Identification by Affinity Chromatography of the Eukaryotic Ribosomal Proteins That Bind to 5 S Ribosomal Ribonucleic Acid

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Rat liver 5 S ribosomal RNA was oxidized with periodate and coupled to its 3' terminus to Sepharose 4B through an adipic acid dihydrazide spacer. The ribosomal proteins that bind to that nucleic acid were isolated by affinity chromatography and identified by electrophoresis in polyacrylamide gels. The eukaryotic 5 S rRNA binding proteins were L6 and L19: small molecular weight species, 18 and 28 S, and two smaller ones, 5 and 5.8 S. The latter two are components of the large ribosomal subparticle. An attempt is being made to identify, by affinity chromatography, the ribosomal proteins that interact with the several species of rRNA. The initial experiments, described here, were designed to characterize the proteins that bind to 5 S rRNA. The nucleic acid was oxidized with periodate and coupled to its 3' terminus to Sepharose 4B through an adipic acid dihydrazide spacer.

Ribosomes are composed of proteins and nucleic acids arranged in a specific three-dimensional configuration. Solution of the structure of these particles requires not only knowledge of the chemistry of the constituents but also information on their interaction. Rat liver ribosomes contain some 70 to 80 proteins (1-5) and 4 molecules of RNA—two high molecular weight species, 18 and 28 S, and two smaller ones, 5 and 5.8 S. The latter two are components of the large ribosomal subparticle. An attempt is being made to identify, by affinity chromatography, the ribosomal proteins that interact with the several species of rRNA. The initial experiments, described here, were designed to characterize the proteins that bind to 5 S rRNA. The nucleic acid was oxidized with periodate and coupled to its 3' terminus to Sepharose 4B through an adipic acid dihydrazide spacer.

**EXPERIMENTAL PROCEDURES**

**Preparation of Ribosomes, Ribosomal Subunits, and Ribosomal Proteins—Subunits were prepared from rat liver ribosomes (8) on a large scale by centrifugation in a zonal rotor (9), and the proteins extracted from the subparticles with 67% acetic acid in 10 mM Tris/HCl containing 33 mM magnesium acetate (10, 11). After extraction, the proteins were precipitated with acetone, collected by centrifugation, and stored at -20°C. The proteins of the 40 S subunit of rat liver ribosomes did not bind to the 5 S rRNA affinity column, nor did the proteins of either the large or small subparticle of Escherichia coli ribosomes.

**Preparation of 5 S rRNA—Rat liver 5 S rRNA was prepared from 80 S ribosomes by extraction with phenol and sodium dodecyl sulfate (12). The ribosomes (18 mg/ml) were suspended in Buffer A (50 mM Tris/HCl, pH 7.3; 0.1 mM NaCl, 1 mM EDTA) containing 2% sodium dodecyl sulfate, and an equal volume of freshly distilled phenol saturated with Buffer A containing 3% sodium dodecyl sulfate was added. The mixture was stirred at 4°C for 1 h and the aqueous layer was collected by centrifugation. The interphase was re-extracted with phenol. The process was repeated twice more. The rRNA in the pooled aqueous extracts was precipitated at -20°C with 2 volumes of 95% ethanol and 1/10 volume of 20% sodium acetate (pH 6.0). The precipitated rRNA was washed three times with 95% ethanol, dried by lyophilization, and dissolved in Buffer (10 mM Tris/HCl, pH 7.3; 1 mM EDTA) to give a concentration of about 5 mg/ml. The bulk of the high molecular weight rRNA (18 and 28 S) and the 5 S RNA hydroxylated to 28 S rRNA was precipitated by adding 1/4 volume of 5 M NaCl and keeping the solution at 0°C overnight. The precipitate was removed by centrifugation and the rRNA in the supernatant was precipitated with 95% ethanol and washed twice, dried by lyophilization, and dissolved in Buffer B (10 mM Tris/HCl, pH 7.4; 0.15 M NaCl; 1 mM EDTA). The rRNA solution (about 7000 A260 units) was applied to a column (2.5 × 30 cm) containing DEAE-cellulose that had been equilibrated with Buffer B. The rRNA was eluted at 12-15°C with a 4-liter linear gradient of 0.15 to 0.7 M NaCl in Buffer B. The flow rate was 50 ml/h and 10-ml fractions were collected. The elution of rRNA was monitored by determining absorption of fractions at 260 nm; the identity of the rRNA in the fractions was established by polyacrylamide gel electrophoresis (see below). The fractions containing 5 S rRNA were pooled and the rRNA was concentrated by ethanol precipitation. The rRNA was washed twice with 95% ethanol, dried by lyophilization, and dissolved in Buffer C (0.1 mM Tris/HCl, pH 8.0; 0.5 M NaCl; 0.01 M MgCl2). and 450 A260 units were applied to a column (1.5 × 90 cm) containing Sephacryl S-200 that had been equilibrated with Buffer C. Elution (10 ml/h) was at 4°C with Buffer C and 4-ml samples were collected.

**Preparation of Sepharose 4B-Adipic Acid Dihydrazide Conjugate—Adipic acid dihydrazide was synthesized and coupled to Sepharose 4B (13) which had been activated with cyanogen bromide (14); the product was washed thoroughly with water and with 0.2 M NaCl. Unreacted groups on the Sepharose were blocked by treatment for 1 h with 0.1 M ethanethiolamine and absorbed material was removed by washing extensively with 0.1 M sodium acetate, pH 5.0. The activated Sepharose 4B-dihydrazide conjugate was stored in the sodium acetate buffer at 4°C.

**Periodate Oxidation of 5 S rRNA—The 5 S rRNA was oxidized with 4.65 mM sodium periodate (15); treatment was for 1 h at 18°C in the dark. Excess periodate was removed by washing the rRNA a number of times with 0.1 mM sodium acetate (pH 5.0) and by repeated precipitation at -20°C with 2 volumes of 95% ethanol and 1/10 volume of 20% sodium acetate (pH 7.0). The precipitated, oxidized 5 S rRNA was stored at -2°C.

**Coupling of Oxidized 5 S rRNA to Sepharose-Adipic Acid Dihydrazide—The oxidized 5 S rRNA was coupled (7) to the Sepharose 4B-adipic acid dihydrazide (5 mg of oxidized 5 S rRNA in 0.1 M sodium acetate, pH 5.0, per g of dry gel). The 5 S RNA-Sepharose was packed in a column (1 × 10 cm), washed with 2.0 M KCl at 22°C until no substances that absorbed at 260 nm was in the eluate, and then equilibrated with Buffer D, i.e. binding buffer (20 mM Tris/HCl, pH 7.4; 0.5 M KCl; 20 mM MgCl2; 0.1 M Na2EDTA; 0.1 M 2-mercaptoethanol). About 85% of the oxidized 5 S RNA was actually coupled with the Sepharose-adipic acid dihydrazide. The estimate was made from the recovery of the oxidized rRNA, which was checked at each stage by UV absorption. The yields were in the range of 70-80% of the theoretical.

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ery of unreacted 5 S rRNA, i.e. the amount of material that absorbed at 260 nm found in the column wash.

Chromatography of Ribosomal Proteins on 5 S rRNA-Sepharose Affinity Column—Ribosomal proteins (0.5 mg/ml of Buffer D) were applied to the 5 S rRNA-Sepharose column (1 × 10 cm) and washed with Buffer D, at a flow rate of 8 ml/h, until there was no absorbance at 280 nm. Several column volumes of Buffer D were required to remove all the unbound ribosomal protein. Affinity chromatography was at 32°C; the binding buffer had a conductivity of 37 mS at that temperature. Bound proteins were eluted with a 30-ml linear gradient formed of equal parts of binding buffer and Buffer E, i.e. dissociation buffer (20 mM Tris/HCl, pH 7.4; 2 M KCl; 5 mM EDTA; 6 mM 2-mercaptoethanol). As a control, the ribosomal protein samples were passed through a column containing activated Sepharose 4B-adipic acid dihydrazide, but to which no rRNA had been coupled. After affinity chromatography, the column containing 5 S rRNA-Sepharose was equilibrated with Buffer D and kept at 4°C.

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate—Ribosomal proteins from affinity chromatography were analyzed by gel electrophoresis in sodium dodecyl sulfate (16) using the Laemmli procedure (17), but with 15% polyacrylamide. The same procedure, but without sodium dodecyl sulfate, was used to identify, and determine the purity of, 5 S rRNA. For the analysis of rRNA, the gels were stained for 6 h with 0.1% methylene blue, 0.2 M sodium acetate, 0.2 M acetic acid (19); destaining was with 10% acetic acid.

Two-dimensional Polyacrylamide Gel Electrophoresis—Ribosomal proteins were identified by micro-two-dimensional polyacrylamide gel electrophoresis (18) using a modification (19) of the usual procedure (20, 21). The proteins in fractions from affinity chromatography were precipitated with 15% trichloroacetic acid, washed with acetone, and dissolved in sample buffer (19) before electrophoresis.

Determination of the Concentration of Protein—The concentration of protein in samples was determined (22) using bovine serum albumin as a standard. If the samples contained 2-mercaptoethanol or high concentrations of salts, they were dialyzed against 2% acetic acid, and concentrated by lyophilization before the determination.

RESULTS AND DISCUSSION

Preparation of 5 S rRNA—Ribosomal RNA was extracted from rat liver 80 S ribosomes with phenol and sodium dodecyl sulfate. The high molecular weight rRNA (18 and 28 S) was precipitated with molar NaCl. Most of the 5.8 S rRNA, which is associated to 28 S by hydrogen bonds, is also precipitated with NaCl. The 5 S rRNA was purified from the supernatant by chromatography on DEAE-cellulose and filtration through Sephacryl S-200. The 5 S rRNA binds to DEAE-cellulose (18 and 28 S do not) and can be eluted with NaCl (at about 0.35 M). The 5 S rRNA fraction from ion exchange chromatography on DEAE-cellulose was resolved further by Sephacryl S-200 filtration and three peaks were obtained. The first peak was shown by polyacrylamide gel electrophoresis to contain RNA larger than 5 S (Fig. 1, Fraction 35); the second peak had pure 5 S rRNA (Fractions 40, 44, 50, and 53); and the third peak material of about 4 S—probably transfer RNA and, perhaps,

RNA fragments (Fractions 56 and 60). The 5 S rRNA in Fractions 44 to 53 was pooled and used to construct the 5 S rRNA-Sepharose affinity columns.

Affinity Chromatography of Ribosomal Proteins on 5 S rRNA-Sepharose—A simple strategy was adopted to identify the proteins that bind to 5 S rRNA (6, 7): ribosomal proteins were chromatographed on a column on which rat liver 5 S rRNA was immobilized by coupling its 3' terminus to Sepharose 4B. When the proteins of the 60 S subunit of rat liver ribosomes were passed through the 5 S rRNA-Sepharose column (Fig. 2), the majority were not retained (Peak A). However, a small fraction of the proteins (about 7%) did bind; that subset was eluted (Peak B) with buffer containing a high concentration of salts and EDTA (dissociation buffer).

A preliminary identification of the proteins in Peak A (not retained) and Peak B (eluted with dissociation buffer) was made by one-dimensional electrophoresis in gels containing sodium dodecyl sulfate (Fig. 3). The bound fraction (Peak B) contained two prominent bands: one had a molecular weight of approximately 33,000; the other of about 25,000. There were, in addition, several less prominent bands. The stained gel of the proteins that bound to 5 S rRNA was scanned at 540 nm and compared with a gel of all the proteins of the 60 S ribosomal subunit (Fig. 3). Most of the proteins of the large subunit have been assigned to one or another of the 21 bands; the assignments were made by analysis of single purified RNA fragments (3). Eleven of the bands contain only a single protein. The protein with a molecular weight of 33,000 was likely to be L6 since it could be aligned with a band that contained only one protein. That band is the most prominent one—it comprises about 42% of the bound protein—and appears to be removed completely from the mixture of all the 60 S ribosomal proteins by affinity chromatography on 5 S rRNA-Sepharose (Fig. 3). The second prominent protein (M_r = 25,000) makes up approximately 23% of the protein bound to 5 S rRNA—migrated in a band that contained both L14 and L19; thus, identification required two-dimensional gel electrophoresis (see below). The minor proteins could not be identified with any certainty since they coincided with bands that contained two or more proteins (Fig. 3).

A more precise identification of the proteins that were bound to 5 S rRNA was made by two-dimensional gel electrophoresis (Fig. 4). The identity of L6 was confirmed, and the second prominent 5 S rRNA-binding protein was shown to be
Eukaryotic 5 S rRNA Binding Proteins

It is not certain if L27 or L27', or both, are among the minor proteins that associate with 5 S rRNA. L27 and L27' are not resolved by two-dimensional polyacrylamide gel electrophoresis and, while they can be separated by electrophoresis in gels containing sodium dodecyl sulfate, in this instance, the identification is complicated since L23', another minor protein, migrates in the same band as L27.

activated Sepharose. In a more important experiment, it was found that no 40 S ribosomal protein associated with immobilized 5 S rRNA; nor did the proteins of either the small (30 S) or large (60 S) subparticle of E. coli ribosomes. The results were reassuring in that they indicated that the binding of L6 and L19 to 5 S rRNA was likely to be specific, not merely the chromatography of a basic protein on a nucleic acid. The failure of any 40 S protein to bind to 5 S rRNA suggests that that interaction (i.e. of 40 S ribosomal proteins with 5 S rRNA) does not participate in the association of ribosomal subunits. The failure of any E. coli ribosomal proteins to bind to rat liver 5 S rRNA suggests that rat liver L6 and L19 are not structurally homologous with E. coli L5, L18 and L25—the proteins that bind to E. coli 5 S rRNA (23-25). The results are not entirely unexpected since Wrede and Erdmann have shown (26) that E. coli L18 and L25 bind to yeast 5.8 S rRNA and not to 5 S rRNA. That and other evidence indicates the homologous ribosomal nucleic acids are eukaryotic 5.8 S and prokaryotic 5 S (26, 27).

The eukaryotic 5 S rRNA binding proteins, as revealed by affinity chromatography, are L6 and L19. A 5 S rRNA-protein complex (5 S RNP) can be extracted from the large subunit of eukaryotic ribosomes with EDTA (28-31). The single protein in the complex was identified as L5 (31) and it was assumed that it was one of, or the only, protein associated with 5 S rRNA in the ribosome. No L5 was bound to immobilized 5 S rRNA in the experiments described here. The results of the two sets of observations can be reconciled in several ways. In none of the EDTA experiments was the identification of the 5 S rRNA sufficiently stringent so as to rule out confusion with 5.8 S rRNA; thus, the EDTA particle might contain L5 and 5.8 S rRNA. The possibility was tested: the 5 S RNP was prepared (31) and the rRNA was identified by polyacrylamide gel electrophoresis—5 S and 5.8 S rRNA standards were analyzed at the same time. The result was certain: the particle contains 5 S rRNA. Moreover, we confirmed that the single ribosomal protein in the complex is L5 (results now shown). A second possibility is that EDTA unfolded the ribosomal subparticle and caused the proteins to lose their specific locations on ribosomal RNA. EDTA will randomize the distribution of proteins in E. coli ribosomal subparticles (32). A third possibility is that the 5 S RNP particle prepared with EDTA preserves the physiological

Fig. 3. Electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate of 60 S ribosomal subunit proteins separated by affinity chromatography on 5 S rRNA-Sepharose. TP60, a mixture of 40 µg of all the 60 S ribosomal subunit proteins; A, 45 µg of 60 S ribosomal subunit proteins that did not bind to 5 S rRNA-Sepharose (Peak A in Fig. 2); B, 3 µg of the proteins that bound to 5 S rRNA-Sepharose (Peak B in Fig. 2). The proteins that bound to 5 S rRNA-Sepharose (Peak B) were scanned at 540 nm. A diagram, giving assignments of all the proteins of the 60 S ribosomal subunit to individual bands, is at the top, and at the bottom is a tracing of the scan of the gel.

L19 (not L14). There were, in addition, five less prominent spots: L7, L23', L27/L27', L35', and L39 (see below).

Experiments of the kind described here must be closely controlled, if for no other reason, because basic ribosomal proteins can easily associate nonspecifically with nucleic acids. No 60 S ribosomal proteins (nor any other ribosomal proteins for that matter) bound to the activated Sepharose 4B-adipic acid dihydrazide conjugate indicating the proteins did not merely associate by ion exchange chromatography on unsubstituted hydrazide groups or on reactive groups on the CNBr-
binding whereas affinity chromatography on 5 S rRNA-Sepharose gives spurious results. The circumstances in which the chromatography was done—the ionic conditions were selected to favor detection of even weak binding of protein to 5 S rRNA—and the control experiments mitigate against the third possibility. There is another possibility and that is that the binding site for L5 is at or near the 3' end of 5 S rRNA and that site is perturbed by periodate oxidation or coupling to the adipic acid dihydrazide or both.

Ionic strength is critical in the association of proteins with nucleic acids (33). At relatively low ionic strength, nonspecific binding can occur and at relatively high ionic strength proper binding can be inhibited. The ionic strength of the binding buffer used in the affinity chromatography experiments described here was 0.38, close to that used by Traub and Nomura (33). At relatively low ionic strength, nonspecific binding can occur and at relatively high ionic strength proper binding may or may not occur with E. coli 5 S rRNA and can be identified on two-dimensional gel plates (26). The latter are generally considered nonspecific contaminants. The rat liver ribosomal proteins that bind weakly to 5 S rRNA (i.e. L7, L23', L27/L27', L35, and L39) result from protein-protein interaction, i.e. they may bind to L6 and L19 associated directly with 5 S rRNA. The proteins may or may not associate with L6 and L19 in the ribosome. It is also possible that the binding of L6 or L19 or both alters the conformation of 5 S rRNA and creates binding sites for the other proteins: that process does occur with E. coli 16 S rRNA (34, 35). Since the binding of the minor proteins would require prior binding of L6 and L19, smaller amounts would be expected. Finally, one must recognize that proteins that are associated physiologically with 5 S rRNA might not bind during affinity chromatography because the structure of the nucleic acid has been altered, just as some proteins might bind factitiously because binding sites have been created by alterations of structure.

The experiments establish the feasibility of identifying by affinity chromatography the eukaryotic ribosomal proteins that bind to rRNA, just as had been demonstrated before (6, 7) for prokaryotic protein-rRNA interactions.

Acknowledgment—This paper is dedicated to Dieter Roth.

REFERENCES
Identification by affinity chromatography of the eukaryotic ribosomal proteins that bind to 5 S ribosomal ribonucleic acid.
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