

One argument for an analogy between prokaryotic 5S RNA and eukaryotic 5.8S RNA comes from the similarities in the synthesis of these two RNA species. Both are synthesized as part of a large ribosomal RNA precursor molecules. But the location of the RNAs within the RNA precursor molecules differ. The prokaryotic 5S RNA occurs at the 3' end of the precursor RNA molecule, while eukaryotic 5.8S RNA is located between the sequences for the large ribosomal RNAs. The synthesis of eukaryotic 5S RNA is quite different from the synthesis of eukaryotic 5.8S RNA and prokaryotic 5S RNA since it is a primary transcription product.

Functions of *E. coli* 5S RNA

An understanding of the function of *E. coli* 5S RNA is important in determining the functional relationships among the low molecular weight ribosomal RNAs of eukaryotes and prokaryotes. *E. coli* 5S RNA is essential for ribosome activity. This is shown most clearly in experiments in which *E. coli* 50S subunits were reconstituted in the absence of 5S RNA. These ribosomes have reduced activity in a number of different functional assays (Dohme and Nierhaus, 1976b). Activity of the peptidyl transferase center is greatly reduced and no significant factor-dependent tRNA binding to the A-site was observed.

Several different functions have been proposed for *E. coli* 5S RNA. An early proposal suggested that during protein synthesis the growing
polypeptide chain becomes attached to the 3'-end of the 5S RNA and is subsequently transferred to aminoacyl-tRNA (Raacke, 1971). Experiments in which E. coli 5S RNA modified at the 3'-end was used to reconstitute 50S ribosomes showed that the ribosomes retained full activity, proving that an intact 3'-end is not required for the biological function of E. coli 5S RNA (Fahnestock and Nomura, 1972).

Another proposed function of E. coli 5S RNA is binding of tRNA to the A-site of the ribosome (Sprinzl et al., 1976). The oligonucleotide TUCG, which is common to all tRNAs, inhibits enzymatic binding of aminoacyl-tRNA to the A-site of E. coli ribosomes. The oligonucleotide may inhibit tRNA binding by interaction with the complementary sequence CGAA found in E. coli 5S RNA (Erdmann, 1976). A similar sequence is present in eukaryotic 5.8S RNAs but is absent in eukaryotic 5S RNAs.

These observations led to the proposal of functional analogy between eukaryotic 5.8S RNAs and prokaryotic 5S RNAs (Erdmann, 1976). Eukaryotic 5S RNAs contain the conserved sequence (Pyrimidine) GAU, which is complementary to the AUCCG sequence found in eukaryotic initiator tRNAs, and Erdmann (1976) thus proposed that eukaryotic 5S RNA is involved in the binding of initiator tRNA to the ribosome. These conclusions were, however, contradicted by the recent report (Pace et al., 1982) that E. coli 5S RNA with the CGAA sequence enzymatically removed is as active as intact 5S RNA in restoring the activity of reconstituted ribosomes. The results of these experiments showed that the phylogenetically conserved sequence CGAA, is not essential for peptide bond formation and elongation directed by poly (U). However, such a system does not assay
for proper initiation and termination and this conserved region may be necessary for functions with natural mRNAs.

Affinity Chromatography Methods

A number of different investigators have used immobilized RNA columns to study the interaction of ribosomal proteins and ribosomal RNAs. A procedure for immobilizing RNA to a Sepharose matrix was developed by Burrell and Horowitz (1975). In this procedure, the RNA is attached to the Sepharose matrix through its 3'-end. *E. coli* 5S RNA immobilized in this manner was used to study the binding of *E. coli* 50S proteins (Burrell and Horowitz, 1977). Only a small number of *E. coli* 50S ribosomal proteins were bound to the immobilized RNA and these proteins were identified as L5, L18, and L25. These same proteins have been shown to interact with *E. coli* 5S RNA by a number of experimental approaches, suggesting that the interaction which occurs between proteins and the immobilized *E. coli* RNA is similar to the interaction which occurs in the intact ribosome. Therefore, such an experimental approach may be useful in the investigation of rRNA-protein interactions which occur in other ribosomes.

In this current study, the interactions between low molecular weight ribosomal RNAs isolated from *E. coli* and *S. cerevisiae* and ribosomal proteins isolated from *E. coli*, *S. cerevisiae*, and rat liver were studied using chromatography on immobilized RNAs. One purpose of these experiments was to identify which low molecular weight eukaryotic rRNA, 5S or 5.8S RNA, is the functional analog of *E. coli* 5S RNA. If either eukaryotic rRNA bound the same set of proteins that were bound by *E. coli* 5S RNA, a functional analogy might be indicated. Functional homol-
ologies between ribosomal proteins also might be identified in such experiments. Ribosomal proteins from different organisms which are bound to the same rRNA might be functionally analogous.
MATERIALS AND METHODS

Materials

Cyanogen bromide (CNBr)-activated Sepharose 4B, Sephadex G10, G50, and G100 were purchased from Pharmacia, Inc. DEAE cellulose (DE-32) was purchased from Whatman Inc. Three quarter log phase cells of *E. coli* B, grown in enriched medium, were purchased from Grain Processing Co. (Muscatine, Iowa). Poly (U) (Type II, potassium salt, MW 100,000) and bovine serum albumin were from Sigma Chemical Co. Uridylate oligonucleotide, (Up)sU, was from Miles Chemical Company. Enzymes, deoxyribonuclease I (RNase free, bovine pancreatic) and bacterial alkaline phosphatase (BAPC), were from Worthington Biochemical Corp. Coomassie Blue R250 was a product of Canalco, Inc. Coomassie Blue G250 was from J.T. Baker Company, and Pyronin Y was from Polyscience Inc.

For experiments in which urea solutions were required, reagent grade urea was used and 8M urea solutions were deionized using Amberlite MB3 from Mallinkrodt Chemical Works. Density grade sucrose (RNase free) used in all gradients, and ultra pure urea, used in glycerol derivatization experiments, were from Schwarz/Mann. Reagent grade phenol from Fisher Scientific Co. was redistilled prior to use and stored in the dark at 4°C. PEG 6000 (Carbowax) was also a product of Fisher Scientific Co. MES buffer (2-(N-morpholino)ethane-sulfonate) was from P-L Biochemicals and 1-Ethyl-3-(3-(dimethyl-
amine)propyl carbodiimide hydrochloride was the product of Pierce Chemical Co.

Reagent grade acrylamide and N,N'-methylene-bis-acrylamide (Fisher) were recrystallized from chloroform and acetone, respectively (Loening, 1967). N,N,N',N'-tetramethylethylenediamine (TEMED) was an Eastman Kodak product. Gel tubes were silanized with 1% (v/v) Column Coat from Miles Laboratories.

For isolation of yeast rRNAs, Red Star or Budweiser's baker's yeast was used. Yeast extract, peptone and yeast nitrogen base (without amino acids) used for growth of yeast cells were from Difco Laboratories.

Dialysis membranes were from Fisher Scientific Co. For work with eukaryotic proteins, spectra/Por 3 membrane (MWCO 3500) was used to reduce loss of low molecular weight proteins. All dialysis membranes were boiled in 0.0054M Na₂EDTA, 0.014M NaHCO₃ and 0.007M 2-mercaptoethanol and then washed several times with deionized water. Dialysis membranes were stored in deionized water at 4°C until used.

Methods

Isolation of E. coli ribosomal subunits

Ribosomes were isolated from 3/4 log phase cells of E. coli B. Cells were suspended in TMA2 (0.01M Tris-HCl, pH 7.4, 0.01M MgCl₂, 0.06M NH₄Cl, 0.006M 2-mercaptoethanol) containing 3μg/mL of deoxyribonuclease I (RNase free) and disrupted by passage through a French pressure cell at 12,000-18,000 p.s.i. The extract was clarified by
centrifugation at 30,000 g for 30 minutes and the resulting supernatant was centrifuged for 12 hours at 4°C in a Ti 45 rotor at 35,000 rpm (100,000 g) in a Beckman L8 ultracentrifuge. The crude ribosomal pellets were resuspended in TMA2 containing 0.5M NH₄Cl. Forty mL of this suspension was carefully layered over 30mL of 1M sucrose prepared in the same 0.5M NH₄Cl containing buffer. The ribosomal preparation was again centrifuged at 4°C for 12 hours in a Ti 45 rotor at 35,000 rpm. The clear ribosomal pellet was suspended in TMA2 and stored at -20°C.

Ribosomes were dissociated into subunits by dialysis against ribosome dissociation buffer (0.01M potassium phosphate, pH 7.4, 0.01M 2-mercaptoethanol, 0.0001 M MgCl₂) for 14-18 hours. The subunits were separated, by zonal centrifugation in a Ti 15 zonal rotor, by the method of Eikenberry et al. (1970), using a hyperbolic gradient of 7.4% to 38% sucrose in TMA4 (0.01M Tris-HCl, pH 7.4, 0.0001 MgCl₂, 0.06M NH₄Cl, 0.006M 2-mercaptoethanol). The sample was introduced into the center of the rotor in an inverse gradient formed by mixing equal volumes, of 7.4% sucrose solutions and the ribosome suspension. After loading the sample into the rotor, 700mL of TMA4 buffer was introduced in the center of the rotor as an overlay. While loading the rotor the speed was 2,000 rpm and this was increased to 31,000 rpm after the sample had been introduced. Centrifugation was continued for 10 hours at 5°C. Unloading of the rotor was accomplished by displacement of the gradient from the outer edge of the rotor using a 60% sucrose solution. Twenty mL fractions were collected and the fractions
containing 30S and 50S subunits were pooled. The Mg\(^{2+}\) concentration was raised to 0.01M and the subunits were precipitated by addition of 100mg/mL of PEG 6000 (Dohme and Nierhaus, 1976a). After stirring for 45 minutes, at 4-8°C, the suspension was centrifuged for 30 minutes at 30,000 g. Precipitated subunits were resuspended in TMA2 and stored at -20°C until used for preparation of protein samples.

The purity of subunits was examined on linear 5-20% sucrose gradients prepared in TMA4. Centrifugation was for 90 minutes at 4°C in a SW 50.1 rotor at 50,000 rpm (240,000 g) in a Beckman L8 ultracentrifuge. Less than 5% cross-contamination was detected in either subunit preparation.

**Growth of *S. cerevisiae A364A***

*Saccharomyces cerevisiae*, strain A364A, was obtained from Jonathan R. Warner (Albert Einstein College of Medicine). Cells stored on agar slants were inoculated into YPD medium (1% yeast extract, 2% peptone, 2% glucose) and grown at 37°C for several days, with daily transfers into fresh medium. Cells were then transferred to synthetic medium (0.67% yeast nitrogen, without amino acids, 1% succinic acid, 0.002% adenine, 0.005% uracil, 0.005% histidine, 0.005% tyrosine, 2% glucose) (Warner and Gorenstein, 1978). For large scale growth, 50mL of the cell suspension (Klett = 300) were inoculated into 500mL of synthetic medium and growth of the cells was monitored using a Klett-Summerson colorimeter with a red filter (#66). Cells were collected by centrifugation when a Klett reading of 100 was obtained.
and then were washed twice with deionized water. The cell paste was stored at -20°C. Typically, it took 10-12 hours to reach a Klett reading of 100, which corresponded to $3 \times 10^7$ cells/mL. The yield of cells from one 500mL inoculation was 2.0g.

**Isolation of yeast ribosomal subunits**

Yeast ribosomes were prepared using a modification of the procedure described by Warner and Gorenstein (1978). Several methods for disruption of yeast cells were tried and disrupting the cells with glass beads in a Sorvall Omni Mixer was found to be the most efficient. Cells were suspended in TMN (0.05M Tris-acetate, pH 7.4, 0.05M NH₄Cl, 0.012M MgCl₂, 0.001M dithiothreitol) using a volume equal to 2-4 times the weight of cells. Glass beads (0.45mm) equivalent to 6 times the weight of cells were added to the cell suspension and the cells were disrupted by stirring for two minutes in the Omni Mixer at 15,000 rpm, while keeping the sample on ice. The stirring was repeated 4 times. To remove glass beads and cell debris, the mixture was centrifuged at 20,000 g for 15 minutes and the supernatant was further clarified by centrifugation at 30,000 g for another 15 minutes. Centrifugation was repeated until the supernatant was completely clear. Forty mL of the supernatant were layered over 30mL of a 10% sucrose solution prepared in HKB (0.02M Tris-HCl, pH 7.4, 0.5M KCl, 0.005M MgCl₂) and centrifuged at 40°C in a Beckman L8 ultracentrifuge for 16 hours in a Ti 45 rotor at 35,000 rpm. The ribosomal pellet from the centrifugation was suspended in HKB buffer, using a tissue homogenizer, gently stirred for
30-45 minutes in the cold, and clarified by centrifugation at 20,000 g for 15 minutes.

Ribosomes prepared by this procedure were rapidly degraded when stored at -20°C. Therefore, they were used immediately for the isolation of ribosomal subunits, using a modification of the method of Sherton et al. (1974). The ribosome suspension was incubated with 1mM puromycin in HKB buffer for 15 minutes at 37°C, then separated into subunits in a Ti 15 zonal rotor using a 7.4% to 38% sucrose gradient prepared in HKB buffer. After the sample was loaded into the rotor, centrifugation was continued at room temperature for 17 hours at 13,500 rpm. The gradient was displaced by a 60% sucrose solution and 20mL fractions were collected. Fractions containing 40S and 60S subunits were pooled, the Mg\(^{2+}\) concentration was raised to 0.01M and 0.7 volume of 95% ethanol was added to precipitate the subunits at -20°C. After 12 hours, the precipitated subunits were collected by centrifugation and then resuspended in TMN buffer with gentle stirring at 4-8°C for 30-45 minutes. The suspension was clarified by centrifugation at 15,000 g. There were some problems with the recovery of 40S subunits, a typical recovery was 60%. Recovery of the 60S subunits was greater than 80%. A number of different procedures were tried to increase recovery, including suspension of the subunits in low Mg\(^{2+}\) buffer and suspension in a urea containing buffer. Neither of these methods improved recovery. The method described gave the best results.
Purity of the subunits was examined on linear 10% to 30% sucrose gradients prepared in HKB buffer; no significant cross contamination of either subunit was found. Contamination was also determined by examination of the two-dimensional gel electrophoresis patterns of the ribosomal proteins prepared from the subunits. Again no significant contamination of either subunit preparation was observed.

Isolation of rat liver ribosomal subunits

Ribosomes were isolated from the livers of female Sprague-Dawley rats, using the method of McConkey (1974). The livers were homogenized with a motor driven pestle in 0.01M Tris-HCl, pH 7.4, 0.01M KCl, 0.001M MgCl₂, 0.001M dithiothreitol, using 3-5mL of buffer per gram of tissue. One tenth volume of 3M KCl in 0.02M MgCl₂ was slowly added to disrupt cytoplasmic aggregates and the suspension was centrifuged at 15,000 g for 15 minutes. Brij 58 and sodium deoxycholate were added to the supernatant to a final concentration of 0.5%. Ribosomes were then pelleted through a sucrose pad containing 1.75M sucrose in 0.05M Tris-HCl, pH 7.4, 0.1M KCl, 0.001M MgCl₂, 0.001M dithiothreitol, by centrifugation in a Beckman L8 ultracentrifuge at 45,000 rpm for 16 hours in a Ti 45 rotor at 4°C. The ribosomal pellet was resuspended in the same buffer, lacking sucrose, by means of a tissue homogenizer and the resulting suspension was stirred in the cold for 45 minutes before being clarified by centrifugation for 30 minutes at 20,000 g.

Ribosomal subunits were prepared by the same procedure used to obtain yeast ribosomal subunits, except that 1mM dithiothreitol was included in all buffers. Fractions containing 40S and 60S subunits
were pooled, and after the Mg\textsuperscript{2+} concentration was raised to 0.01M, subunits were precipitated by the addition of 1 volume of cold 95% ethanol. The precipitated subunits were collected by centrifugation and suspended in 0.05M Tris-HCl, pH 7.4, 0.1M KCl, 0.001M MgCl\textsubscript{2}, 0.001M dithiothreitol using a tissue homogenizer. The suspension was then treated as already described for yeast subunits. Purity of subunits was examined on sucrose gradients. The 60S subunits seemed to be contaminated with 40S subunits. These were reincubated with 1mM puromycin and purified by centrifugation in a VTi 50 rotor at 26°C for 40 minutes into a linear 10% to 30% sucrose gradient prepared in 0.02M Tris-HCl, pH 7.4, 0.003M MgCl\textsubscript{2}, 0.5M KCl, 0.001M dithiothreitol. The 60S subunits were pooled and precipitated with ethanol as already described.

**Preparation of ribosomal proteins**

Proteins were extracted from ribosomal subunits by the acetic acid procedure described by Hardy et al. (1969). The Mg\textsuperscript{2+} concentration of the ribosome suspension was raised to 0.1M, two volumes of cold glacial acetic acid were added and the solution was stirred for 45 minutes at 4-8°C. Precipitated RNA was removed by centrifugation. The supernatant, containing the ribosomal proteins, was dialyzed against 1% acetic acid for 15-24 hours and the preparation was then lyophilized. Prior to use in chromatography experiments, lyophilized *E. coli* proteins were dissolved in 4M or 8M urea at a concentration of 5mg/mL. Eukaryotic ribosomal proteins were prepared in a similar manner except that Spectra/Por 3 membranes were used for dialysis. This
prevented the loss of low molecular weight ribosomal proteins. The
eukaryotic proteins were dissolved in 8M urea containing 1mM
dithiothreitol, since eukaryotic proteins are known to be susceptible
to aggregation as a result of disulfide bond formation (Warner and
Gorenstein, 1978). Protein concentration was determined by the dye
binding assay of Bradford (1976), using bovine serum albumin as the
standard.

Electrophoretic separation of ribosomal proteins

Disc gel electrophoresis (one dimension) of ribosomal proteins
was performed at pH 4.5 in 10% (w/v) acrylamide containing 0.15% (w/v)
methylene-bis-acrylamide, according to the procedure of Leboy et al.
(1964). To remove artifacts of sulfhydryl oxidation, samples were
treated with 0.006M 2-mercaptoethanol in pH 8.1 Tris-HCl buffer at 0°C
for 3-5 hours just prior to electrophoresis (Hardy et al., 1969).
Early experiments were carried out with cylindrical gels, 0.5cm x 9.5cm
or 16cm, using a 1cm stacking gel which contained 2.5% acrylamide.
Electrophoresis was run at 4°C at 2.5mA/gel until the tracking dye,
Pyronin Y, had just run off the gel. In later experiments, electro­
phoresis was performed on 11cm x 20cm gel slabs in the apparatus
described by Reid and Bieleski (1968). The gel was 0.15cm thick and
had sample wells to accommodate up to 12 samples. For gel slabs, a
2cm stacking gel was used with a 9cm separation gel.

Generally, gels were stained with 0.25% Coomassie Blue R250 in
7.5% acetic acid: 50% methanol for 3-12 hours and destained in 7.5%
acetic acid: 50% methanol. The destaining solution was changed
several times and complete destaining took about 24 hours (Howard and Traut, 1973). In some later experiments, the gels were stained in a solution of 0.08% (w/v) Coomassie Blue G250 prepared using 0.8N sulfuric acid, 0.8N KOH and 12% trichloracetic acid. Using this stain, protein spots were visible in 3-5 hours and no destaining was required (Blakesley and Boezi, 1977).

Early experiments involving the two-dimensional electrophoresis of ribosomal proteins were performed in the apparatus described by Kaltschmidt and Wittmann (1970a). The first dimension (0.5 x 17cm) was run on 4% acrylamide gels containing 8M urea in the electrode buffer system described by Howard and Traut (1973), which contained 0.006M disodium EDTA, 0.77M boric acid and 0.06M Tris at pH 8.2. The protein sample was polymerized in the center of the gel and electrophoresis was carried out at 2.7mA/gel for 20 hours at 40°C. Gels were then dialyzed for one hour against 1 L of 8M urea containing 0.013M acetic acid and 0.012M KOH and then polymerized to the top of the gel slab for electrophoresis in the second dimension. The gel for the second dimension (20 x 20 x 0.5cm) was polymerized with 18% acrylamide. The buffer for the second dimension contained 0.186M glycine and 0.026M acetic acid. Gels were run for 22-24 hours (room temperature) at 105 volts.

For later experiments, a smaller apparatus was designed, which allowed smaller amounts of protein to be analyzed. The length of gels in the first dimension was reduced to 9.5cm while the size of the second dimension gel were reduced to 10 x 10 x 0.2cm. The size of both the upper and lower buffer reservoirs was the same as in the
apparatus described by Kaltschmidt and Wittmann (1970a). The gel solutions and buffers used were the same as those described earlier. Electrophoresis in the first dimension (4°C) was performed at 2.5mA/gel for 30 minutes; the current was then increased to 5mA/gel for another 6-7 hours. In the second dimension, electrophoresis (room temperature) was at 80 volts for 16 hours with *E. coli* proteins or 12 hours with yeast proteins. Gels were stained in manner previously described for one-dimensional gels.

*E. coli* ribosomal proteins were identified by reference to the standard map described by Kaltschmidt and Wittmann (1970b) and yeast ribosomal proteins were identified as described by Bollen et al. (1981).

For analysis of rat liver proteins, the method of Lastick and McConkey (1976) was used; this is a modification of the Kaltschmidt-Wittmann procedure. The buffers used in the separations were similar to those used in the original procedure. The separation in the first dimension again was in 4% acrylamide 6M urea gels using electrode buffer (0.06M Tris, 0.78M boric acid and 0.003M EDTA). The sample was not polymerized in the center of the gel, but layered on the top of the gel in a buffer containing 5% 2-mercaptoethanol. After electrophoresis at 65 volts for 22 hours at 4-8°C, the first dimension gels were soaked for less than 5 minutes in a solution containing 6M urea, a high concentration of acetic acid (0.35M) and 5% 2-mercaptoethanol. The gel was then sealed to the top of the acrylamide slab with agarose. Separation in the second dimension was done using 15% acrylamide gels in a 0.093M glycine and 0.13M acetic acid
buffer (pH 4.05). Electrophoresis in the second dimension was at room temperature for 7-8 hours at a constant voltage of 100 volts. The gels were again stained with Coomassie Blue stain as described earlier. Proteins were identified according to the proposed uniform nomenclature for mammalian ribosomal proteins (McConkey et al., 1979).

**Isolation of yeast 5S and yeast 5.8S RNA**

Commercial baker’s yeast (*Saccharomyces cerevisiae*) was the source of purified rRNAs used in the preparation of RNA-Sepharose columns. A sequential 3 step extraction procedure based on the method of Rubin (1975) was used. Yeast cells were suspended in buffer A (0.1M sodium acetate, pH 5, 1% SDS), typically 1 pound of yeast was suspended in one liter of buffer and a volume of 72% phenol (v/v) equal to one half the volume of the cell suspension was added. This mixture was shaken vigorously at room temperature for 1 hour and the phases were separated by centrifugation. The upper aqueous layer, which contains mostly tRNA, and the phenol layer were discarded. The cell pellet was then resuspended in a volume of buffer B (0.1M sodium acetate, pH 5, 0.02M EDTA, 1% SDS) equal to the volume of buffer used in the first extraction, and one half volume 72% phenol was added. This suspension was again shaken for 1 hour at room temperature. After centrifugation to separate the phases, the aqueous layer containing primarily 5S rRNA was saved and the phenol layer was discarded. The pellet was resuspended in buffer B as before, 72% phenol was added, and the mixture shaken at 42°C for 1 hour. The aqueous layer, containing 5.8S rRNA was separated as before and the phenol layer and
residual pellet were discarded. RNA was precipitated from each aqueous layer by the addition of two volumes of 95% ethanol, after increasing the salt concentration by the addition of 1/10 volume of 20% potassium acetate, pH 5. The precipitated RNA was collected by centrifugation and again treated with 72% phenol as described. Treatment with phenol was repeated until no denatured protein was present at the interface between the aqueous and phenol layers. 5S and 5.8S rRNA were further purified by chromatography on Sephadex G100 or Sephacryl S200 equilibrated with 0.14M sodium acetate, 1M NaCl, pH 4.5. Purity of each RNA sample was determined by polyacrylamide gel electrophoresis as described later.

Isolation of E. coli 5S RNA

E. coli 5S RNA was extracted from either isolated ribosomes or whole cells. Enough bentonite and sodium dodecyl sulfate were added to the sample to give a final concentration of 1 mg/mL and 0.5%, respectively (Hills and Horowitz, 1966). An equal volume of 90% phenol was added and the mixture was shaken at room temperature for 1 hour. The layers were separated by centrifugation and the aqueous layer was again treated with phenol until no denatured protein was present at the interface between the two layers. After addition of 1/10 volume of 20% potassium acetate, pH 5, RNA was precipitated from solution by addition of 2 volumes of 95% ethanol. Precipitated RNA was collected by centrifugation, dissolved in 0.02M potassium phosphate, pH 7.7, and applied to a DEAE-cellulose (DE-32) column which had been equilibrated with the same buffer. This chromatographic
procedure was used to remove high molecular weight RNAs from the sample (Monier and Feunteun, 1971). The column was washed with 0.02M potassium phosphate, pH 7.7, until the A_{260} of the effluent was negligible and then with 0.02M potassium phosphate, pH 7.7, containing 0.35M NaCl. The 5S RNA was eluted with 0.02M potassium phosphate, pH 7.7, buffer containing 1M NaCl and further purified on either Sephadex G100 or Sephacryl S200. Purity of the 5S RNA preparation was examined by polyacrylamide gel electrophoresis.

**Electrophoresis of RNA samples**

Purity of the RNA samples was examined by electrophoresis in 10% acrylamide gels using 0.02M Tris-acetate, pH 8, 0.001M EDTA, 4M urea buffer (Rubin, 1973). Electrophoresis in the gel slab apparatus described for one dimensional protein gels was for 5-6 hours at 12mA. Gels were stained overnight with 1% Pyronin Y in 15% acetic acid and destained in 7.5% acetic acid for several hours.

**Preparation of adipic acid dihydrazide-Sepharose**

Adipic acid dihydrazide was synthesized from diethyladipate and hydrazine hydrate according to the procedure of Lamed et al. (1973). One hundred mL of diethyladipate and 200mL of hydrazine hydrate were refluxed for 3 hours. The resulting adipic acid dihydrazide crystals were collected and recrystallized twice from 85% ethanol.

Cyanogen bromide-activated Sepharose 4B was washed with 0.1M sodium carbonate, pH 9.6, using 50mL for each gram of Sepharose 4B. The gel was suspended in 0.1M sodium carbonate, pH 9.6 at a concentration of 1 gram dry weight of Sepharose 4B per 4mL of buffer.
This suspension was mixed for 12-15 hours at 4°C with 10mL of a saturated solution of adipic acid dihydrazide for each gram dry weight of Sepharose 4B (Lamed et al., 1973). The resulting adipic acid dihydrazide-Sepharose was washed with 0.2M NaCl until a negative test for dihydrazide was obtained with a 2,4,6-trinitrobenzene sulfonate reagent (Cuatrecasas, 1970). The dihydrazide-Sepharose was then washed with water and suspended in 0.1M sodium acetate, pH 5, and stored at 4°C until used. The volume of the gel suspension was 5mL for each gram dry weight of Sepharose 4B.

To determine whether CNBr-activated groups remaining unsubstituted after coupling with adipic acid dihydrazine interfered with the binding of RNA or proteins, in one experiment these reactive groups were blocked by reaction with ethanolamine. Adipic acid dihydrazide-Sepharose was mixed with 0.1M ethanolamine-HCl, pH 7.5, using 5mL of ethanolamine solution for each g of Sepharose suspended in 15mL of water (Bartkowiak and Pawelkiewicz, 1972). The reaction was allowed to continue for 12 hours at 4°C and the gel was washed as in the standard procedure previously described and then suspended in 0.1M sodium acetate, pH 5.

To block unreacted adipic acid dihydrazide groups remaining after coupling of periodate oxidized RNA, Sepharose-RNA was reacted with 0.52M acetaldehyde, pH 5, using 10mL for each g of Sepharose-RNA. After the reaction, the gel was equilibrated with binding buffer and used in protein binding experiments. The volume of the gel suspension was 5mL for each gram dry weight of Sepharose 4B.
Coupling of RNA to adipic acid dihydrazide-Sepharose through the 3' terminus.

The 3' terminus of the RNA was oxidized using an excess of sodium periodate according to the procedure of Fahnestock and Nomura (1972). RNA was dissolved in 0.1M sodium acetate, pH 5, at a concentration of 1.67 mg/mL and then incubated with 0.048mL of 0.1M sodium periodate for each mL of RNA solution. The oxidation reaction was continued for 1 hour in the dark at room temperature and then stopped by the addition of 2 volumes of cold 95% ethanol. After 2 hours at -20°C, the RNA precipitate was collected by centrifugation and dissolved in 0.1M sodium acetate, pH 5, at a concentration of 2-3 mg/mL. To couple the RNA to adipic acid dihydrazide-Sepharose, oxidized RNA was added to a suspension of dihydrazide-Sepharose in 0.1M sodium acetate, pH 5. The mixture was agitated for 16-24 hours at 4°C on a rotating table (Burrell and Horowitz, 1977). Typically, 2.5mL of gel suspension (0.5g dry weight) was mixed with 5mg of oxidized RNA. The product was collected by centrifugation in a clinical centrifuge and the supernatant containing noncovalently bound RNA was removed. Remaining noncovalently bound RNA was removed from the gel by extensive washing at 4°C with 2M KCl, until the A_{260} of the supernatant was below 0.2. The amount of RNA covalently attached was determined by subtracting the amount of RNA recovered in the combined 2M KCl washes from the total RNA originally added to the adipic acid dihydrazide-Sepharose. Using 5mg of oxidized RNA and 2.5mL of Sepharose gel suspension (0.5g dry weight), about 90% of the RNA became covalently attached to the
Sepharose matrix. The amount of RNA was determined from the A$_{260}$ using a value of A$_{260} = 24$.

**Coupling of RNA to adipic acid dihydrazide-Sepharose through the 5' terminus**

First, the 3'-end of the purified RNA was oxidized using a 120 fold molar excess of NaIO$_4$ in the manner already described, except that the oxidation reaction was allowed to continue for 2 hours at room temperature. After precipitation, the RNA was dissolved in 0.1M sodium borate (pH 9) at a concentration of 2.0mg/mL and the resulting 3'-terminal dialdehyde groups were substituted by addition of 2.9mM methylamine-HCl (in ethanol) to a final concentration of 0.97mM (Fahnestock and Nomura, 1972). The incubation with methylamine was continued at 0°C for 1 hour. The resulting methylamine-RNA adduct was reduced by the addition of 0.05M KBH$_4$ (in 0.1M sodium borate buffer) using a volume of KBH$_4$ equal to the volume of methylamine; incubation was continued at 0°C for another hour. The reduction reaction was stopped by addition of 0.0125mL of 0.1M EDTA and 1.0mL of 0.5M sodium acetate (pH 3.5) for each mg of RNA. After a 5 minute incubation on ice, the RNA was precipitated with 2.5 volumes of cold 95% ethanol. The precipitated RNA was collected by centrifugation and dissolved in 2% potassium acetate (pH 5) - 0.05M methylamine and then washed 5 times by ethanol precipitation. After the final wash, the RNA was dissolved in 0.1M sodium acetate (pH 5) at a concentration of 5 mg/mL.

Oxidized RNA which had not reacted with methylamine was removed by binding to dihydrazide-Sepharose. The RNA was mixed, at 4°C, for at least 12 hours with adipic acid dihydrazide-Sepharose in 0.1M
sodium acetate, pH 5. For each mg of RNA, 0.6mL (0.2g dry weight) of settled dihydrazide-Sepharose gel was used. After 12 hours, the Sepharose gel was centrifuged in a clinical centrifuge and the supernatant containing RNA not covalently attached to the matrix was removed and saved. The adipic acid dihydrazide Sepharose was then washed with 2M KCl to remove additional noncovalently bound RNA, until the A260 of the washes were less than 0.15. The washes were combined with the unbound RNA fraction and dialyzed against deionized water to remove KCl which interferes with ethanol precipitation of RNA. After dialysis for 24 hours with several changes of water, the sample was precipitated by addition of 1/10 volume of 20% potassium acetate (pH 5) and 2 volumes of cold 95% ethanol. Typically, 90% of the RNA originally mixed with the Sepharose was recovered, indicating that most of the RNA had reacted with methylamine.

Methylamine-substituted RNA was derivatized with glycerol using a procedure, similar to one developed by Ho et al. (1981) for sorbitol derivatization of RNA. In the present procedure, the amount of urea used was increased from 240mg to 400mg for each mg of RNA and glycerol was substituted for sorbitol. RNA was dissolved in 0.25M sodium-2-(N-morpholino)ethane sulfonate (pH 5.5) at a concentration of 2.5mg/mL. For each mL of RNA solution, 0.600g of 1-ethyl-3-[3-(dimethylamine)propyl]carbodiimide, 2.4mL of glycerol and 1.00g of ultra pure urea were combined. The resulting syrup was incubated for 2 hours at room temperature. At the end of the incubation, the sample was diluted with an equal volume of 0.02M Tris-HCl, pH 7.4, 0.5M NaCl, applied to a Sephadex G50 column (53cm x 2cm), and eluted with the
same buffer. Three-mL fractions were collected, fractions containing RNA were pooled and the RNA precipitated with ethanol.

The RNA was then collected, dissolved in TM2 (10mM Tris-HCl, pH 7.4, 10mM magnesium acetate) and renatured by heating the sample at 60°C for 5 minutes and then slowly cooling to room temperature (Aubert et al., 1968). Renatured glycerol derivatized RNA was precipitated with ethanol and dissolved in 0.1M sodium acetate, pH 5, at a concentration of 1.67mg/mL. The glycerol substituent was then oxidized with periodate using a 120 fold molar excess of reagent. At the end of the 2 hour incubation, the RNA was again precipitated by the addition of ethanol. The precipitated RNA was collected and dissolved in 0.1M sodium acetate, pH 5. The sample was then bound to dihydrazide-Sepharose by mixing at 4°C for 12 hours with dihydrazide-Sepharose as described previously for the coupling of RNA through its 3'-terminus. The RNA-Sepharose gel was then washed with 2M KCl to remove noncovalently bound RNA. About 40-50% of the RNA became covalently attached to the Sepharose.

Preparation of poly(U)-Sepharose

To obtain efficient attachment of poly(U) to Sepharose, the poly(U) was first fragmented by mild acid hydrolysis according to the procedure of Belitsina and Spirin (1979). Poly(U) was dissolved in 0.05M HCl at a concentration of 2mg/mL and incubated at room temperature for 10 minutes. This produced fragments 100-150 nucleotides long. The solution was neutralized with NaOH and the poly(U) was precipitated by addition of 1/10 volume of 20% potassium acetate, pH 5,
and 2 volumes of cold 95% ethanol. 3'-Terminal phosphate was removed by incubating the fragmented poly (U) (5 mg/mL), with 0.008 mg/mL bacterial alkaline phosphatase in 0.01M Tris-HCl, pH 8, 0.001M MgCl₂, 0.001M 2-mercaptoethanol at 37°C for 2 hours. Protein was removed by two phenol extractions and poly (U) was precipitated by addition of 2 volumes of 95% ethanol. To oxidize the 3' end, poly (U) was dissolved in water at a concentration of 10mg/mL and incubated for 1 hour at room temperature in the dark with 8 mg/mL NaI₂O₃. Oxidized poly (U) was recovered by ethanol precipitation and dissolved in 0.1M sodium acetate, pH 5. Covalent binding to adipic acid dihydrazide-Sepharose was carried out as described for RNA. Approximately 75% of the poly (U) was covalently attached by this procedure.

**Preparation of Oligo (U)₆-Sepharose**

5mg of oligo (U)₆ was oxidized using 10 fold molar excess of NaI₂O₃. Unreacted NaI₂O₃ was separated from oligo (U)₆ on a Sephadex G10 column equilibrated with 0.1M sodium acetate, pH 5. The oxidized oligo (U)₆ was mixed with adipic acid dihydrazide-Sepharose as already described. About 74% of the oligo (U)₆ was covalently attached to the matrix.

**Chromatography of ribosomal proteins on RNA-Sepharose**

All chromatography experiments were carried out at 4-8°C. RNA-Sepharose was poured into a small column, 0.9 x 3 cm, which contained 5-10mg of immobilized RNA. The column was equilibrated at 4°C with binding buffer. In early experiments, binding buffer was 0.005M
potassium phosphate, pH 7.4, 0.02M MgCl₂, 0.006M 2-mercaptoethanol and either 0.2M or 0.3M KCl. For all experiments with yeast ribosomal proteins or rat liver ribosomal proteins, the binding buffer used contained 0.02M Tris-HCl, pH 7.4, 0.02M MgCl₂, 0.001M dithiothreitol and 0.2M KCl to 0.45M KCl.

Before application to columns of immobilized RNA, the ribosomal protein mixture was diluted with binding buffer to a concentration of about 0.5 mg protein/mL and dialyzed for at least 12 hours against the same buffer. Some protein precipitated during dialysis and this was removed by centrifugation. Two-dimensional gel electrophoresis of the E. coli 50S ribosomal proteins remaining soluble showed that all proteins except L20 were present in the protein mixture applied to the column. However, reduced levels of proteins L4, L5, and L21 were present. Proteins also precipitated during dialysis of rat liver and yeast ribosomal proteins, but there was no preferential loss of individual proteins. Several attempts to increase the solubility of the ribosomal proteins, were unsuccessful.

Ribosomal proteins which had been dialyzed against the appropriate binding buffer, were applied to the column at a concentration of 0.2-0.5 mg/mL. For most experiments, a total of 5-10mg of ribosomal protein was used. After sample application, the columns were first washed with 30mL of binding buffer to elute proteins not bound to the RNA-Sepharose. Bound proteins were then eluted with a 30-50mL linear salt gradient formed by mixing equal volumes of binding buffer and dissociation buffer (0.005M potassium phosphate, pH 7.4, or
0.02M Tris-HCl, pH 7.4, 0.005M EDTA, 2M KCl and 0.006M 2-mercaptoethanol or 0.001M dithiothreitol). Columns were run at a flow rate of 8-12 mL/hour and two mL fractions were collected. The protein content of each fraction was determined by method of Bradford (1976). In several later experiments, a final wash with 0.01M Tris-HCl, pH 7.4, 4M LiCl, 8M urea was used to remove tightly bound proteins (Metspalu et al., 1980). Fractions were pooled as indicated in each experiment and dialyzed at 4°C against 3 or more changes of 1% acetic acid for 20 hours. Each sample was then lyophilized and dissolved in 8M urea for electrophoretic analysis. Not all of the protein applied to the column was recovered, even when the columns were washed with buffers containing 4M LiCl and 8M urea. In most experiments, the recovery of ribosomal protein was 60%-70%.
RESULTS

General Procedure and E. coli, Yeast and Rat Liver Ribosomal Protein Preparation

Chromatography of ribosomal proteins on RNA columns was done at 4-8°C. The RNA containing columns were prepared as described in Methods and then equilibrated with binding buffer usually containing 0.2M or 0.3M KCl. Ribosomal proteins were applied to the RNA containing column and after the column was washed with binding buffer to remove unbound protein, bound protein was eluted with a high salt/EDTA containing dissociation buffer (see Methods for details). When washing the column with binding buffer, most of the protein came directly through the column having no interaction with the immobilized RNA, however a small amount of protein was eluted slowly by the binding buffer after the major protein peak. These proteins, which showed weak interactions with the RNA, were pooled separately and identified as the intermediate fraction. Columns were generally used two or three times for protein binding experiments and then discarded. Each time the same proteins were bound and there was no significant decrease in the amount of protein bound. Immediately after each use the column was reequilibrated with binding buffer. After a column was used more than three times, anomalous results were obtained. Either no protein was bound, or protein was bound so tightly that it was never eluted from the RNA-Sepharose.
E. coli 50S ribosomal proteins were not totally soluble in the binding buffers used. The method for obtaining the greatest solubility of the proteins has been described in Materials and Methods. Using this method, only about 5% of the total protein sample was insoluble in the binding buffer. When the soluble E. coli 50S ribosomal proteins were compared to the total E. coli 50S ribosomal proteins (proteins extracted from 50S subunits and dissolved directly in 8M urea), using two dimensional gel electrophoresis, a reduction in the amounts of proteins L4, L5, and L21 in the soluble protein mixture was observed (Figure 2). Proteins prepared from E. coli 30S ribosomal subunits, yeast ribosomes and rat liver ribosomes also had a limited solubility. But in these cases there was no preferential loss of any proteins.

To increase the solubility of the protein samples, several other methods of protein preparation were tried. It had been reported that improved solubility of ribosomal proteins could be achieved by dialysis against pH 6 buffer which contained little or no salt (Amils et al., 1978). Dialysis against such a buffer was carried out and no precipitate was formed, but when the KCl concentration of the protein solution was adjusted to 0.2M prior to chromatography, protein precipitated from solution. In another experiment, the protein solution was dialyzed directly against binding buffer after acetic acid extraction, without lyophilization. Still a precipitate was formed. Both of these experiments failed to increase the solubility of the proteins.
Figure 2. Two-dimensional gel electrophoresis of *E. coli* 50S ribosomal proteins. [Proteins were extracted from isolated 50S sub-units using acetic acid, lyophilized and then dissolved in 4M urea which dissolved all the protein. Proteins were separated by two-dimensional gel electrophoresis in the reduced size gel apparatus described in Methods. The proteins are labeled according to the nomenclature of Kaltschmidt and Wittman (1970b). L20 was not visible and L5, L21, L26, L27, L28, L29, L31, L32 and L33 were always very lightly stained.]
In all experiments, the recovery of ribosomal protein from the columns of immobilized RNA was low, 50-70%. The recovery of protein did not depend on the type of proteins, the type of RNA, or the numbers of times the column had been used. Numerous attempts were made to increase the recovery of protein. Column flow rates were decreased and the concentration of the protein sample applied to the column was decreased, still there was no increase in the amount of protein recovered. In later experiments, the columns were washed with 8M urea, 4M LiCl, 10mM Tris-HCl, pH 7.4. This buffer has been reported to remove tightly bound proteins (Metspalu et al., 1980), but no significant amount of protein was eluted under these conditions.

Even though recovery of total protein from columns was low, there did not seem to be a loss of any specific proteins. The protein fraction that showed no interaction with the RNA (unbound fraction) was compared to the protein fraction eluted with high salt/EDTA buffer (bound fraction), using two-dimensional gel electrophoresis. The sum of the proteins present in these two fractions was the same as that in the column input. The proteins missing from the unbound fraction or found at reduced levels, were present in the bound or intermediate fraction. These results suggest that the low recovery of protein represented a nonspecific binding of protein to the columns, and not the specific binding of particular proteins.

To insure that the proteins were interacting only with the RNA and not the Sepharose matrix, E. coli 50S proteins and yeast 60S
proteins were chromatographed on adipic acid dinydrazide-Sepharose (no bound RNA). No *E. coli* 50S ribosomal proteins and only about 1% of the yeast 60S ribosomal proteins were bound to the matrix. These experiments were done using binding buffer containing 0.2M KCl and under these conditions greater than 10% of *E. coli* 50S proteins and of yeast 60S were bound to columns containing *E. coli* 5S RNA. Failure of these proteins to bind to columns lacking RNA indicated that the proteins were interacting directly with the RNA and not the Sepharose matrix. The recovery of protein from these columns was only 68% with *E. coli* 50S proteins, and 50% with yeast 60S proteins.

Binding of Large Subunit Ribosomal Proteins to Columns of Immobilized RNA

Prokaryotic ribosomes have only one low molecular weight ribosomal RNA, 5S RNA. In contrast, eukaryotic ribosomes have two low molecular weight ribosomal RNAs, 5S RNA and 5.8S RNA. The purpose of this study was to attempt to identify the eukaryotic ribosomal RNA (rRNA) which might be the functional analog of prokaryotic 5S rRNA. An affinity chromatography method using immobilized rRNAs was used to study this question. If either eukaryotic 5S or 5.8S rRNA was able to bind the same set of ribosomal proteins bound by prokaryotic 5S rRNA, a functional analogy might be established.

**Binding of E. coli 50S ribosomal proteins to E. coli 5S rRNA**

Previous work by Burrell and Horowitz (1977), confirmed in this study, had shown that immobilized *E. coli* 5S rRNA was able to bind a
significant amount of *E. coli* 50S ribosomal proteins at 0.3M KCl; 5.3% of the protein recovered was eluted by the high salt/EDTA dissociation buffer (Table 1). Bound proteins were eluted as a single peak. The proteins in this bound fraction were identified as L18 and L25, with lesser amounts of L5, by coelectrophoresis on two-dimensional gels with a light background of total *E. coli* 50S ribosomal proteins (Figure 2, 3, 4). Small amounts of protein L2 were also visible on two-dimensional gels (Figure 4). These same proteins were identified in a complex with *E. coli* 5S rRNA by a number of different methods, including sucrose density centrifugation (Horne and Erdmann, 1972), gel electrophoresis (Gray et al., 1973) and mild RNAse digestion of 50S ribosomal subunits (Chen-Schmeisser and Garrett, 1977).

To further investigate the interaction of *E. coli* 5S RNA with *E. coli* 50S proteins, binding buffer containing 0.2M KCl was used. Under these conditions there was an increase in the amount of protein retained by the column, with about 15% of the protein recovered in the fraction eluted by the high salt/EDTA dissociation buffer (Table 1). Bound protein was eluted as two separate peaks (Figure 5), and the proteins in each peak were identified by two dimensional gel electrophoresis. Protein L2 was found in the first peak (bound I); with small amounts of L17 also bound in some experiments (Figure 6A and B). Spots other than L2 and L17 were also visible on two-dimensional gels of this protein fraction. In almost all cases, there was an extra spot directly below L2 (see Figure 6A). This spot did
Table 1. Effect of KCl concentration on binding of *E. coli* 50S ribosomal proteins to immobilized RNA

Affinity chromatography experiments were performed as described in Figure 3, using binding buffer containing either 0.2M KCl or 0.3M KCl. Protein was determined by the method of Bradford.

<table>
<thead>
<tr>
<th>RNA</th>
<th>% Bound&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ribosomal proteins bound&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0.3M KCl</td>
<td>0.2M KCl</td>
</tr>
<tr>
<td>E. coli 5S</td>
<td>5.3</td>
<td>6.8 (peak 1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.4 (peak 2)</td>
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<tr>
<td>Poly (U)</td>
<td>none</td>
<td>13.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>oligo (U)&lt;sub&gt;6&lt;/sub&gt;</td>
<td>-</td>
<td>none</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fraction of the protein recovered.

<sup>b</sup> Identified by two dimensional gel electrophoresis using the standard nomenclature of Kaltschmidt and Wittmann (1970b).

<sup>c</sup> The binding of proteins enclosed by parenthesis was variable.

<sup>d</sup> When binding buffer containing 0.25M KCl was used, 2% of the protein was in the bound fraction. The major protein bound to the RNA was tentatively identified as L2.

<sup>e</sup> L2 was in the intermediate fraction indicating some interaction with poly (U).
Figure 3. Affinity chromatography of *E. coli* 50S proteins, on *E. coli* 5S RNA-Sepharose at 0.3M KCl. A mixture of *E. coli* 50S proteins, 7.4mg in 20mL of binding buffer (0.005M potassium phosphate, pH 7.4, 0.02M MgCl₂, 0.006M 2-mercaptoethanol) containing 0.3M KCl was applied to a column (1x4cm) to which 4.3mg of *E. coli* 5S RNA had been coupled. 2.0mL fractions were collected at a flow rate of 12mL/hr, at 4-8°C. The column was washed with binding buffer to remove protein that did not bind to the RNA. Bound proteins were eluted with a linear KCl gradient formed by mixing 25mL of binding buffer and 25mL of dissociation buffer (0.005M potassium phosphate, pH 7.4, 2M KCl, 0.005M EDTA, 0.006M 2-mercaptoethanol). Protein concentration was determined by the method of Bradford (1976). Fractions were pooled as follows: unbound (1-16), intermediate (17-37) and bound (50-59). (- - -) KCl concentration.
Figure 4. Two-dimensional gel electrophoresis of *E. coli* 50S proteins bound to *E. coli* 5S RNA-Sepharose, at 0.3M KCl. [A] Bound fraction (proteins eluted by high salt/EDTA buffer, see Figure 3), 30μg analyzed alone. B) bound proteins (30μg) analyzed with a background of total 50S protein (75μg).]
Figure 4 (continued)
Figure 5. Affinity chromatography of *E. coli* 50S proteins on *E. coli* 5S RNA-Sepharose at 0.2M KCl. [A mixture of total 50S proteins, 10.5mg in 20mL of binding buffer, was chromatographed, as in Figure 3, on *E. coli* 5S RNA-Sepharose which contained 4.3mg of immobilized RNA. Fractions were pooled as follows: unbound (1-16), intermediate (17-40), bound I (41-46) and bound II (50-55). (---) KCl concentration.]
Figure 6. Two-dimensional gel electrophoresis of *E. coli* 50S proteins bound to *E. coli* 5S RNA at 0.2M KCl. [A] bound I (30μg) from Figure 5, analyzed alone. B) bound I (30μg) mixed with 75μg of total 50S proteins. C) bound II (50μg) from Figure 5, analyzed alone.]
Figure 6 (continued)
not correspond to any known *E. coli* 50S ribosomal proteins. This same spot was also present in gels of some total *E. coli* 50S ribosomal protein preparations and became darker the longer the protein was stored in urea and may reflect some protein modification caused by urea. A second unidentifiable spot was also found in the region of L6/L11 (See Figure 6A). This probably corresponds to a similar spot observed by Burrell and Horowitz (1977) which was not identified as a ribosomal protein. The proteins in the second peak, (bound II) were identified as L18 and L25 (Figure 6C). Proteins L2 and L17 were not retained in significant amounts by immobilized *E. coli* 5S rRNA when the binding buffer contained 0.3M KCl (compare Figures 4 and 6).

The results of binding experiments with *E. coli* 50S proteins showed that when binding buffer containing 0.3M KCl was used only proteins L18 and L25 were specifically bound to *E. coli* 5S RNA. At lower salt concentrations (0.2M KCl), there was additional binding of proteins L2 and L17. These proteins seemed to bind to *E. coli* 5S with less affinity than L18 and L25 since they were eluted at a lower salt concentration.

**Binding of *E. coli* 50S ribosomal proteins to yeast 5.8S and yeast 5S RNA**

The binding of *E. coli* 50S proteins to yeast 5S and yeast 5.8S ribosomal RNA was first studied using the same 0.3M KCl binding buffer (0.005M potassium phosphate, pH 7.4, 0.02M MgCl₂, 0.3M KCl, 0.006M 2-mercaptoethanol) that was used for initial binding experiments with
**E. coli** 5S RNA. The ionic conditions were similar to those used in the reconstitution of ribosomal subunits (Traub and Nomura, 1969). Under these conditions there was very little binding of **E. coli** 50S proteins to yeast 5S RNA (Figure 7 and Table 1). A similar elution profile was obtained with yeast 5.8S RNA (results not shown). This was in contrast to results obtained with **E. coli** 5S RNA in which 5-6% of the protein recovered was in the bound fraction.

The bound fraction from a yeast 5S RNA containing column was analyzed on discontinuous gels (one dimensional) and a number of faint bands were observed (results not shown). Proteins present in these bands could not be identified because of the small amounts available. Intermediate fractions were also analyzed on gels and two dark-staining bands were noted, one near the top of the gel and the second band migrating somewhat faster. Again, there was not enough protein to analyze on two-dimensional gels, so positive identification could not be made.

When the salt concentration in the binding buffer was lowered to 0.2M, KCl both 5S and 5.8S yeast ribosomal RNAs bound significant amounts of **E. coli** 50S proteins (Table 1). The results of one experiment with immobilized yeast 5S RNA are shown in Figure 8; a similar elution profile was obtained with yeast 5.8S RNA (results not shown).

Proteins in the bound fraction were analyzed by two-dimensional gel electrophoresis and identified by coelectrophoresis with a light background of total **E. coli** 50S ribosomal proteins. The same two major proteins were bound to both yeast 5S and yeast 5.8S RNA. These
Figure 7. Affinity chromatography of *E. coli* 50S proteins on yeast 5S RNA-Sepharose at 0.3 M KCl.

A mixture of *E. coli* 50S proteins, 10 mg in 7.0 mL of binding buffer, was chromatographed as in Figure 3, on a 1 x 4 cm column containing 7.9 mg of immobilized yeast 5S RNA. Fractions were pooled as follows: unbound (1-6), intermediate I (7-17), intermediate II (18-44), bound (44-62). (---) KCl concentration.
Figure 8. Affinity chromatography of *E. coli* 50S proteins on yeast 5S RNA-Sepharose at 0.2M KCl.  
8.2mg of *E. coli* 50S proteins in 7.0mL of binding buffer were chromatographed as in Figure 3, on a 1x3cm column to which 5.9mg of yeast 5S RNA had been coupled. Fractions were pooled as follows: unbound (1-8), intermediate I (9-18), intermediate II (20-35), and bound (36-45). (----) KCl concentration.
were identified as L2 and L17 (Figure 9). The unbound fractions (proteins showing no interaction with the RNA) from columns containing yeast 5S and yeast 5.8S RNA were also examined on two-dimensional gels. Proteins L2 and L17 were missing from these fractions (data not shown).

Varying amounts of protein L17 were bound to the yeast ribosomal RNAs. In one experiment, with yeast 5.8 RNA, the bound fraction contained only one protein which was identified as L2 (Figure 10A). The intermediate fraction (eluted with binding buffer after emergence of the unbound fraction) from this same experiment was also analyzed on two-dimensional gels and it contained only one major spot which was identified as L17 with small amounts of L16 also bound (Figure 10B). The results this experiment suggest that L2 binds more strongly to yeast 5.8S RNA than does L17. Small variations in the experimental conditions changed L17 from a protein which was only eluted by high salt/EDTA dissociation buffer, to a protein which had only slight affinity for the RNA.

It has been reported that yeast 5.8S RNA is capable of forming a complex with E. coli 50S proteins L18 and L25 which can be isolated from a sucrose gradient (Wrede and Erdmann, 1977). In our experiments, these proteins might have been bound so tightly to immobilized yeast 5.8S RNA that the high salt/EDTA dissociation buffer was not able to elute the proteins. The use of 8M urea and 4M LiCl (in 0.01M Tris-HCl, pH 7.4) to remove tightly bound ribosomal proteins from immobilized RNA columns has recently been reported (Metspalu et al.,
Figure 9. Two-dimensional gel electrophoresis of E. coli 50S proteins bound to yeast 5S RNA-Sepharose at 0.2M KCl. [Electrophoresis was carried out as described in Methods, using the larger gels. A) bound fraction (85µg) from Figure 8, analyzed alone. B) bound proteins (85µg) mixed with 120µg of total 50S proteins.]
Figure 10. Two-dimensional gel electrophoresis of fractions from the chromatography of *E. coli* 50S proteins on yeast 5.8S RNA-Sepharose. [Affinity chromatography was performed using binding buffer containing 0.2M KCl. A) bound proteins (30μg) analyzed alone. B) intermediate fraction (28μg), analyzed alone.]
Figure 10 (continued)
1980). This buffer was, therefore, used in attempts to remove tightly bound \textit{E. coli} 50S proteins from immobilized yeast 5.8S RNA.

A sample of \textit{E. coli} 50S proteins was applied to a yeast 5.8S RNA column using binding buffer containing 0.2M KCl and the column was washed with an additional 30mL of binding buffer. A 30mL gradient formed by mixing equal volumes of binding buffer and dissociation buffer was used to elute proteins in the usual manner. The column was then washed with 15-20mL of 8M urea/4M LiCl buffer. The high salt/EDTA buffer eluted 6.2% of the protein recovered from the column and 0.4% of the protein recovered was eluted by the urea/LiCl buffer. Proteins from both of these fractions were analyzed on two-dimensional gels. As expected, L2 and L17 were found in the fraction eluted by the high salt/EDTA dissociation buffer. A number of light protein spots were visible after two-dimensional gel electrophoresis of the proteins eluted with the urea/LiCl buffer. These were identified as proteins L1, L3, L4, L5, L6, L11, and L17 (gels not shown). When proteins from the unbound fraction were examined on two-dimensional gels, no reduction in the amounts of proteins L18 and L25 was evident. These results again indicate that immobilized yeast 5.8S RNA is not capable of binding \textit{E. coli} 50S ribosomal proteins L18 and L25.

\textbf{Binding of \textit{E. coli} 50S ribosomal proteins to poly (U) and oligo (U)\textsubscript{6}}

Since \textit{E. coli} 50S ribosomal proteins L2 and L17 were bound to yeast 5S, yeast 5.8S and \textit{E. coli} 5S RNA, experiments were undertaken to investigate the specificity of this binding using the nonspecific
polynucleotide poly (U) coupled to agarose. Binding of \textit{E. coli} 50S proteins to immobilized poly (U) was studied at 0.2M, 0.25M, and 0.3M KCl. Results similar to those obtained with yeast 5S and yeast 5.8S RNA were obtained. There was very little protein bound to immobilized poly (U) when binding buffer containing 0.3M KCl was used. As the salt concentration of the binding buffer was lowered there was an increase in the amount of ribosomal protein retained by the column. About 2% of the protein was bound when buffer containing 0.25M KCl was used and about 13.4% of the protein was bound when buffer containing 0.2M KCl was used (Table 1). In both experiments, the protein was eluted by the high salt/EDTA buffer as a single peak, similar to experiments with yeast 5S and yeast 5.8S RNA.

The proteins from each fraction were analyzed on discontinuous gels (results not shown). While no proteins were bound at 0.3M KCl, the intermediate fraction from this trial had one dark band with mobility similar to L2. At 0.25M KCl, the bound fraction had one dark band, also with mobility similar to L2, and the intermediate fraction had a single band with mobility similar to L17. The bound fraction from the experiment at 0.2M KCl showed two bands that were tentatively identified as L2 and L17. The proteins bound at 0.2M KCl were positively identified as proteins L2, L16, and L17 by two-dimensional gel electrophoresis (Figure 11). The unbound fraction was also analyzed on two-dimensional gels and proteins L2 and L17 were missing, indicating that poly (U) was able to bind all of the L2 and L17 present in the protein sample applied to the column. There was not
Figure 11. Two-dimensional gel electrophoresis of *E. coli* 50S proteins bound to poly(U)-Sepharose at 0.2M KCl. [Bound proteins (40µg), analyzed alone.]
enough protein in the bound fractions from experiments at higher salt concentration to analyze on two-dimensional gels, so proteins in these fractions could not be positively identified.

To further investigate the specificity of the binding of *E. coli* 50S proteins to poly (U), a column was prepared which was treated with ethanolamine and acetaldehyde (see Methods). The binding of *E. coli* 50S proteins to this column was investigated at 0.2M KCl. The poly (U) was still able to bind *E. coli* 50S proteins, but at a reduced level (about 2% of protein was in the bound fraction). The proteins in the bound fraction were analyzed on discontinuous gels and L2 appeared to be the major protein present, with many more lightly stained proteins, showing that L2 interacts directly with the poly (U) and not with reactive groups present on the dihydrazide-Sepharose matrix.

Columns containing immobilized oligo (U)₆ were used to further investigate the binding specificity of *E. coli* 50S proteins. Using binding buffer containing 0.2M KCl, no proteins were bound to the oligo (U)₆ column (Table 1). This suggests that the binding of ribosomal proteins requires oligonucleotides greater than 6 bases in length.

**Preparation of Sepharose-RNA immobilized through the 5' end**

Failure of proteins L18 and L25 to bind to yeast 5.8S RNA was in contrast to results obtained by other experimental approaches (Wrede and Erdmann, 1977). This might be due to the method of RNA immobilization. Attachment of RNA to the Sepharose matrix through the 3' end
might disrupt protein binding sites near the 3' end. An alternate method was developed to immobilize the RNA through the 5' end, to determine if this might affect the binding of *E. coli* 50S proteins. Briefly, the method involves blocking the 3' of the RNA with methylamine, after periodate oxidation. The 5' phosphate is then derivatized with glycerol and the glycerol is oxidized with periodate to yield an aldehyde through which the RNA is covalently coupled to adipic acid dihydrazide-Sepharose (see Methods section for details).

In preparation of columns of glycerol derivatized RNA, it was important to establish that the RNA attached to the Sepharose matrix only through its 5' end. Portions of the RNA were removed at various stages during column preparation and the RNA was tested for its ability to covalently attach to dihydrazide-Sepharose. The results of these experiments are summarized in Table 2.

Without oxidation, very little RNA was bound to the dihydrazide-Sepharose (Table 2, line 1), as previously reported (Burrell and Horowitz, 1977). After periodate oxidation of the 3' end, greater than 90% of RNA was covalently attached to the dihydrazide-Sepharose (Table 2, line 2). The oxidized RNA was treated with methylamine and then reduced with KBH₄ to substitute the 3'-dialdehyde groups. Less than 5% of this RNA sample became covalently attached (Table 2, line 3), indicating that most of the dialdehyde groups formed during the oxidation reaction had reacted with methylamine. Even though only a small fraction of the RNA appeared to have unsubstituted dialdehyde groups at the 3' end, it was necessary to remove these unsubstituted
Table 2. Binding of RNA to adipic acid dihydrazide Sepharose

Except where otherwise noted 0.5mg (12A260 units) of RNA were mixed with 0.1g of swollen dihydrazide-Sepharose (in 0.1M sodium acetate, pH 5) for at least 12 hours at 4°C. The gel was washed extensively with 2M KCl to remove any RNA not covalently attached. For a detailed description of the various treatments see Methods.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>E. coli 5S RNA</th>
<th>Yeast 5S RNA</th>
<th>Yeast tRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>-</td>
<td>1.3</td>
<td>10.0</td>
</tr>
<tr>
<td>Periodate oxidation of 3' end</td>
<td>91.9</td>
<td>91.3</td>
<td>91.0</td>
</tr>
<tr>
<td>Methylamine addition and reduction</td>
<td>4.1</td>
<td>2.1</td>
<td>0a</td>
</tr>
<tr>
<td>Glycerol derivatization (2 hrs); No oxidation</td>
<td>0</td>
<td>0</td>
<td>6.1</td>
</tr>
<tr>
<td>Glycerol derivatization (2 hrs); 2 hrs oxidation</td>
<td>41.8b(149)</td>
<td>34.2b (28)</td>
<td>33.0b (88)</td>
</tr>
<tr>
<td>Glycerol derivatization (2 hrs); 4 hrs oxidation reaction</td>
<td>-</td>
<td>30.8c</td>
<td>28.5</td>
</tr>
</tbody>
</table>

a12 A260 units of the methylamine treated sample was oxidized with periodate and no RNA was covalently attached.

bThe number in parentheses indicates the total A260 units mixed with an appropriate amount of dihydrazide-Sepharose.

cFor this experiment 31 A260 units of RNA was used.
RNAs prior to glycerol derivatization. This was accomplished by coupling the unsubstituted RNA to dihydrazide-Sepharose.

The next step involved derivatization of the RNA with glycerol. The procedure used was similar to one used to couple sorbitol to RNA, developed by Ho et al. (1981). In the present modification, using glycerol, the concentration of urea was increased to 8.8M and different derivatization times were tested. No direct measure of glycerol incorporation was made, but the extent of modification was estimated from the ability of the RNA to bind to dihydrazide-Sepharose after oxidation. Increasing the time of glycerol derivatization does not increase the amount of RNA which could be covalently attached to the matrix. Derivatization of E. coli tRNA with glycerol was continued for two, four or six hours and then followed by a two hour reaction with periodate. For each sample about 45% of the RNA was able to covalently bind to the dihydrazide-Sepharose matrix. Also, increasing reaction time with periodate does not increase the amount of RNA bound (Table 2, lines 5,6). 30-40% of the RNA was covalently attached to the Sepharose matrix under the best conditions.

The inability of the remaining (oxidized) glycerol-treated RNA to attach to the Sepharose matrix might be explained in several ways. The simplest explanation is that the amount of RNA used exceeded the binding capacity of the Sepharose. This possibility was tested by attempting to attach unbound (oxidized) glycerol-treated yeast tRNA, recovered from a previous experiment, to fresh dihydrazide-Sepharose.
Only 6.7% of the RNA was bound to the fresh matrix indicating that most of the RNA was incapable of binding to dihydrazide-Sepharose.

Two other explanations for the failure of 60% of (oxidized) glycerol-treated RNA to bind to the Sepharose matrix are possible. One is that only 40% of the RNA was derivatized with glycerol. A second possibility is that all of the RNA was derivatized but only 40% was oxidized under the conditions used. To distinguish between these possibilities, (oxidized) glycerol treated yeast tRNA which had not bound to the dihydrazide-Sepharose was reoxidized; only 2.2% of this RNA bound to the Sepharose. Since further oxidation did not increase the amount of RNA bound to the Sepharose, it is likely that only 40% of the RNA was derivatized by glycerol.

Binding of *E. coli* 50S ribosomal proteins to 5'-immobilized *E. coli* 5S RNA

The binding of *E. coli* 50S proteins to *E. coli* 5S RNA immobilized through the 5' end was studied using binding buffer containing 0.3M, 0.25M and 0.2M KCl. At 0.3M KCl, very little protein was bound (i.e. eluted by the high salt/EDTA dissociation buffer) and no individual proteins could be identified by two-dimensional gel electrophoresis (Table 3). The proteins of the unbound fraction were also examined on two-dimensional gels, and there did not seem to be a reduction in the amount of any of the *E. coli* 50S proteins (data not shown). This was in contrast to experiments with *E. coli* 5S RNA attached to the matrix through the 3' end (Figure 3, 4, and Table 1), where the binding of proteins L18 and L25 was observed.
Table 3. Binding of *E. coli* 50S proteins to RNA immobilized through the 5′ end

Affinity chromatography was performed as described in Figure 3, using RNAs attached through the 5′ end.

<table>
<thead>
<tr>
<th>RNA</th>
<th>0.3M KCl</th>
<th>% Bound</th>
<th>0.25M KCl</th>
<th>0.2M KCl</th>
<th>Ribosomal proteins bound 0.3M KCl</th>
<th>0.25M KCl</th>
<th>0.2M KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli 5S</td>
<td>1.4</td>
<td>5.3</td>
<td>8.8</td>
<td>none identifiable</td>
<td>L2, L21, L25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>L2, L16, L17, L18, L21, L25</td>
<td></td>
</tr>
<tr>
<td>Yeast 5S</td>
<td>1.8</td>
<td>-</td>
<td>9.4</td>
<td>L2, L16, L17</td>
<td>-</td>
<td>L2, L16, L17, L18, L21, L25</td>
<td></td>
</tr>
<tr>
<td>Yeast 5.8S</td>
<td>1.8</td>
<td>-</td>
<td>3.5</td>
<td>L2, L16, L17</td>
<td>-</td>
<td>L2, L16, L17, L21</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>The intermediate fraction (includes proteins eluted slowly by the binding buffer) was also analyzed by two-dimensional gel electrophoresis. The major proteins were identified as L2, L17, L19, and L25.
When binding buffer containing 0.25M KCl was used, there was an increase in the amount of protein retained by the RNA, with 5.3% of the protein eluted in the bound fraction. The proteins in the bound fraction were only lightly stained on two-dimensional gels but proteins L2, L21 and L25 could be identified (data not shown). Analysis of the unbound fraction by two-dimensional gel electrophoresis again showed no loss of any proteins. The proteins in the intermediate fraction were identified as L2, L17, L18 and L25.

At 0.2M KCl, a significant amount of E. coli 50S proteins was bound to 5'-immobilized E. coli 5S RNA (Table 3). The protein was eluted by a shallow gradient of high salt/EDTA buffer as two peaks with the first peak being much larger than the second (Figure 12). The proteins present in each peak were examined by two-dimensional gel electrophoresis. By comparison to the pattern of total 50S ribosomal proteins, proteins L2, L16, L17, L18, L25 and L21 were identified in both peaks (shown for the first peak in Figure 13). Proteins L16, L17, L18 and L25 were found in the intermediate fraction. Protein L2 was totally absent from the unbound fraction and some reduction in the amount of L17 was also observed.

**Binding of E. coli 50S ribosomal proteins to 5'-immobilized yeast 5S and yeast 5.8S RNA**

When yeast 5S and yeast 5.8S RNA were covalently attached to the Sepharose matrix through the 3' end, there was no binding of E. coli ribosomal proteins L18 and L25. But these proteins were bound to
Figure 12. Affinity chromatography (at 0.2M KCl) of *E. coli* 50S proteins on *E. coli* 5S RNA immobilized through the 5' end. A mixture of total *E. coli* 50S proteins, 5.0mg in 10.0mL of binding buffer containing 0.2M KCl was applied to a 1x3cm column containing 2.6mg of immobilized *E. coli* 5S RNA. Chromatography was as described in Figure 3, using a 60.0mL linear salt gradient to elute the proteins. Fractions were pooled as follows: unbound (1-17), intermediate (18-33), bound I (34-38), bound II (40-45) and bound III (46-75). (---) KCl concentration.
Figure 13. Two-dimensional gel electrophoresis of *E. coli* 50S proteins bound to *E. coli* 5S RNA immobilized through the 5' end (see Figure 12). [30µg of the proteins in the bound I fraction were applied to the gel.]
E. coli 5S RNA. Using yeast 5S and yeast 5.8S RNA attached to the Sepharose matrix through their 5' ends, the binding of E. coli 50S proteins was studied to see if L18 and L25 could now bind to either yeast RNA.

Again it was observed that at 0.3M KCl there was very little binding of E. coli 50S proteins to either RNA (Table 3). When the salt concentration in the binding buffer was lowered to 0.2M KCl, there was an increase in the amount of protein bound to the RNAs (Table 3). Both yeast 5S and yeast 5.8S RNA bound a similar group of proteins. Proteins L2, L16, L17 and a small amount of L18 were identified by two-dimensional gel electrophoresis, along with several other spots. The bound fraction from the yeast 5S RNA column also contained significant amounts of proteins L21 and L22 (Figure 14); while only L21 was bound to yeast 5.8S RNA (Figure 15). Two other spots were also visible, one directly below L2 and another slightly to the left (Figures 14, 15). Neither of these spots correspond to any known ribosomal proteins.

Binding of yeast 60S ribosomal proteins to 3'immobilized RNAs

The binding of yeast 60S proteins to immobilized yeast 5S, yeast 5.8S and E. coli 5S RNA was studied. The yeast proteins bound by these different RNAs were identified in an attempt to establish functional analogies among them. If E. coli 5S RNA bound the same set of yeast proteins that was bound by either of the two yeast RNAs, then a functional analogy might be established.
Figure 14. Two-dimensional gel electrophoresis of *E. coli* 50S proteins bound at 0.2M KCl to yeast 5S RNA attached to Sepharose through the 5' end. [Proteins were identified by comparison to a total 50S protein pattern.]
Figure 15. Two-dimensional gel electrophoresis of \textit{E. coli} 50S proteins bound at 0.2M KCl to yeast 5.8S RNA attached to Sepharose through the 5' end.
Binding buffer containing either 0.3M KCl or 0.2M KCl was used in these studies. At 0.3M KCl, very little protein was retained by any of the RNAs. For all three RNAs tested, only about 1-3% of the protein recovered from the column was eluted in the bound fraction (Table 4).

The yeast 60S proteins which were bound to the RNAs at 0.3M KCl were identified by two-dimensional gel electrophoresis. Proteins were numbered according to the proposed standard nomenclature for yeast ribosomal proteins of Bollen et al. (1981). Both the bound fraction and the unbound fraction (proteins that had no interaction with the RNA) were examined on two-dimensional gels. Proteins in the bound fraction were classified as major or minor RNA binding proteins, based on the intensity of staining. The darkest staining protein spots were classified as major RNA binding proteins and lighter protein spots were classified as minor RNA binding proteins. On two-dimensional gels of the proteins in the unbound fraction, the major RNA binding proteins were either totally absent or present at greatly reduced levels.

The major RNA binding proteins from yeast 60S ribosomal subunits were the same for yeast 5S RNA, yeast 5.8S RNA, and E. coli 5S RNA. These were identified as L2/3 and L23 shown for E. coli 5S RNA (3'-attached) in Figure 16. In the electrophoresis system used, there was poor resolution between proteins L22 and L23 and also between L2 and L3; results from gel electrophoresis of unbound protein fractions aided in identification of the bound proteins. There was a reduction in the amount of protein L23 on gels of the unbound fraction
Table 4. Effect of KCl concentration on the binding of yeast 60S proteins to immobilized RNAs.

Affinity chromatography of yeast 60S proteins was done as described in Figure 3, using binding buffer containing 0.3M KCl or 0.2M KCl. Bound proteins were identified by two-dimensional gel electrophoresis.

<table>
<thead>
<tr>
<th>RNA</th>
<th>% Bound</th>
<th>Ribosomal proteins bound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.3M KCl</td>
<td>0.2M KCl</td>
</tr>
<tr>
<td>E. coli 5S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3'-attached)</td>
<td>2.8</td>
<td>16.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli 5S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5'-attached)</td>
<td>3.4</td>
<td>11.0</td>
</tr>
<tr>
<td>Yeast 5S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3'-attached)</td>
<td>2.9</td>
<td>12.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast 5.8S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3'-attached)</td>
<td>1.8</td>
<td>20.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 16. Two-dimensional gel electrophoresis of yeast 60S proteins bound to *E. coli* 5S RNA-Sepharose (3'-attached) at 0.3M KCl. [Electrophoresis was as described in Methods. A) bound proteins (20µg), analyzed alone. B) bound proteins (20µg) mixed with 20µg of total 60S yeast proteins.]
(results not shown), indicating that L23 rather than L22, was most likely one of the bound proteins. It was still difficult to distinguish between L2 and L3, so the protein is designated L2/3.

In addition to the major RNA binding proteins L23 and L2/3, several other more lightly stained proteins were also visible on gels of proteins bound to RNA at 0.3M KCl. In experiments with E. coli 5S RNA these were identified as L5, L10 and L12 (Figure 16). Yeast 5S RNA bound protein L21 almost as tightly as proteins L23 and L2/3; L5, L10, L12, L17, L18, and L35 were also bound but in lesser amounts (Table 4). The gel pattern of the proteins bound to yeast 5.8S RNA was somewhat smeared, (results not shown) but the major proteins identified were L2/3 and L23; lesser amounts of proteins L5, L10, L12, and L3 were also bound (Table 4).

The binding of yeast 60S proteins to immobilized RNAs was also studied at 0.2M KCl. The amount of protein bound at the lower salt concentration was much larger than the amount bound at 0.3M KCl (Table 4). Greater than 12% of the protein recovered from the column was in the bound fraction (Table 4). Again, the proteins bound to RNA were identified using two-dimensional gel electrophoresis. The two-dimensional gel patterns of the proteins bound to yeast 5S and E. coli 5S RNA columns were quite similar (Figure 17). The major proteins identified as binding to both RNAs include L2/3 and L23, which are also bound to the RNAs at 0.3M KCl. Also identified as major RNA binding proteins were L10 and L21 (Table 4 and Figure 17). For yeast
Figure 17. Two-dimensional gel electrophoresis of yeast 60S proteins bound to immobilized RNAs at 0.2M KCl. (A) Proteins bound to *E. coli* 5S RNA (35μg), analyzed alone. B) Proteins bound to *E. coli* 5S RNA (35μg) mixed with 20μg of total yeast 60S proteins. C) Proteins bound to yeast 5S RNA (30μg), analyzed alone. D) Proteins bound to yeast 5.8S RNA (35μg), analyzed alone.
Figure 17 (continued)
Figure 17 (continued)
5S RNA, L30 was also classified as a major RNA binding protein (Figure 17C). Again a number of lighter spots were also visible on two-dimensional gels. The minor proteins binding to _E. coli_ 5S RNA were identified as L5, L17, L18, L30 and L37 (Figure 17A and Table 4). The lighter spots present in the protein fraction bound to the yeast 5S RNA column were identified as L12, L17, L18, L19, and L37 (Table 4 and Figure 17C). Two-dimensional gels of proteins in the unbound fraction from each column showed that the major RNA binding proteins were either absent or were present in greatly reduced amounts.

Im mobilized yeast 5.8S RNA was able to bind a greater amount of yeast 60S proteins at 0.2 M KCl than either _E. coli_ 5S RNA or yeast 5S RNA (Table 4), and there was a larger number of different proteins in this bound fraction. The major RNA binding proteins were identified as L2/3, L10, and L23 (Table 4 and Figure 17D); these were also bound to yeast 5S and _E. coli_ 5S RNA. In addition L12, was identified as a major RNA binding protein. There were several protein spots on two-dimensional gels which stained only slightly lighter than the major 5.8S RNA binding proteins. These were identified as L17, L18, L19, L21, and L30, with trace amounts of L5 and L37.

**Binding of yeast 60S ribosomal proteins to 5'-attached _E. coli_ 5S RNA**

The binding of yeast 60S ribosomal proteins to _E. coli_ 5S RNA was studied using RNA attached to the Sepharose matrix through its 5' end. In experiments with _E. coli_ 50S proteins, _E. coli_ 5S RNA immobilized
through the 5' end was able to bind the same set of proteins that were bound when *E. coli* 5S RNA was immobilized through its 3' end (Table 3 and Figures 12 and 13).

Binding of yeast 60S proteins to *E. coli* 5S RNA was studied using binding buffer containing 0.3M KCl or 0.2M KCl. At 0.3M KCl, the amount of protein bound was about the same as that bound to *E. coli* 5S RNA attached to the Sepharose matrix through its 3' end (Table 4). Yeast ribosomal proteins in this bound fraction were identified as L2/3 and L23 (Table 4). These were the same proteins bound to the RNA when it was attached to dihydrazide-Sepharose through its 3' end. Using binding buffer containing 0.2M KCl, there was an increase in the amount of protein bound by immobilized *E. coli* 5S RNA (Table 4). While the amount of the protein retained by the column was somewhat less than the amount of protein bound when the RNA was attached through its 3' end, the same proteins, L2/3, L10, L21, and L23, were bound by the RNA (Figure 18). The results of these experiments with yeast 60S proteins again showed that immobilized *E. coli* 5S RNA bound the same set of proteins when it was attached to the Sepharose matrix through either its 3' end or its 5' end.

**Binding of rat liver 60S ribosomal proteins to immobilized RNAs**

Since immobilized yeast 5S and yeast 5.8S RNAs are able to bind significant amounts of yeast 60S proteins, it was of interest to determine if these RNAs are also capable of binding ribosomal proteins from a higher eukaryotic species. Therefore, the interaction of rat
Figure 18. Two-dimensional gel electrophoresis of yeast 60S proteins bound at 0.2M KCl to 
E. coli 5S RNA attached to Sepharose through its 5' terminus. [About 50μg of 
bound proteins were analyzed and then compared to a total yeast 60S protein 
pattern.]
liver ribosomal proteins with yeast 5S and yeast 5.8S RNA was also studied. Other workers have examined the binding of rat liver 60S proteins to immobilized rat liver 5S RNA and rat liver 5.8S RNA (Ulbrich and Wool, 1978, Ulbrich et al., 1979). The results of our experiments on rat liver ribosomal protein interaction with yeast 5S and yeast 5.8S RNA are compared to those of these other groups.

Initially, binding buffer containing 0.3M KCl was used. Both yeast 5S RNA and yeast 5.8S RNA bound a large amount of rat liver 60S ribosomal proteins under these conditions (Table 5). The bound fraction from the yeast 5S RNA column contained 28.8% of the protein recovered from the column, and the bound fraction from the yeast 5.8S RNA column contained 41.2% of the recovered protein (Table 5 and Figure 19). The yeast 5.8S RNA column was washed with 8M urea, 4M LiCl, 10mM Tris-HCl, pH 7.4 to remove tightly bound proteins. Protein analysis of the column fractions indicated that a significant amount of protein was eluted by this buffer (Figure 19). However, a little protein was detected when the pooled fraction (bound II) was analyzed after dialysis. The urea/LiCl buffer gave a positive reaction in the Bradford protein assay. So, the apparent protein peak (bound II) was due to an artifact of the analysis. When the small amount of protein present in this fraction was analyzed by two-dimensional gel electrophoresis, no proteins were evident on the gels. The identity of the rat liver ribosomal proteins bound to yeast 5S RNA and 5.8S RNA columns at 0.3M KCl is shown in Table 5 and Figure 20A. Proteins are numbered according to the proposed standard nomenclature for mammalian ribosomal proteins (McConkey et al., 1979).
Table 5. Effect of KCl concentration on the binding of rat liver 60S proteins to immobilized yeast 5S and yeast 5.8S RNA

<table>
<thead>
<tr>
<th>RNA</th>
<th>% Bound</th>
<th>Ribosomal proteins bound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.3M KCl</td>
<td>0.4M KCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast 5.8S</td>
<td>41.2a</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aDoes not include protein eluted by 8M urea, 4M LiCl, 10mM Tris-HCl, pH 7.4. For yeast 5S this was 6.6% of the recovered protein and for yeast 5.8S this was 2.5%. In both cases no proteins were visible on two-dimensional gels of this fraction.

Figure 19. Affinity chromatography of rat liver 60S proteins on yeast 5.8S RNA at 0.3M KCl.

[A mixture of total rat liver 60S proteins, 3.0mg in 11.2mL of binding buffer, was applied to a 1x3cm yeast 5.8S RNA-Sepharose column containing 4.4mg of immobilized RNA. Chromatography was done as in Figure 3, using a 30mL linear salt gradient (0.3M-2M KCl) to elute bound proteins. After the gradient was completed (fraction 60), the column was washed with 8M urea, 4M LiCl, 10mM Tris-HCl, pH 7.4 (indicated by the arrow). Column fractions were pooled as follows: unbound (1-18), intermediate (19-32), bound I (33-46), and bound II (47-84). (---) KCl concentration.]
Figure 20. Two-dimensional gel electrophoresis of rat liver 60S proteins bound to yeast 5.8S RNA-Sepharose. [A] Proteins which were bound to yeast 5.8S RNA at 0.3M KCl; proteins are identified in Table 5. B) Proteins which were bound to yeast 5.8S RNA at 0.4M KCl; proteins are identified in Table 5.]
When the salt concentration of the binding buffer was increased to 0.4M KCl, as expected, the amount of rat liver 60S ribosomal protein bound to the yeast RNA columns was reduced (Table 5). Only about 13% of the protein recovered was in the bound fraction. The proteins bound to both yeast 5S RNA and yeast 5.8 RNA were identified by two-dimensional gel electrophoresis as proteins L3, L4, L5, L6, L14, and L35 (Figure 20B and Table 5). In addition to these proteins, L19 was a major 5.8S RNA-binding protein; relatively small amounts of this protein were bound to yeast 5S RNA (Table 5). On the other hand, protein L27 was identified as one of the major proteins bound to yeast 5S RNA, while only a small amount of L27 was bound to yeast 5.8S RNA (Table 5). Analysis of the proteins not bound to yeast 5S RNA or 5.8S RNA showed that proteins L3, L4, and L5 were almost totally absent from this fraction and that the amounts of other RNA binding proteins present were considerably reduced.

The binding of rat liver 60S proteins to yeast 5.8S RNA was also studied at 0.45M KCl. The amount of protein recovered in the bound fraction was reduced to 5.6% under these conditions (Table 5). The number of different ribosomal proteins in this fraction was also greatly reduced. The major proteins bound were identified as L3, L6, L14, and L35; lesser amounts of L19, L24, and L27 were also detected (Figure 21). The proteins in the unbound fraction were also analyzed on two-dimensional gels. L3 was totally missing from this fraction and the amount of proteins L5, L14 and L19 was reduced in comparison to a pattern of total rat liver 60S proteins.
Figure 21. Two-dimensional gel electrophoresis of rat liver 60S proteins bound to yeast 5.8S RNA-Sepharose at 0.45M KCl. [A] bound proteins (30µg) analyzed alone. B) bound proteins (30µg) mixed with 30µg of total rat liver 60S proteins.]
The interaction of low molecular weight rRNAs from the large ribosomal subunit with the small ribosomal subunit may be important for subunit association (Azad, 1978). Therefore, the ability of yeast 5S rRNA, yeast 5.8S rRNA and \textit{E. coli} 5S rRNA to bind small subunit proteins was studied.

**Binding of \textit{E. coli} 30S ribosomal proteins to immobilized RNAs**

Previous work by Burrell and Horowitz (1977) had shown that immobilized \textit{E. coli} 5S rRNA did not bind significant amounts of \textit{E. coli} 30S ribosomal proteins at 0.3M KCl. But some proteins showed a weak affinity for the RNA, and were eluted only slowly by binding buffer. The major protein in this intermediate fraction was identified as S3, with trace amounts of S6, S9, S13, S18 and S19/20 also present (Table 6).

Experiments done with \textit{E. coli} 50S ribosomal proteins showed that very little protein was bound to columns containing immobilized yeast RNAs at 0.3M KCl (Table 1). However, there was a significant amount of protein retained by columns containing immobilized yeast RNAs at 0.2M KCl. This lower salt concentration was used to study the binding of \textit{E. coli} 30S ribosomal proteins to yeast RNAs. Under these conditions, both yeast 5S RNA and yeast 5.8S RNA were able to bind significant amounts of protein, more than 18% of the protein was recovered in the bound fraction (Table 6 and Figure 22).
Table 6. Effect of KCl concentration on the binding of *E. coli* 30S proteins to immobilized RNAs

Affinity chromatography of *E. coli* 30S proteins was performed as described in Figure 3 using binding buffer containing either 0.2M KCl or 0.3M KCl.

<table>
<thead>
<tr>
<th>RNA</th>
<th>% Bound 0.3M KCl</th>
<th>% Bound 0.2M KCl</th>
<th>Ribosomal proteins bound&lt;sup&gt;a&lt;/sup&gt; 0.3M KCl</th>
<th>Ribosomal proteins bound&lt;sup&gt;a&lt;/sup&gt; 0.2M KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli 5S</td>
<td>none</td>
<td>-</td>
<td>none&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>yeast 5S</td>
<td>-</td>
<td>21.3</td>
<td>S4, S9, S20 (major)</td>
<td>S3, S5, S7 (minor)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>S6, S13, S14, S15, S16,</td>
<td>S6, S13, S14, S15, S16 (trace)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>yeast 5.8S</td>
<td>-</td>
<td>18.2</td>
<td>S4, S9, S20 (major)</td>
<td>S6, S13 (minor)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>S6, S13, S14, S15, S16 (trace)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>poly (U)</td>
<td>none</td>
<td>9.6</td>
<td>none&lt;sup&gt;c&lt;/sup&gt;</td>
<td>S6, S9 (major)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S4, S5, S7, S13, S18 (minor)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Protein preparations used for chromatography experiments lacked protein S1, S2 and S4.

<sup>b</sup>Experiments by Burrell and Horowitz (1977). Proteins identified in the intermediate fraction were S3 with lesser amounts of S6, S9, S13, and S18.

<sup>c</sup>S3 was the major protein in the intermediate fraction, with lesser amounts of S13 and S20.
Figure 22. Affinity chromatography of *E. coli* 30S proteins on yeast 5.8S RNA-Sepharose at 0.2M KCl. [A mixture of *E. coli* 30S proteins, 3.5mg in 15.5mL of binding buffer containing 0.2M KCl, was applied to a 1x3cm column containing 5.3mg of immobilized yeast 5.8S RNA. Chromatography was as in Figure 3, using a 30mL linear salt gradient (0.2M KCl + 2.0M KCl) to elute bound proteins. Fractions were pooled as follows: unbound (1-16), intermediate I (17-24), intermediate II (25-42) and bound (33-51). (---) KCl concentration.]
The first protein peak (Figure 22; unbound fraction) was broader than the corresponding peak in experiments with \textit{E. coli} 50S proteins. This trailing suggests the occurrence of weak interactions of \textit{E. coli} 30S proteins with yeast 55 and yeast 5.8S RNAs, as well as the stronger interactions required for the binding of proteins.

Two-dimensional gel electrophoresis of proteins in the bound fraction showed a very complex pattern with a large number of spots present (Figure 23 and 24). Positive identification of the proteins in the bound fraction was made by coelectrophoresis with total 30S proteins. The protein preparation used in these experiments lacked protein S1, which is removed by the high salt treatment used to prepare the ribosomes. Proteins S2 and S11 were also not visible on two-dimensional gels. The major \textit{E. coli} proteins bound to yeast 55 RNA were identified as S4, S9 and S20, with lesser amounts of S3, S5 and S7 also bound. Other lightly stained spots were also visible and were identified as S6, S13, S14, S15/16 and S18 (Figure 23 and Table 6). A similar set of proteins was bound to yeast 5.8S RNA (Table 6 and Figure 24). The major binding proteins were again identified as S4, S9 and S20 with lesser amounts of S5, S14, and S15/16 (Figure 24). Proteins S5 and S7, which bound quite readily to yeast 55 RNA, were hardly visible on two-dimensional gels of the protein fraction bound to yeast 5.8S RNA (compare Figures 23A and 24A).

Binding of \textit{E. coli} 30S ribosomal proteins to poly (U) was studied using binding buffer containing either 0.3M KCl or 0.2M KCl. No proteins were retained by the poly (U) column at 0.3M KCl, but at 0.2M
Figure 23. Two-dimensional gel electrophoresis of *E. coli* 30S proteins bound to yeast 5S RNA-Sepharose. [Affinity chromatography was performed at 0.2M KCl. Electrophoresis was as described in Methods, using the reduced gel apparatus. A) bound proteins (27µg), analyzed alone. B) bound proteins (27µg), mixed with 27µg of total *E. coli* 30S proteins.]
Figure 24. Two-dimensional gel electrophoresis of \textit{E. coli} 30S proteins bound to yeast 5.8S RNA-Sepharose, [when chromatography was performed at 0.2M KCl. Bound proteins (30\micro g), analyzed alone.]
KCl a significant amount of protein bound to poly (U) (Table 6). Again, the elution profile of the protein showed a trailing effect, suggesting weak interactions of some proteins with the poly (U). Proteins in the pooled fractions were identified by two-dimensional gel electrophoresis. The major proteins bound to poly (U) were S6 and S9 with lesser amounts of S4, S5, S7, S13, and S18 also observed (Figure 25).

**Binding of yeast 40S ribosomal proteins to immobilized RNAs**

Immobilized yeast 5S RNA and yeast 5.8S RNA were able to bind *E. coli* 30S ribosomal proteins. It was of interest to determine if these RNAs could also bind yeast 40S ribosomal proteins. In experiments with yeast 60S ribosomal proteins a few specific proteins were bound to *E. coli* 5S RNA, yeast 5S RNA and yeast 5.8S RNA at 0.3M KCl. This same salt concentration was used to study the binding of yeast 40S ribosomal proteins to immobilized RNAs. There was very little protein eluted by the high salt/EDTA buffer under these conditions (Table 7), only 1-3% of the protein recovered from the columns was found in the bound fraction. Washing columns containing immobilized yeast 5S RNA, yeast 5.8S RNA or *E. coli* 5S RNA, with 8M urea, 4M LiCl, 10mM Tris-HCl, pH 7.4 buffer, failed to elute significant additional amounts of protein.

Very little protein was bound to yeast 5.8S RNA and no identifiable proteins were visible on two-dimensional gels. The major protein bound to yeast 5S and *E. coli* 5S RNA was identified as S17 shown for *E. coli* 5S RNA in (Figure 26). Lighter protein spots were
Figure 25. Two-dimensional gel electrophoresis of *E. coli* 30S proteins bound to poly (U)-Sepharose at 0.2M KCl. [Bound proteins (20μg), analyzed alone.]
Table 7. Binding of yeast 40S proteins to immobilized RNAs.

Affinity chromatography of yeast 40S proteins on immobilized RNAs was performed using binding buffer containing 0.3M KCl, as described in Figure 3. Proteins were identified on two-dimensional gels, using standard nomenclature of Bollen et al. (1981).

<table>
<thead>
<tr>
<th>RNA</th>
<th>% bound (0.3M KCl)</th>
<th>Ribosomal proteins bound</th>
<th>Ribosomal proteins missing from unbound fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli 5S</td>
<td>2.3</td>
<td>S17 (major)$^a$</td>
<td>S17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S4, S7, S13, S22, S29 (minor)</td>
<td>S7, S14/15 (reduced)</td>
</tr>
<tr>
<td>Yeast 5S</td>
<td>3.5</td>
<td>S17 (major)</td>
<td>S17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S4, S7, S13 (trace)</td>
<td>S14/15 (reduced)</td>
</tr>
<tr>
<td>Yeast 5.8S</td>
<td>1.1</td>
<td>none identifiable</td>
<td>S17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>S7, S14/15 (reduced)</td>
</tr>
</tbody>
</table>

$^a$Intermediate fraction was also examined on two-dimensional gels. Major proteins present in this fraction were identified as S14/15 and S13 with lesser amounts of S7, S10, S21, S22 and S27.
Figure 26. Two-dimensional gel electrophoresis of yeast 40S proteins bound to *E. coli* 5S RNA at 0.3M KCl. [Bound proteins (40µg), analyzed alone.]
also visible on the gels. For yeast 5S RNA, these lighter protein spots were identified as 54, 57 and a very light spot identified as S13. Two-dimensional gel electrophoresis of the proteins bound E. coli 5S RNA showed the same proteins bound, with the addition of S22 and S29 (Figure 26).

Two-dimensional gels of proteins in the unbound fraction from the yeast 5S RNA and E. coli 5S RNA columns showed reduced levels 57 and S14/15, with S17 totally missing (results not shown). The gel of the unbound fraction from the yeast 5.8S RNA column also showed a slight reduction in the amount of S14/15, and S17 was again missing. The intermediate fraction eluted from the E. coli 5S RNA column was also examined. There were several very darkly stained spots visible on the gel and these proteins were identified as S14/15, S13 with lesser amounts of S7, S10, S21, S22, and S27 (results not shown). All of these results suggest that protein S17 interacts strongly with yeast 5S RNA, yeast 5.8S RNA and E. coli 5S RNA, and that protein S14/15 has a weaker interaction with these same RNAs.
DISCUSSION

Interaction of *E. coli* 50S Ribosomal Proteins with Immobilized RNAs

An affinity chromatography procedure to study the interaction of ribosomal proteins and ribosomal RNAs was developed in this laboratory (Burrell and Horowitz, 1975). This procedure was developed as an alternate approach to the study of protein-RNA interactions which occur in the ribosome. Earlier reports have described the method for preparing immobilized RNA columns and limited studies on the interaction of immobilized *E. coli* 5S RNA with *E. coli* ribosomal proteins (Burrell and Horowitz, 1975, 1977). Specific binding of ribosomal proteins was observed in buffer containing 0.3M KCl and 0.02M MgCl$_2$. Proteins L18 and L25 were strongly bound to the immobilized *E. coli* 5S RNA with small amounts of L5 also bound.

One purpose of the investigation described in this report was to determine which low molecular weight eukaryotic ribosomal RNA is the functional analog of prokaryotic 5S RNA. The rationale behind the experiments was to establish whether eukaryotic 5S RNA or 5.8S RNA binds the same set of ribosomal proteins that bind to *E. coli* 5S RNA, thus indicating a functional analogy. As a first step, the binding of *E. coli* 50S ribosomal proteins to *E. coli* 5S RNA was studied in more detail than previously reported. At 0.3M KCl, proteins L5, L18 and L25 were bound to *E. coli* 5S RNA confirming previous results (Figure 4) (Burrell and Horowitz, 1977). At a lower salt concentration, 0.2M KCl, two additional proteins, L2 and L17 were also bound (Figure 6). The affinity of these proteins for *E. coli* 5S RNA was lower than that of
proteins L5, L18 and L25 and the two groups of proteins were eluted separately by gradient elution from 5S RNA-Sepharose columns (Figure 5).

Using other experimental approaches, a number of investigators have identified an *E. coli* 5S RNA-protein complex containing proteins L5, L18 and L25 (see Introduction). Additional proteins have also been found to interact with *E. coli* 5S RNA. After incubation of *E. coli* 5S RNA with 50S proteins in a buffer containing 0.32M KCl and 0.02 MgCl₂, a nucleoprotein complex containing ribosomal proteins L5, L18, and L25 was isolated by sucrose gradient centrifugation; smaller amounts of proteins L1, L10, L7/12, L27 and L30 were also associated with this complex (Wrede and Erdmann, 1977). In the course of studies on the reconstitution of *E. coli* 50S subunits, it was found that appreciable amounts of protein L2, as well as L5, L18 and L25 bound to 5S RNA at 44°C in 4 mM Mg⁺²-containing buffer (Dohme and Nierhaus, 1976a).

Functional assays have also shown a close relationship between proteins L2, L5, L17, L18 and L25. Components involved in the peptidyltransferase activity of the 50S ribosomal subunit have been identified (Hampl et al., 1981). Protein L2 and L18 as well as L3, L4, L15, L16, and 23S RNA are essential for peptidyltransferase activity. Strong indications for the involvement of proteins L17, L25 and 5S RNA together with proteins L6, L10, L11, L13, L21, L22, and L27 were also obtained. Studies of the order of assembly of ribosomal proteins during reconstitution of active *E. coli* 50S subunits have also revealed interactions among ribosomal proteins. Proteins L2, L5 and
L18 plus L3, L4 and L15 play an essential role in the binding of E. coli 5S RNA to 23S RNA (Rohl and Nierhaus, 1982). This group of proteins is quite similar to the group of proteins required for peptidyltransferase activity.

Protein-protein crosslinking studies have helped to identify those proteins that are in close proximity to each other within the ribosome. Protein L2 has been crosslinked to L5 and L17 among other ribosomal proteins (Kenny and Traut, 1979). Crosslinks between L5 and L17, and L5 and L25 have also been identified (Kenny and Traut, 1979). These results suggest that proteins L2, L5, L17 and L25 are located near each other in the ribosome. Isolation of a protein complex containing L2 and L17 again indicates that a close association occurs between these protein in the ribosome (Wystup et al., 1979).

Other groups have adopted the affinity chromatography methods developed in this laboratory to determine the ribosomal proteins which interact with low molecular weight ribosomal RNAs and tRNAs (Ulbrich et al., 1980a; Metspalu et al., 1982a). Ulbrich et al. (1980a) showed that at room temperature (22°C), in binding buffer containing 0.3M KCl, proteins L5, L18 and L25 were bound to E. coli 5S RNA in agreement with the results of our experiments. Other investigators have used epoxy activated Sepharose 6B coupled to adipic acid dihydrazide to which periodate oxidized RNA was covalently attached (Ustav et al., 1978). This gave a spacer arm of about 30Å, which is three times longer than the spacer used in our experiments. The binding of E. coli 50S proteins to E. coli 5S RNA immobilized by this
method was studied using different ionic conditions and protein concentrations (Metspalu et al., 1982a). At a ribosomal protein concentration of 0.2 mg/mL, and with binding buffer containing 30 mM MgCl₂ and 200 mM KCl, proteins L5, L18, L25 and small amounts of L2 bound to the E. coli 5S RNA. When the protein concentration was increased to 0.4 mg/mL, using the same binding buffer, protein L17 was bound in addition to proteins L5, L18, L25 and L2. This protein concentration (0.4 mg/mL) is very close to the protein concentration used in our experiments, and the same group of proteins were bound to the immobilized RNA. When the ribosomal protein concentration was further increased to 1.8 mg/mL or binding buffer containing 1 mM MgCl₂ and 200 mM KCl was used, there was an increase in the number of proteins associated with E. coli 5S RNA. In addition to L5, L18, L25, L2 and L17, proteins L15, L16, L22, L33, L34 and variable amounts of L3 and L21 were bound (Metspalu et al., 1982a). These results, as well as the results of our experiments, clearly indicate that the binding of E. coli 50S ribosomal proteins to immobilized E. coli 5S RNA depends on the ionic conditions and protein concentration used.

Ribosomal proteins L2, L5, L17, L18 and L25 which were bound to immobilized E. coli 5S RNA have been shown to be functionally related. All of these proteins along with E. coli 5S RNA are involved in peptidyltransferase activity and L2, L5 and L18 are also required for binding of E. coli 5S RNA to 23S RNA in reconstitution experiments. These same proteins (L2, L5, L17, L18 and L25) have also been shown to be closely associated by protein-protein crosslinks. These results again indicate the suitability of the affinity chromatography proce-
dure for the identification of protein-nucleic acid interactions that occur within the ribosome.

From the results of these affinity chromatography experiments it was not possible to determine if bound proteins are interacting directly with RNA or binding indirectly by association with proteins L5, L18 and L25 which have been shown to interact directly with the RNA (Spierer and Zimmermann, 1978b). Metspalu et al. (1982a), designed an experiment to address this problem. Proteins L5, L18 and L25 were removed from the mixture of ribosomal proteins by repeated chromatography on immobilized *E. coli* 5S RNA, using conditions under which only proteins L5, L18 and L25 are bound. The ribosomal protein sample lacking proteins L5, L18 and L25 was then chromatographed on an immobilized *E. coli* 5S RNA column in buffer containing 1 mM MgCl₂ and 200 mM KCl. Under these conditions L2, L15, L16, L17, L22, L33 and L34 were bound to the RNA. These proteins clearly recognized specific sequences of the RNA or structural features that are present even in the absence of proteins L5, L18 and L25.

To determine whether the *E. coli* 50S ribosomal proteins that bind to *E. coli* 5S RNA also interact with eukaryotic 5S or 5.8S RNA the binding of *E. coli* 50S proteins to yeast 5S RNA and yeast 5.8S RNA was first investigated at 0.3M KCl. Neither of the yeast RNAs bound significant amounts of *E. coli* 50S proteins, in contrast to the results of binding experiments with *E. coli* 5S RNA where L18, L25, and L5 were bound (Figure 7). However, at lower salt concentrations (0.2M KCl) proteins L2, L17 and variable amounts of L16 were bound to both
yeast 5S RNA and yeast 5.8 RNA (Figures 8, 9, 10). Even at the lower salt concentration, immobilized yeast 5S RNA and yeast 5.8S RNA failed to bind E. coli ribosomal proteins L5, L18 and L25.

These results are similar to those of affinity chromatography experiments described by others (Ulbrich et al., 1978, 1979; Toots et al., 1979). Ulbrich et al. (1978, 1979) failed to detect the binding of any E. coli 50S ribosomal proteins to rat liver 5S RNA and rat liver 5.8S RNA at 0.3M KCl. Toots et al. (1979) also failed to detect the binding of E. coli 50S ribosomal proteins to mammalian 5S RNA or 5.8S RNA at 0.32M KCl and 0.02M MgCl₂. At lower salt concentrations, binding of E. coli ribosomal proteins to yeast 5.8S RNA and bovine liver 5.8S RNA was observed (Toots et al., 1979). At 0.2M KCl protein L2 was bound to yeast 5.8S RNA together with lesser amounts of L17 and L19. Using the same conditions, a somewhat different group of proteins was bound to bovine liver 5.8S RNA. The major binding protein was identified as L21, with lesser amounts of proteins L2 and L17 as well as L1, L3, L4, L19 and L20 also bound.

When the salt concentration was lowered to 0.1M KCl, the E. coli ribosomal proteins bound to yeast 5.8S RNA were identified as L2, L17 and L19 together with small amounts of L18. A similar group of proteins was bound to bovine liver 5.8S RNA at 0.1M KCl. The major binding proteins were identified as L2, L17, L19, L20 and L21 with lesser amounts of L1, L3, L4 and L5 bound (Toots et al., 1979). Binding of E. coli 50S proteins to eukaryotic 5S RNA at these lower salt concentrations was not studied. The results of these experiments are
3'-dialdehyde exposed and able to covalently attach to the dihydrazide Sepharose. However, when methylamine treated yeast tRNA was reoxidized, it failed to bind to the dihydrazide-Sepharose, indicating that the methylamine adduct is stable under the condition used for the oxidation reaction (Table 2, line 3). In another experiment, RNA to which glycerol had been coupled was mixed with dihydrazide-Sepharose prior to the oxidation reaction. Again there was no covalent attachment of the RNA (Table 2, line 4), suggesting that the 3'-end of the RNA remains blocked by methylamine and any covalent attachment of the RNA to dihydrazide-Sepharose must have occurred through its 5'-end.

When the binding of *E. coli* 50S proteins to 5'-immobilized RNAs was studied no significant protein binding was observed at 0.3M KCl (Table 3). This is in contrast to the binding of proteins L18, L25 and L5 to 3'-immobilized *E. coli* 5S RNA. At 0.2M KCl the set of proteins bound to 5'-immobilized *E. coli* 5S RNA was similar to that bound to 3'-immobilized 5S RNA. Proteins L2, L17, L18 and L25 were bound in both cases (Figures 6, 13) with additional proteins L16 and L21 bound when *E. coli* 5S RNA was immobilized through its 5'-end. When *E. coli* 5S RNA was immobilized through its 3'-end, bound proteins were eluted by the dissociation buffer in two peaks (Figure 5), the first peak containing proteins L2 and L17 and the second peak containing the more tightly bound proteins L18 and L25. No such separation was obtained when 5'-immobilized *E. coli* 5S RNA was used. Proteins L18 and L25 seem to have a reduced affinity for 5'-immobilized *E. coli* 5S RNA.

The binding of *E. coli* 50S ribosomal proteins to yeast 5S and yeast 5.8S RNA immobilized through the 5'-end was also studied. The
The main purpose of these experiments was to determine if the *E. coli* 5S RNA binding protein L5, L18 and L25 would bind to the 5'-bound yeast rRNAs. No significant amount of *E. coli* 50S proteins was bound to either of these yeast rRNAs at 0.3M KCl, but proteins L2, L16, L17, L21 and small amounts of L18 were bound to both yeast RNAs at 0.2M KCl (Figures 14, 15, and Table 3). 5'-immobilized yeast 5S RNA also bound protein L22. These proteins are similar to those bound to yeast 55S RNA and yeast 5.8S RNA immobilized through their 3'-ends (compare Table 1 and Table 3). Again there was no significant binding of proteins L18, L25 and L5 to 5'-immobilized yeast 5S or yeast 5.8S RNA; these proteins were bound to 5'-immobilized *E. coli* 5S RNA. It is interesting to note that protein L21 was bound to 5'-immobilized yeast 55S RNA, yeast 5.8S RNA and *E. coli* 5S RNA, but failed to bind to these RNAs when they were immobilized through their 3'-ends. Perhaps there is a change in the tertiary structure of the RNAs as a result of the 5'-attachment, exposing a binding site for protein L21.

An interaction of yeast 5.8S RNA with *E. coli* 50S proteins L18 and L25 could not be detected even when 5'-immobilized RNA was used. Since the recovery of protein from the immobilized RNA columns was always somewhat low, it is possible that proteins L18 and L25 remained bound to the RNA column even after elution with dissociation buffer. Perhaps these proteins had such a strong affinity for the RNA that the high salt/EDTA buffer was not sufficient for elution. The use of 8M urea/4M LiCl buffer to remove tightly bound ribosomal proteins from RNA has recently been reported (Metspalu et al., 1980). This buffer was used in an attempt to remove tightly bound *E. coli* 50S proteins.
main purpose of these experiments was to determine if the \textit{E. coli} 5S RNA binding protein L5, L18 and L25 would bind to the 5'-bound yeast rRNAs. No significant amount of \textit{E. coli} 50S proteins was bound to either of these yeast rRNAs at 0.3M KCl, but proteins L2, L16, L17, L21 and small amounts of L18 were bound to both yeast RNAs at 0.2M KCl (Figures 14, 15, and Table 3). 5'-immobilized yeast 5S RNA also bound protein L22. These proteins are similar to those bound to yeast 5S RNA and yeast 5.8S RNA immobilized through their 3'-ends (compare Table 1 and Table 3). Again, there was no significant binding of proteins L18, L25 and L5 to 5'-immobilized yeast 5S or yeast 5.8S RNA; these proteins were bound to 5'-immobilized \textit{E. coli} 5S RNA. It is interesting to note that protein L21 was bound to 5'-immobilized yeast 5S RNA, yeast 5.8S RNA and \textit{E. coli} 5S RNA, but failed to bind to these RNAs when they were immobilized through their 3'-ends. Perhaps there is a change in the tertiary structure of the RNAs as a result of the 5'-attachment, exposing a binding site for protein L21.

An interaction of yeast 5.8S RNA with \textit{E. coli} 50S proteins L18 and L25 could not be detected even when 5'-immobilized RNA was used. Since the recovery of protein from the immobilized RNA columns was always somewhat low, it is possible that proteins L18 and L25 remained bound to the RNA column even after elution with dissociation buffer. Perhaps these proteins had such a strong affinity for the RNA that the high salt/EDTA buffer was not sufficient for elution. The use of 8M urea/4M LiCl buffer to remove tightly bound ribosomal proteins from RNA has recently been reported (Metspalu et al., 1980). This buffer was used in an attempt to remove tightly bound \textit{E. coli} 50S proteins
from a column containing immobilized yeast 5.8S RNA. A small amount of protein was eluted with 8M urea/4M LiCl, and ribosomal proteins L1, L3, L4, L5, L6, L11, and L17 were identified in this fraction. The *E. coli* ribosomal proteins not bound to RNA in this experiment were also examined by two-dimensional gel electrophoresis, and there was no apparent reduction in proteins L18 and L25 present in this fraction. Again, the binding of *E. coli* proteins L18 and L25 to immobilized yeast 5.8S RNA could not be detected.

The results of these experiments failed to identify the eukaryotic low molecular ribosomal RNA which might be the functional analog of *E. coli* 5S RNA. Neither immobilized yeast 5S RNA or immobilized yeast 5.8S RNA were able to bind significant amounts of proteins L5, L18 and L25, which were bound to immobilized *E. coli* 5S RNA. The salt concentration of the binding buffer was changed and the site of RNA attachment to the Sepharose matrix was changed but still proteins L18 and L25 were not bound to immobilized yeast RNAs. In a recent review, Erdmann et al. 1980, reported the results of experiments using immobilized RNAs. All prokaryotic 5S RNAs tested were able to bind *E. coli* 50S ribosomal proteins L5, L18 and L25. In the same experiments, at 0.32M KCl, immobilized yeast 5S RNA and yeast 5.8S RNA failed to bind any *E. coli* 50S ribosomal proteins. Although yeast 5.8S RNA free in solution is able to form a complex with *E. coli* 50S proteins, L18 and L25 (Wrede and Erdmann, 1977), immobilized yeast 5.8S RNA fails to bind these same proteins. The reasons for this discrepancy are not clear at present.
Since E. coli 50S ribosomal proteins L2 and L17 were bound to all three low molecular weight ribosomal RNAs, the specificity of this binding was tested by studying the interaction of ribosomal proteins with the nonspecific polynucleotide, poly (U) coupled to dihydrazide-Sepharose. Similar to results with immobilized yeast RNAs, no E. coli 50S ribosomal proteins were bound to immobilized poly (U) at 0.3M KCl, but proteins L2 and L17 were bound at 0.2M KCl (Table 1). A column of immobilized oligo (U)$_6$ was also prepared, and this failed to bind any E. coli 50S ribosomal proteins at 0.2M KCl (Table 1). Apparently the binding of E. coli ribosomal protein L2 and L17 to immobilized polynucleotides requires a salt concentration of less than 0.3M and oligonucleotides greater than 6 nucleotides long.

Other investigators have also reported the binding of proteins L2 and L17 to immobilized polynucleotides (Sarapuu and Villems, 1982). At several different salt concentrations immobilized poly (U), poly (C), poly (A) and phage MS2 RNA bound proteins L2 and L17. These were the only E. coli 50S ribosomal proteins bound in significant amounts under the conditions tested. Transfer RNA immobilized to epoxy-activated Sepharose 6B also bound proteins L2 and L17 in addition to proteins L15, L16, L18, L22, L33 and L34 (Ustav et al., 1977a; Metspalu et al., 1982b).

These results suggest that the binding of E. coli proteins L2 and L17 to immobilized polynucleotides is simply a nonspecific interaction between the highly negatively charged phosphate backbone of the polynucleotide and the basic amino acid residues of the protein. The $\pi_1$ values for ribosomal proteins have been determined and both pro-
teins L2 and L17 are quite basic with pi values greater than 12
(Garrett and Wittmann, 1973). Yet proteins L15, L16, L19, L20, L27,
and L33 also have pi values greater than 12 and these proteins were
not bound to immobilized polynucleotides. Also proteins L2 and L17
are among the few E. coli 50S proteins that can be crosslinked to mRNA
analogues (Pongs et al., 1979). This suggests that the binding of
proteins L2 and L17 to immobilized polynucleotides is not a nonspeci­
fic interaction. But instead, proteins L2 and L17 may be part of the
mRNA binding site on the 50S ribosomal subunit.

Ribosomal protein L16 bound to E. coli 5S RNA, yeast 5S RNA, yeast
5.8S RNA and poly (U) in some experiments but not in others (Table 1).
Two-dimensional gel electrophoresis of the total E. coli 50S ribosomal
proteins indicated that only small amounts of L16 were present in the
protein preparations; L16 always appeared as a very light spot. In
those experiments that failed to show binding of L16, there appeared
to be less L16 in the protein sample, and this may account for the
absence of binding in these experiments.

Interaction of Yeast 60S Ribosomal Proteins
with Low Molecular Weight rRNAs

The technique of affinity chromatography of ribosomal proteins
on columns of immobilized RNA was also used to identify the yeast 60S
ribosomal proteins that interact with either yeast 5S RNA, yeast 5.8S
RNA as well as E. coli 5S RNA. These studies showed that about 3% of
the proteins were bound to these RNAs at 0.3M KCl (Table 4). The
major yeast ribosomal proteins bound to all three RNAs were identified as L2/3 and L23 (Figure 6). This is in contrast to results with *E. coli* 50S ribosomal proteins in which only *E. coli* 5S RNA bound a significant amount of protein at 0.3M KCl. When the salt concentration of the binding buffer was decreased to 0.2M KCl, there was an increase in the amount of yeast ribosomal proteins bound to all three RNAs; more than 12% of the protein was recovered in the bound fraction (Table 4). The major proteins binding to all three RNAs under these conditions included proteins L2/3 and L23 which were bound at 0.3M KCl, and protein L10. Protein L21 was identified as a major protein bound to yeast 5S RNA and *E. coli* 5S RNA, while this same protein was identified as a minor component in the protein fraction bound to yeast 5.85 RNA (Figure 17). In addition, protein L30 was identified as a major protein binding to yeast 5S RNA, while L12 was a major protein bound to yeast 5.8S RNA. Studies of the binding of yeast 60S ribosomal proteins to 5'-immobilized *E. coli* 5S RNA showed that at 0.2M KCl the major proteins bound to the RNA were L2/3, L10, L21, and L23, the same proteins that were bound to *E. coli* 5S RNA when immobilized through its 3'-end (Figure 18).

A recent report has identified yeast ribosomal proteins which bind to yeast 5.8S RNA (Lee et al., 1983). Three methods were used to identify these 5.8S rRNA binding proteins. RNA protein complexes were formed by incubation of total yeast 60S ribosomal proteins with purified yeast 5.8S RNA and isolation of the complex on sucrose gradients. Binding of yeast ribosomal proteins to immobilized yeast 5.8S
to identify proteins which interact with yeast 5.8S RNA. And finally, the interaction of individual proteins with yeast 5.8S RNA was studied using a nitrocellulose filtration assay. Purified individual proteins were incubated with the RNA and only RNA-protein complexes were retained by the membrane. By all three methods proteins L23, L30, and L31 were bound to yeast 5.8S RNA. Three other proteins L29, L35, and L41 were also bound to immobilized yeast 5.8S RNA. Binding of proteins to immobilized yeast 5.8S RNA was done at 25°C in a buffer containing 0.33M KCl and 0.02M MgCl₂ and about 4 to 5% of the proteins were bound. Additional proteins were found in the RNA-protein complex isolated from sucrose gradients, including proteins L12, L19, L20, L25, L33, and L39. The results of these experiments are somewhat different from the results we have obtained. Protein L2/3 was not identified as a binding protein by any of the methods tested, but proteins L12, L19, L21, L23 and L30 which were bound to yeast 5.8S RNA in our experiments were also identified as interacting with yeast 5.8S RNA in the report of Lee et al. (1983). The differences in the results of these experiments may in part be explained by the different methods of RNA and protein preparation. In the work of Lee et al. (1983), the RNA was isolated using 6M urea and then heated prior to covalent attachment to the Sepharose-dihydrazide. In our RNA preparation, no urea was used. Also, ribosomal proteins were extracted from purified ribosomal subunits using 4M urea and 2M LiCl rather than the acetic acid extraction method employed in our experiments. It is possible that these procedures yielded proteins and RNA with different secondary and ter-
tary structures which may explain the discrepancies between the results of Lee et al. (1983) and results we obtained. Also, different ionic conditions and temperature were used in the experiments of Lee et al. (1983). Ionic conditions have a strong effect on protein binding.

A 55 RNA-protein complex has been isolated from yeast 60 ribosomal subunits after EDTA treatment (Mazelis and Petermann, 1973; Nazar, 1979). This complex contained only one protein, identified as YL3 which is one of the few acidic yeast ribosomal proteins. None of the yeast 60S ribosomal proteins found bound to immobilized yeast 5S RNA or yeast 5.8S RNA in our experiments were acidic. It appears that the protein released from the 60S subunit in a complex with yeast 5S RNA does not bind to immobilized yeast 5S RNA under conditions used in our experiments. The difference in these results may be due to the method used to prepare the yeast 60S ribosomal proteins in our experiments. The proteins were extracted from the 60S ribosomal subunit with acetic acid and then dissolved in urea. This may have resulted in a partial denaturation of the proteins, and altered their conformation so that YL3 no longer interacted with yeast 5S RNA. Recently, a method for preparing E. coli ribosomal proteins that uses non-denaturing conditions has been developed (Littlechild and Malcolm, 1978). As judged by proton magnetic resonance, the proteins obtained by these mild procedures have a more complex tertiary structure than acetic acid/urea treated proteins (Morrison et al., 1977). In future experiments, proteins prepared using milder conditions should be used.
The *E. coli* 50S ribosomal proteins which bind to immobilized *E. coli* 5S RNA are necessary for peptidyltransferase activity (Hampl et al., 1981). Some work has also been done on the identification of yeast ribosomal proteins involved in peptidyltransferase activity in yeast ribosomes. Using two derivatives of Phe-tRNA as affinity labels, proteins involved in peptidyltransferase activity were identified. Proteins L2, L4/6, L7/8, L11, L17/18, L19/20, L22/23, L26, L29, L36, L42, and L43 were labelled by these analogues (Perez-Gosalbez et al., 1978). Protein L2/3 and L23 which were bound to immobilized yeast RNAs are part of this group of proteins, indicating possible involvement of these proteins in peptidyltransferase activity. The yeast ribosomal proteins, which bind to immobilized yeast 5S and yeast 5.8S RNA, may be functionally related to *E. coli* 50S ribosomal proteins L5, L18 and L25 which bind to immobilized *E. coli* 5S RNA and are also required for peptidyltransferase activity.

**Interaction of Rat Liver 60S Ribosomal Proteins**

with Low Molecular Weight rRNAs

Immobilized yeast RNAs were also able to bind significant amounts of ribosomal proteins from a higher eukaryotic organism, the rat. The binding of rat liver 60S ribosomal proteins to immobilized yeast 5S and yeast 5.8S RNA was studied at 0.3M KCl, 0.4M KCl and 0.45M KCl. The amount of rat liver 60S proteins retained by the immobilized yeast RNA columns at 0.3M KCl was much greater than the amount of *E. coli* 50S ribosomal proteins or yeast 60S ribosomal proteins bound under the same conditions; 41% of the protein was bound to yeast 5.8S RNA and
29% to yeast 5S RNA (Table 5). When the salt concentration of the buffer was increased to 0.4M KCl, only 13% of the ribosomal protein was bound to both yeast rRNAs (Table 5). At the lower salt concentrations, the binding of ribosomal proteins is quite nonspecific; at 0.3M KCl about one third of the 44 rat liver 60S ribosomal proteins were bound (Table 5). At 0.45M KCl, only 5.6% of the protein was bound by immobilized yeast 5.8S RNA. The four major binding proteins were identified as L3, L6, L14, and L35, with lesser amounts of L19, L24 and L27 also present (Table 5 and Figure 21). The proteins retained by the immobilized RNA at the higher salt concentration were undoubtedly the proteins with the highest affinity for the RNA.

Immobilized yeast 5S RNA and yeast 5.8S RNA bound a similar set of rat liver 60S ribosomal proteins. At 0.4M KCl, proteins L3, L4, L5, L6, L14, and L35 were the major proteins bound to both RNAs, but L27 showed significant binding only to yeast 5S RNA and L19 was bound strongly only by yeast 5.8S RNA (Table 5). This is similar to results of experiments with yeast 60S ribosomal proteins, in which a group of proteins (L2/3, L10, L21, and L23) were bound to both yeast RNAs with one additional protein bound specifically to each yeast RNA.

Other investigators have used affinity chromatography methods to study the binding of rat liver ribosomal proteins to immobilized RNAs. Using the procedure we developed, Ulbrich et al. (1978, 1979, 1980a) have identified rat liver ribosomal proteins that bind to rat liver 5.8S RNA, rat liver 5S RNA and E. coli 5S RNA. All experiments were run at room temperature using binding buffer containing 0.3M KCl and
20 mM MgCl₂. The proteins bound to rat liver 5.8S RNA were identified as L6, L8 and L19 with smaller amounts of proteins L14, L17, L18, L27/L27' and L35 also bound. When rat liver 5.8S RNA was covalently attached to epoxy-activated Sepharose 6B through a 30 Å spacer arm, it bound rat liver 60S ribosomal proteins L5, L6, L7, and L19 (Metspalu et al., 1978; Saarma et al., 1980). These chromatography experiments were done at 4°C using buffer containing 0.15M KCl and 20 mM MgCl₂. Differences in the results from these two laboratories cannot be easily explained. However, the proteins that were bound to rat liver 5.8S RNA were found to bind to yeast 5.8S RNA in our experiments, although the affinity of some of the proteins for the RNAs was different. While proteins L5, L6, L7, L19 and L8 were identified as having a high affinity for rat liver 5.8S RNA, the interaction with yeast 5.8S RNA was much weaker. These proteins were not strongly bound to yeast 5.8S RNA at 0.45M KCl, but did bind to yeast 5.8S RNA at lower salt concentrations. The differences between binding of rat liver 60S ribosomal proteins to immobilized yeast 5.8S RNA and rat liver 5.8S RNA cannot be explained solely by differences in the primary sequence of these RNAs since the sequences of these RNAs are quite similar (Ulbrich et al., 1979). But it should be noted that different E. coli 50S ribosomal proteins were bound to immobilized yeast 5.8S RNA and bovine liver 5.8S RNA even though the sequences of these RNAs are quite similar (Toots et al., 1979).

HeLa 60S ribosomal proteins L7 and L23' have been crosslinked to the 3' end of HeLa 5.8S RNA (Svoboda and McConkey, 1978). Failure of
rat liver protein L7 to bind either rat liver 5.8S RNA or yeast 5.8S RNA when immobilized to adipic acid dihydrazide-Sepharose may be explained by steric hindrance. Covalent attachment of the RNA to the Sepharose matrix through its 3' end using a short spacer as in our experiments and those of Ulbrich et al. (1979) may sterically hinder the binding of L7. When 5.8S RNA was attached to the Sepharose matrix through a longer spacer arm, significant binding of L7 to the RNA was observed (Metspalu et al., 1978).

Complexes formed between yeast 5.8S RNA and rat liver 60S ribosomal proteins have been prepared (Lee and Henry, 1982). The yeast 5.8S RNA was incubated at 37°C with a mixture of rat liver 60S ribosomal proteins in a buffer containing 0.34M KCl and 20 mM MgCl₂ and the RNA-protein complex was isolated from a sucrose density gradient. The proteins in this complex were identified as L6, L8, L19, L35 and L35a (Lee and Henry, 1982). Proteins L6, L19 and L35 were also bound to immobilized yeast 5.8S RNA in our experiments, even though the ionic conditions and temperature were somewhat different, 0.45M KCl and 4°C vs 0.34M KCl and 37°C. There are some differences in the identity of rat liver proteins bound to immobilized yeast 5.8S RNA and to yeast 5.8S RNA free in solution. These differences may be due to structural changes in the RNA due to immobilization or differences in the preparation of the proteins and the RNA. The rat liver ribosomal proteins bound to the yeast 5.8S RNA free in solution and immobilized yeast 5.8S RNA are similar to rat liver ribosomal proteins bound to immobilized rat liver 5.8S RNA. Clearly, there must be conserved
structural features present in the yeast 5.8S RNA which are recognized by rat liver 60S ribosomal proteins.

Rat liver 60S ribosomal proteins binding to rat liver 5S RNA attached to Sepharose 4B have been identified as L6 and L19 with smaller amounts of L7, L23', L27/27', L35' and L39 also bound (Ulbrich and Wool, 1978). Proteins L5, L6, L19 with lesser amounts of proteins L7, L8 and L35 were identified as binding to rat liver 5S RNA attached to epoxy activated Sepharose 6B through a longer spacer arm (Saarma et al., 1980). Earlier, affinity chromatography experiments using immobilized rat liver 5S RNA had failed to detect the binding of protein L5 to the immobilized RNA (Ulbrich and Wool, 1978; Metspalu et al., 1978). In later experiments, 8M urea/4M LiCl was used to elute tightly bound ribosomal proteins from a rat liver 5S RNA-containing column (Metspalu et al., 1980). The rat liver 5S RNA column was first washed with dissociation buffer and then the 8M urea/4M LiCl buffer was applied. Only protein L5 was eluted by this buffer. Again these results show some similarity to the results of rat liver ribosomal protein binding to yeast 5S RNA (Table 5), but some differences in the affinity of the proteins for the RNA are also apparent. Protein L19 which is bound strongly to rat liver 5S RNA shows only weak interaction with yeast 5S RNA.

A 5S RNA-protein complex is released from rat liver 60S ribosomal subunits by treatment with EDTA (see Introduction). The complex contains a single protein, identified as L5 (Tereao et al., 1980). Metspalu et al. (1980) also found protein L5 tightly associated with rat liver 5S RNA in affinity chromatography experiments. The complex formed between immobilized
rat liver 5S RNA and protein L5 was able to bind a molecule of rat liver 5.8S RNA (Metspalu et al., 1980), indicating that protein L5 has binding sites for both 5S RNA and 5.8S RNA. In our experiments, rat liver protein L5 was bound to both yeast 5S RNA and yeast 5.8S RNA, but less tightly than to rat liver rRNAs since it was eluted with high salt/EDTA buffer; none was recovered in the 8M urea/4M LiCl wash (Table 5).

As in experiments with E. coli ribosomal proteins, these experiments do not distinguish between proteins which interact directly with immobilized RNA and proteins which interact indirectly through other proteins bound to the RNA. In several recent reports, the binding of individual purified rat liver proteins to isolated rat liver 5S RNA and rat liver 5.8S RNA was studied by the nitrocellulose membrane filtration procedure (Ulbrich et al., 1980b; Todokoro et al., 1981). Radiolabeled RNA was incubated with purified proteins and only the RNA-protein complexes were retained on the filter. In all cases, individual proteins which were bound strongly by the immobilized RNA were also bound to free RNA in this filter assay. But most proteins which showed only weak interaction with immobilized RNAs failed to bind to the free RNAs in these experiments. This suggests that the latter proteins do not interact directly with the RNA, but instead interact with other proteins present in the complex. It is also possible that the proteins that interact strongly with the RNA cause a change in the RNA structure which is required before other proteins can bind. In these experiments purified protein L5 which has been identified in a complex with RNA released from the ribosome,
failed to bind to rat liver 5S RNA (Ulbrich et al., 1980b). Although several different methods were used to isolate protein L5 and different ionic conditions were tried no significant binding of the protein was observed (Ulbrich et al., 1980b). Perhaps the association of protein L5 and 5S RNA is dependent on the prior binding of other proteins.

Structure and Functions of RNA-Protein Complexes

The *E. coli* 50S ribosomal proteins L5, L18 and L25 that bind to immobilized *E. coli* 5S RNA are also part of a 5S RNA-protein complex isolated from native 50S ribosomal subunits (see Introduction), suggesting that the interaction of proteins and immobilized RNAs mimics the situation that occurs within the intact ribosome. Therefore, it is likely that the yeast ribosomal proteins that bind to immobilized yeast ribosomal RNAs also interact with the RNA within the native ribosome. Although there have been reports of yeast ribosomal proteins that interact with yeast 5.8S RNA (Lee et al., 1983), no reports have identified yeast ribosomal proteins that interact with yeast 5S RNA. Information on domains which may exist within the yeast ribosome are important since no one yet has reported the reconstitution of eukaryotic ribosomal subunits. Yeast ribosomal proteins L2/3, L10, L21, and L23 which bind to both yeast 5S and yeast 5.8S RNA may be part of a subribosomal domain in native ribosomes.

The fact that heterologous complexes of immobilized yeast RNAs and rat liver proteins can be formed suggests a conservation of structure in the components of eukaryotic ribosomes. Several models
for the secondary structures of ribosomal RNAs have been proposed and there is a high degree of homology in the models proposed for the low molecular weight ribosomal RNAs from both prokaryotic and eukaryotic organisms. But yet very little information exists on the tertiary structure of these RNAs within the native ribosome. The fact that most of the rat liver ribosomal proteins that bind to immobilized rat liver 5S and 5.8S RNAs also bind to the corresponding yeast RNAs suggests that similar tertiary structures exist for low molecular weight ribosomal RNAs in diverse and unrelated eukaryotic species.

Recent experiments have been designed to gain an understanding of the function of the subribosomal protein-RNA complexes formed from 5S and 5.8S RNA and ribosomal proteins. One proposed function of *E. coli* 5S RNA is binding of tRNA to the A site within the ribosome (Erdmann, 1976). Therefore, the binding of tRNA to protein-RNA complexes formed from rat liver 5S and 5.8S RNAs has been studied to see if eukaryotic low weight ribosomal RNAs serve a similar function (Chan et al., 1982; Saarma et al., 1981). Both rat liver 5S RNA-protein complexes and rat liver 5.8S RNA-protein complexes were able to bind about one mole of deacylated tRNA for each mole of RNA-protein complex (Saarma et al., 1981). Chan et al. (1982) studied the binding of purified tRNAs to the RNA-protein complexes. Purified initiator (tRNA$_{Met}^f$) tRNA and elongator tRNAs (tRNA$_{Phe}^m$ and tRNA$_{Met}^m$) were bound to both 5S and 5.8S RNA-protein complexes (Chan et al., 1982). The same RNA-protein complexes were also able to bind the tenary elongation complex EF-1alpha•GTP•Phe•tRNA$_{Phe}^m$ and the tenary initiator complex eIF-2•GTP•Met•tRNA$_{Met}^f$, supporting the idea that the low molecular
weight ribosomal RNAs are part of the tRNA binding domain in the intact eukaryotic ribosome.

A hypothesis suggests that the binding of tRNA to the ribosome involves interaction of the sequence TUCG, found in all noninitiator tRNAs, with the CGAA sequence found in prokaryotic 5S RNA (Erdmann, 1976). A similar sequence is found in eukaryotic 5.8S RNAs, but eukaryotic 5S RNAs lack this sequence. Instead eukaryotic 5S RNA have, in a similar position, the sequence GAUC which is complementary to the GAUC sequence found in eukaryotic initiator tRNAs. Prokaryotic initiator tRNAs lack this sequence (Erdmann, 1976). It has been proposed that in eukaryotes 5.8S RNA functions in the binding of elongator tRNA and 5S RNA in the binding of initiator tRNAs (Erdmann 1976). Direct experimental evidence to support this proposal is still lacking. It is interesting to note that in our experiments similar eukaryotic ribosomal proteins were bound to both yeast RNAs, but one protein shows specificity of binding to only one RNA. Yeast 60S ribosomal proteins L2/3, L10, L21 and L23 were bound to both yeast 5S RNA and yeast 5.8S RNA at 0.2M KCl. Under the same conditions L30 was bound strongly to yeast 5S RNA and L12 was bound strongly to yeast 5.8S RNA. Perhaps within the ribosome protein L30, in association with 5S RNA, is involved in binding initiator tRNA and L12 in association with 5.8S RNA is involved in elongator tRNA binding. Similar results were obtained with rat liver ribosomal proteins. At 0.4M KCl proteins L4, L5, L6, L14, L35 were bound to both yeast RNAs, with L27 bound strongly only to yeast 5S and L19 bound strongly to yeast 5.8S
RNA. It seems clear that the eukaryotic low molecular ribosomal RNAs are located close together within the ribosome since they bind similar proteins. Yet, the binding of specific proteins to only one RNA, suggests separate domains within the ribosome for the differential binding of elongator and initiator tRNAs.

The results of these experiments may also help to identify functional analogies between eukaryotic ribosomal proteins. In experiments with prokaryotic ribosomes, 5S RNA-protein complexes were reconstituted using *E. coli* 5S RNA and *B. stearothermophilus* 50S ribosomal proteins (Zimmermann and Erdmann, 1978). This complex contained *B. stearothermophilus* proteins BL5 and BL22 which are functionally equivalent to *E. coli* ribosomal proteins L18 and L25. In our experiments, we have formed homologous protein-RNA complexes with yeast RNA and yeast ribosomal proteins and heterologous complexes containing yeast RNA and rat liver ribosomal proteins. The primary sequences of the yeast ribosomal proteins and the rat liver ribosomal proteins bound to yeast RNAs should be compared, to determine if any sequence homology exists between the proteins from these organisms.

Interaction of Small Subunit Ribosomal Proteins with Low Molecular Weight rRNAs

Since interaction of the low molecular weight ribosomal RNAs of the large subunit with the small ribosomal subunit may be important for ribosome subunit association (Azad, 1978), the binding of small subunit ribosomal proteins to low molecular weight rRNAs of the large
subunit was also studied. At 0.2M KCl, large amounts of *E. coli* 30S proteins were bound to yeast 5S RNA and yeast 5.8S RNA. The major bound proteins were identified as S4, S9, S20 with smaller amounts of a number of other proteins (Table 6 and Figures 23, 24). Under the same conditions, the major proteins bound to poly (U) were identified as S6 and S9 (Figure 25). The binding of *E. coli* 30S ribosomal proteins to *E. coli* 5S RNA was studied by Burrell and Horowitz (1977) using binding buffer containing 0.3M KCl. No proteins were bound to the RNA, but the fraction eluted late by the binding buffer contained several proteins. The major protein was identified as S3 and lesser amounts of proteins S6, S9, S13, S18 and S19/20 were found.

The results of these experiments are in contrast to results of experiments with *E. coli* 50S ribosomal proteins. The same two *E. coli* 50S ribosomal proteins (L2 and L17) were bound to yeast 5S RNA, yeast 5.8S RNA and poly (U), but these RNAs bound different *E. coli* 30S proteins. Protein S9 was bound to yeast 5S RNA, yeast 5.8S RNA and poly (U) and it also showed weak interaction with *E. coli* 5S RNA. Proteins S4 and S20 interacted strongly with yeast rRNAs, but these proteins had little interaction with *E. coli* 5S RNA or poly (U). Protein S6, which is one of the major proteins bound to poly (U) (Table 6), interacted only weakly with yeast rRNAs, and S3, which interacted with *E. coli* 5S RNA had little interaction with yeast rRNAs or poly (U). Since proteins S4 and S20 were bound tightly only to yeast rRNAs and showed little interaction with poly (U), the binding of these proteins may represent a specific interaction of the yeast rRNAs with *E. coli* small subunit proteins.
Ustav et al. (1977b) studied the binding of *E. coli* 30S proteins to *E. coli* 5S RNA coupled to epoxy-activated Sepharose 6B through a 30Å spacer arm. The major protein bound was identified as S4 with lesser amounts of proteins S6, S7, S9, S13, S18 and S20. All of these proteins were also identified in our experiments as having interactions with immobilized yeast rRNAs (Table 6).

If interactions between low molecular ribosomal RNAs from the large subunit and ribosomal proteins from the small subunit are important for ribosome subunit association then the proteins involved in these interactions should be located at the interface between the subunits. Protein-protein crosslinking experiments have identified several proteins on the two subunits that can be crosslinked and are presumably located at the ribosome interface (Cover et al., 1981). Proteins S4, S6, S9 and S13 which were bound to immobilized RNAs (Table 6) were among the 30S proteins which were crosslinked to 50S proteins. The 50S proteins involved in these crosslinks include proteins L2, L5 and L17 (Cover et al., 1981) which were also bound to immobilized *E. coli* 5S RNA. It seems likely that some of the *E. coli* 30 ribosomal proteins which bound to the immobilized RNAs may be involved in ribosome subunit association.

When the binding of yeast 40S ribosomal proteins to immobilized yeast 5S RNA, yeast 5.8S RNA and *E. coli* 5S RNA was studied, very little protein was bound to any of the RNAs at 0.3M KCl (Table 7). The major protein bound to all three RNAs was identified as S17 with lesser amounts of proteins S14/15 (Table 7).
The binding of rat liver 40S ribosomal proteins to immobilized rat liver 5S RNA, rat liver 5.8S RNA and *E. coli* 5S RNA has been studied by Ulbrich et al. (1978, 1979, 1980a). No rat liver 40S ribosomal proteins were bound to rat liver 5S RNA. But rat liver 40S ribosomal proteins S13 and S9 were bound to rat liver 5.8S RNA and S9 was also bound to *E. coli* 5S RNA. From these results, it seems clear that the low molecular weight rRNAs are able to interact with specific small subunit proteins from eukaryotic ribosomes, an interaction which may be important in subunit association.
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