RNA binding proteins of the large subunit of bovine mitochondrial ribosomes

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Received October 20, 1987; Revised and Accepted February 24, 1988

ABSTRACT

RNA binding properties of proteins from the large subunit of bovine mitochondrial ribosomes were studied using four different approaches: binding of radiolabeled RNA to western blotted proteins; disassembly of the intact 39 S ribosomal subunits with urea; binding of ribosomal proteins to RNA in the presence of urea; and binding of proteins extracted with lithium chloride to RNA. Results from these four approaches allowed us to identify a set of six proteins (L7, L13, L14, L21, L26, and L44) which appear to be strong RNA binding proteins. Seven additional proteins (L8, L11, L28, L35, L40, L49, and L50) were identified as secondary RNA binding proteins. RNA binding properties of the proteins in both of these sets were compared with the topographic disposition and susceptibility towards lithium chloride extraction of the individual proteins. Proteins from the first set are good candidates for early assembly proteins since they have a high affinity for RNA, are generally found in 4M lithium chloride core particles, and are among the most buried proteins in the 39 S subunit.

INTRODUCTION

Mammalian mitochondrial ribosomes (mitoribosomes) represent a unique class of ribosomes as revealed from their physicochemical and functional properties (1-3). These mitochondrial ribosomes are approximately the same size as bacterial ribosomes but they contain nearly twice as much protein and only about half of the RNA found in bacterial ribosomes (4). They contain 85 different proteins (5), which as revealed from immunological and electrophoretic analyses represent a distinct set from either bacterial or cytoplasmic ribosomal proteins (r-proteins) (6, 7). All of the proteins are products of cytoplasmic protein synthesis (8), and consequently, all must be imported for coordinate assembly with mitochondrial RNAs. The requirement for the coordinate participation of both the mitochondrial and nuclear genomes and the exceptionally high content and large number of proteins in mitoribosomes raises numerous questions about their assembly and structural organization. Of particular interest are those proteins involved directly in RNA-protein interactions since these are likely candidates for early

assembly proteins. In addition, such proteins may comprise a subset of proteins homologous to RNA binding proteins in other classes of ribosomes (9, 10, 11).

The work reported here is the first to identify rRNA binding proteins in mitochondrial ribosomes. Mammalian mitochondrial ribosomes present a particular challenge, in this respect, since they are so protein rich. Attempts to study RNA:protein interactions in these mitochondrial ribosomes are hindered by the large number of proteins and their tendency to aggregate when classical approaches to study ribosome assembly are followed. We used four alternative approaches in this study to identify RNA binding proteins in the large subunit (39S) of bovine mitochondrial ribosomes: (a) binding of radiolabeled RNA to proteins on western blots, (b) disassembly of 39 S mitoribosomal subunits by treatment with urea to identify proteins which remain associated with the RNA, (c) binding of proteins to RNA in the presence of urea, and (d) binding of lithium chloride extracted proteins to RNA in solution. Through a combination of these approaches, it appears that 6 out of the total 52 proteins in the large subunit of bovine mitoribosomes interact directly with the RNA. The RNA binding properties of these proteins are correlated with their other properties, including topographic disposition and susceptibility towards "salt stripping".

MATERIALS AND METHODS

Isolation of ribosomal subunits

Bovine mitochondrial ribosomes were isolated from liver mitochondria as described previously (5). Mitoribosomal subunits were derived from 55 S ribosomes by centrifugation in 10-30% linear sucrose gradients in solution C (5 mM MgCl₂, 300 mM KCl, 5 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.5). *E. coli* ribosomes were prepared from *E. coli* (K-12 strain 1200 su⁻) as described (12). Crude ribosomes were washed in solution A (350 mM NH₄Cl, 10 mM MgCl₂, 10 mM Tris-HCl, pH 7.4) and the ribosomal subunits were derived from 70 S ribosomes by zonal centrifugation in a 10-30% linear sucrose gradient in solution B (5 mM MgCl₂, 500 mM KCl, 5 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.4).

Extraction of ribosomal proteins and two-dimensional gel electrophoresis

Mitochondrial ribosomal proteins were extracted with 9 M urea, 3 M LiCl (5) and *E. coli* ribosomal proteins were extracted with acetic acid (13). The conditions for two-dimensional gel electrophoresis (2D PAGE) have been described (5). Briefly, electrophoresis in the first dimension was in 3.5%

polyacrylamide stacker gels in 60 mM KAc buffer, pH 6.7 with 9 M urea and in 4.6% polyacrylamide separator gels in 340 mM KAc buffer, pH 4.3 with 9 M urea. The electrophoresis in the second dimension was in a 10% polyacrylamide slab gel containing 0.5% SDS, 5 M urea, and 100 mM sodium phosphate, pH 7.2.

Isolation and radioiodination of rRNA

Mitoribosomal 16 S RNA was extracted from 39 S subunits of bovine mitoribosomes and from intact mitochondria. RNA isolated from mitochondria by the guanidinium isothiocyanate method (14) was fractionated on 10-30% linear sucrose density gradients in solution D (0.5% SDS, 100 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 7.4) and the 16 S RNA was collected and further purified by a second gradient centrifugation.

E. coli 16 S RNA was isolated from "salt washed" ribosomes by treatment with solution D containing 2% SDS and the RNA was purified by centrifugation on 10-30% linear sucrose density gradients in solution D. Mitochondrial and E. coli rRNAs were radioiodinated with ^{125}I using IODO-GEN (Pierce Co., Rockford, IL) as an oxidizing agent (15). Labeling reactions were carried out in 20 mM NaAc buffer, pH 4.5 for 30 min. at 40°C.

Binding of RNA to r-proteins on western blots.

Gels containing ribosomal proteins separated by 2 D PAGE were incubated for 15 min. in 25 mM Tris, 190 mM glycine buffer pH 8.3, containing 0.05% SDS. The proteins were electrophoretically transferred to nitrocellulose in the same Tris-glycine buffer, but lacking SDS. After protein transfer, the nitrocellulose blots were incubated twice for 30 min. at room temperature in buffer E (5 mM MgCl_2 , 5 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.2, and 100 or 300 mM KCl, depending on the stringency desired). Following a 15 min. incubation in solution E containing 0.4% glycine, individual blots were incubated with 100-200 pmoles of ^{125}I -labeled 16 S RNA (approx. $1 - 2 \times 10^5$ cpm/pmole) for 1 hour at room temperature in 100 ml of solution E. The blots were washed in solution E three times for 30 min. at room temperature, air dried and exposed to Kodak XAR-5 film. RNA binding proteins were identified by superimposing the autoradiograms on the blots after staining the proteins with fast green (16).

Control protein RNA binding assays were performed with horse heart cytochrome c or ribosomal proteins extracted from E. coli 30 S subunits. Multiple aliquots containing 3.3 ug of each protein were spotted onto nitrocellulose using a Schleicher and Schüell dot blot apparatus. Nitrocellulose was cut into strips containing two sets of triplicate spots of each

protein. The strips were incubated in solution E as described above and probed with radioiodinated E. coli 16 S RNA (270 pmoles, specific radioactivity 8,000 cpm/pmole).

Partial disassembly of 39 S mitoribosomal subunits by urea

Ribosomal 39 S subunits were treated with 6 M urea in solution F (100 mM KCl, 5 mM MgCl₂, 5 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.2) at a concentration of 700 pmoles/ml for 10 min. at 4°C. The mixture was centrifuged at 4°C for 1 hour at 218,000 x g to sediment the urea resistant core particles. This centrifugation is sufficient to clear particles with sedimentation coefficients higher than 6.6 S (17). The supernatant was saved, and the pellet was re-extracted and centrifuged as before. Supernatants were combined and analyzed by 2D PAGE. Proteins remaining in the core particles were extracted with 9 M urea, 3 M LiCl and also analyzed by 2D PAGE.

Binding of mitoribosomal proteins to RNA in the presence of urea

Mitoribosomal proteins were extracted with 9 M urea, 3 M LiCl as described above, except that the RNA was not removed by centrifugation. Instead, the extracted sample containing both the proteins and RNA, was dialyzed against a solution containing 9 M urea, 0.01% aminoethanethiol and 67 mM KAc pH 6.7. The proteins from this sample were analyzed directly by 2D PAGE and compared to a control sample in which the RNA was removed by digestion with RNase A.

Lithium chloride extraction and radiolabeling of mitoribosomal proteins

Proteins from the large mitoribosomal subunits were extracted by lithium chloride as described (17). The subunits (10 A₂₆₀ U, at a concentration of 1.1 nmoles/ml) were serially extracted with 1 M and 2 M LiCl, each for 1 hour at 4°C. The mixtures were then centrifuged at 4°C for 1 h at 218,000 x g, and the supernatants were dialyzed overnight against 0.7 M LiCl, 70 mM KCl, 1 mM EDTA, and 20 mM Tris-HCl, pH 7.4. Individual fractions of proteins were radiolabeled with ¹²⁵I using IODO-GEN as an oxidizing agent (18). The radiolabeled proteins were extensively dialyzed in 0.5 ml aliquots against solution G (20 mM MgCl₂, 200 mM KCl, 5 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.4). After dialysis, radioactivities of the protein solutions ranged from 1.3 - 2.5 x 10⁶ cpm/ml.

Binding of lithium chloride extracted mitoribosomal proteins to RNA

The ¹²⁵I-labeled r-protein samples were centrifuged for 1 h at 218,000 x g to remove aggregates with sedimentation coefficients higher than 5 S (17). Supernatants containing soluble proteins were saved and typically 250 ul of this protein solution was mixed with 1.2 A₂₆₀ U of 16 S mitoribosomal

RNA in solution G and chromatographed on a Sephacryl S 200 column (1 x 27 cm). The elution profile was monitored at 254 nm and by counting the ^{125}I radioactivity of the effluent. To minimize protein losses, the column was primed with 70 ml of solution G containing 10 $\mu\text{g/ml}$ RNase free BSA and 0.05% glycine, and washed with solution G prior to sample application. The RNA-protein complexes eluting in the void volume were pooled and mixed with 3 A_{260} U of carrier 39 S subunits. The MgCl_2 concentration was raised to 40 mM and complexes were precipitated for 20 min. with 2 volumes of cold ethanol (19). The precipitate was resuspended in water, and the proteins were extracted with 9 M urea, 3 M LiCl and analyzed by 2D PAGE. Gels were stained with Coomassie Brilliant Blue R-250, dried and autoradiographed. The RNA binding proteins were identified by superimposing the autoradiograms on the stained gels.

RESULTS

Binding of mitoribosomal RNA to r-proteins on western blots

The 39 S subunits of bovine mitoribosomes contain 52 different r-proteins which are poorly soluble as a group, mostly hydrophobic, having similar isoelectric points and molecular weights. In addition, mitoribosomes are of very low abundance (20) making the purification of individual mitochondrial r-proteins a difficult task. To overcome these difficulties we used a new procedure which allows the detection of DNA and RNA binding proteins by blotting electrophoretically separated proteins on nitrocellulose and probing them with radiolabeled nucleic acids (21-24). To ensure that our experiments were done under conditions which promote specific interactions, we performed control experiments using r-proteins from *E. coli* 30 S subunits and cytochrome c and probed these with radiolabeled *E. coli* 16 S RNA. Cytochrome c, a globular protein of a size and isoelectric point similar to the majority of ribosomal proteins serves as a good control for nonspecific binding. When cytochrome c is probed with the bacterial RNA, the substantial interaction which can be detected at low salt conditions (50 mM KCl), is dramatically reduced as a function of ionic strength, indicating that this binding is mainly due to nonspecific ionic interactions with this basic protein (Fig. 1). In contrast, the bacterial 16 S RNA binds well to the *E. coli* r-proteins under the whole range of conditions tested (50-400 mM KCl), with optimal binding occurring at 100 mM KCl (Fig.1). Taking into account conditions used by others for ribosome reconstitution studies (25, 26), we determined that 300 mM KCl in solution E is the best KCl concentra-

Figure 1. Binding of ^{125}I -labeled *E. coli* 16 S RNA to *E. coli* r-proteins (o) and cytochrome c (●) dot blotted onto nitrocellulose, as a function of KCl concentration in solution E.

tion to differentiate between specific and nonspecific interactions. These conditions are also similar to those used by others for probing r-proteins from *E. coli*, chloroplasts (21) and yeast (22) on western blots. In control experiments, we used these conditions to bind ^{125}I -labeled 16 S RNA from *E. coli* to 30 S r-proteins from *E. coli* on western blots and we detected RNA binding to proteins S4, S9, S12, S13, S14, S17, S18, S19 (Fig. 2). All of these proteins have been identified by others (27) as RNA binding proteins. Under somewhat different conditions, Rozier and Mache (21) detected RNA binding to proteins S5 and S7, suggesting that the ability of certain ribosomal proteins to interact with rRNA is sensitive to preparation conditions. In addition, primary RNA binding proteins which have a major non-electrostatic component to their binding interaction such as S8 (28) fail to bind RNA on Western blots. Accordingly, this method may also fail to detect certain mitochondrial RNA binding proteins.

Proteins isolated from the large subunit of mitoribosomes were separated by 2D PAGE and blotted onto nitrocellulose membranes (Fig. 3C). Mitochondrial ^{125}I -labeled 16 S RNA from 39 S ribosomal subunits was incubated with similar blots of r-proteins under either low stringency (solution E containing 100 mM KCl) (Fig. 3A) or high stringency conditions (solution E containing 300 mM KCl) (Fig. 3B). Under low stringency conditions, 19 proteins bound the RNA (L6, L7, L8, L10, L11, L13, L14, L18, L21, L23, L25, L26, L28, L35, L40, L44, L49, L50, L51/52). Of these proteins only 9 (L6, L7, L8, L13, L14, L21, L26, L28, L44) bind RNA at the higher stringency.

Figure 2. Binding of ^{125}I -labeled *E. coli* 16 S RNA to *E. coli* 30 S r-proteins separated by 2D PAGE and blotted onto nitrocellulose. (A), western blot of stained r-proteins; (B), autoradiogram of western blot probed with RNA; (C), schematic diagram showing proteins which bind RNA (solid spots). The second-dimension positions and molecular weights ($\times 10^3$ daltons) of several marker proteins are shown at the right of the schematic diagram.

gency, indicating that for most of the 19 previously observed mitochondrial r-proteins, ionic interactions contribute heavily to the stability of RNA-protein complexes. A high contribution of ionic interactions to the binding is also apparent for 7 of the latter proteins (L6, L8, L13, L21, L26, L28, and L44) since they bind RNA less under this higher salt condition (Fig. 3B). Two spots (L7, L14), however, appear to be of even higher

Figure 3. Binding of ^{125}I -labeled mitochondrial 16 S RNA to mitochondrial 39 S r-proteins separated by 2D PAGE and blotted onto nitrocellulose. (A) and (B), autoradiograms of western blots probed with RNA in the solution E containing 100 mM KCl or 300 mM KCl, respectively; (C), representative blot of stained 39 S ribosomal proteins and (D), schematic diagram showing the proteins which bind RNA under high stringency conditions (solid spots), and low stringency conditions (solid and hatched spots).

intensity in autoradiograms from high stringency experiments, indicating that the binding of RNA to these proteins involves other forces in addition to simple ionic interactions.

Effect of urea treatment on mitoribosomal 39 S subunits

In order to learn more about the nature of the interactions between the RNA and the proteins that stabilize 39 S ribosomal subunits, we used 6 M

Figure 4. 2D PAGE analysis of r-proteins from urea treated 39 S subunits. (A), proteins dissociated from subunits in the presence of 6 M urea; (B), proteins of 6M urea stable core particles (C), control gel of mitochondrial 39 S ribosomal proteins present in this preparation; (D), schematic diagram showing proteins present exclusively in the core particles (solid spots).

urea to disassemble the particles in solution F (Methods). Mitoribosomal 39 S subunits were treated with 6 M urea and 100 mM KCl in solution F and the split proteins were separated from the urea stable core particles. Under these conditions, moderate ionic as well as moderate hydrophobic interactions between proteins and RNA should be disrupted. 2D PAGE was used to analyze the proteins in both the split protein and core fractions.

Figure 5. Identification of 39 S r-proteins which bind to mitoribosomal 16 S RNA in the presence of 9 M urea by 2D PAGE. (A), 39 S ribosomal proteins extracted by 9 M urea and 3 M LiCl; (B), proteins which remain dissociated from the rRNA after the removal of LiCl by dialysis against sample buffer, and (C), schematic diagram showing proteins missing on the gel shown on the picture B (solid spots), i.e. associated with RNA.

Most of the proteins are released from the 39S subunits by this treatment, yielding a well defined subset of proteins which are not associated with the core particles (Fig 4A). Ten proteins remain exclusively associated with the core particles under these conditions (proteins L7, L11, L13, L14, L21, L26, L40, L49, L50 and L51/52), indicating that these proteins must be binding to RNA by very strong ionic and/or hydrophobic interactions (Fig. 4B). All of these proteins were identified above as RNA binding proteins on western blots under the low stringency conditions and five (L7, L13, L14,

L21 and L26) were also scored under the high stringency conditions. These results are summarized in the schematic diagram (Fig. 4D) where the proteins were assigned to one or the other category depending on their enrichment in one or the other gel, relative to the unfractionated proteins in control gels (Fig. 4C). These findings are in accord with the LiCl stripping results (17) where proteins L7, L11, L13, L14, L21 and L49 (8) were found in the "core particle" fraction.

Binding of mitoribosomal proteins to mitoribosomal rRNA in the presence of urea

The set of proteins identified in the 6 M urea core particles is likely to include those which interact directly with the RNA. In order to determine which of these proteins can bind to RNA with strong ionic forces, we dissociated the proteins from the RNA in the presence of 9 M urea and 3 M LiCl. The LiCl was removed from the sample containing the r-proteins and RNA by dialysis against 9 M urea and 67 mM KAc, pH 4.3. The proteins, maintained in solution by 9M urea, were allowed to interact with the RNA at low ionic strength (67 mM KAc). This incubation mixture was analyzed directly by 2D PAGE. The complexes of proteins associated with the RNA fail to enter the gel during the first dimension electrophoresis (Fig. 5B). Missing proteins are scored as RNA-binding proteins if they are present in control gels (Fig. 5A). In this manner, we have selected a set of 14 r-proteins which can interact directly with RNA by ionic interactions (L1, L3, L7, L11, L13, L14, L21, L26, L35, L40, L44, L45, L49, L50). In the control gel, protein L7 occupies a position directly under protein L6 which is found in ribosome samples digested with RNase A. All of these proteins were found to be associated with the RNA either in the core particles produced by urea disassembly or by binding RNA directly on western blots.

Binding of LiCl group-fractionated mitoribosomal proteins to mitoribosomal 16 S RNA.

Above we have used three alternative procedures to obtain information on RNA binding proteins in 39S subunits of bovine mitoribosomes. To confirm and extend the above observations, we also studied RNA binding to proteins which can be extracted from 39S subunits by LiCl (17). Proteins prepared by this gentle extraction procedure generally remain soluble (29). It is thought that proteins isolated by this method retain more secondary and tertiary structure, resembling a more "native" conformation, rendering them more suitable for ribosome reconstitution experiments (30, 31).

Following this approach, we used 1M and 2M LiCl to sequentially extract

Figure 6. Isolation of complexes of mitochondrial 16 S RNA with ^{125}I -labeled mitochondrial 39 S r-proteins on a Sephacryl S 200 column. (A), elution profile of ^{125}I -labeled 39 S r-proteins extracted with 2 M LiCl; (B), elution profile of mixture of 16 S RNA with ^{125}I -labeled 39 S r-proteins extracted with 2 M LiCl. Arrows indicate position corresponding to the void volume of the column as measured by Dextran Blue 2000.

the majority of the proteins from 39S subunits. The extracted proteins were labeled with ^{125}I , dialyzed against solution G and cleared of aggregates by centrifugation. The labeled proteins were incubated with 16 S RNA in solution G containing 20 mM MgCl_2 and 200 mM KCl. RNA-protein complexes formed under these ionic conditions were separated from unbound proteins by gel filtration on a column of Sephacryl S 200. Figure 6 shows the results obtained for the proteins extracted with 2 M LiCl. The elution profile for the protein fraction alone shows a broad peak far from the void volume indicating the absence of high molecular weight aggregates (Fig 6A). In contrast, when 16S mitochondrial rRNA is mixed with the proteins, we observe a peak of radioactivity migrating with the RNA in the void volume (Fig. 6B). Similar results were obtained with the proteins extracted by 1 M LiCl.

Figure 7. 2D PAGE analysis of 39 S r-proteins which were bound to RNA in the complexes isolated on a Sephacryl S 200 column. (A), autoradiogram of the gel with ^{125}I -labeled proteins extracted with 2 M LiCl; (B), autoradiogram of the gel with proteins which were eluted from the column in complex with RNA; (C), stained gel of r-proteins from the 39 S subunits preparation which were used for LiCl extractions of r-proteins, and (D), schematic diagram showing RNA binding proteins (solid spots). Dotted spots on schematic diagram indicate positions of contaminating proteins from 28 S mitoribosomal subunit.

To identify ^{125}I -labeled proteins bound to RNA, material eluting in the void volume fractions was extracted in the presence of carrier 39 S subunits and the proteins were analyzed by 2D PAGE (Fig. 7). In this manner, we identified a set of 19 RNA binding proteins from the 2 M LiCl extract (L1, L3, L4, L5, L8, L9, L10, L12, L16, L17, L18, L19, L22, L24, L26, L32, L35, L44, L47) (Fig. 7B, D). In addition to these proteins, we detect an additional protein, L28, eluting with the ribonucleoprotein complex when 1M LiCl extracted proteins are used (data not shown). It should be noted that some of the proteins in the ribonucleoprotein complexes

eluting in the void volume may not be RNA binding proteins, but, by virtue of the experimental design, may instead bind directly to other proteins in association with the RNA. A small fraction of mitochondrial r-proteins (L7, L21, L41, and L49) remains associated with the RNA core particle after LiCl extraction and another set (L14, L21, and L49) are poorly labeled with iodine; thus these proteins are not included in this analysis despite being shown by our other approaches as being RNA binding proteins.

DISCUSSION

Mitochondrial ribosomes play a critical role in energy production in eukaryotic cells since they are involved in the translation of mRNAs coding for several components of the electron transport and oxidative phosphorylation systems in mitochondria (32, 33). Our laboratory has characterized several unique aspects of the structure and function of mammalian mitoribosomes (4, 5, 12, 34, 35). Virtually nothing is known about their biosynthesis, except that their RNA is encoded within the mitochondrial genome and their proteins are encoded in the nuclear genome (8). The proteins must be transported into the mitochondria for assembly with the rRNA. To identify the set of proteins that may interact with RNA early in assembly, we analyzed the RNA binding properties of the proteins in the large subunit of bovine mitoribosomes. We undertook four approaches which contribute in different and complementary ways to the identification of these proteins (Table 1). The first approach involved using western blots to test the binding of individual proteins to RNA. This approach has the advantage that prior purification of single proteins is not required and binding can be done under different stringency conditions. While we detect 19 proteins which can interact with RNA under low stringency conditions (100 mM KCl), only 9 of them can still bind RNA under high stringency conditions (300 mM KCl). Thus, most of these 19 proteins are binding by weak ionic interactions which are disrupted under the high stringency conditions. The set of nine proteins (L6, L7, L8, L13, L14, L21, L26, L28, and L44) which can be detected at the high stringency condition bind more strongly. Two of the proteins in particular, L7 and L14, seem to have a very high affinity for RNA under these conditions, possibly because of additional, non ionic contributions to their binding.

The second approach used 6 M urea under moderate ionic conditions to interfere with weak hydrophobic interactions and H-bonding between proteins and RNA in intact 39S subunits. Most of the ribosomal proteins are released

Table 1. PROTEINS OF 39S MITORIBOSOMAL SUBUNITS SCORED AS RNA BINDING PROTEINS USING DIFFERENT EXPERIMENTAL APPROACHES

#	WESTERN BLOTS		UREA STUDIES		GEL EXCLUSION CHROMATOGRAPHY	RNA BINDING PROTEINS	
	300 mM KCl	100 mM KCl	6M Urea	9M Urea	LiCl ^a	Strong	Weak
1	-	-	-	*	*		
2	-	-	-	-	-		
3	-	-	-	*	*		
4	-	-	-	-	*		
5	-	-	-	-	*		
6	*	*	-	-	-		
7	*	*	*	*	nd	7	
8	*	*	-	-	*		8
9	-	-	nd	nd	*		
10	-	*	-	-	*		
11	-	*	*	*	-		11
12	-	-	-	-	*		
13	*	*	*	*	nd	13	
14	*	*	*	*	nd	14	
15	-	-	-	-	nd		
16	-	-	-	-	*		
17	-	-	-	-	*		
18	-	*	-	-	*		
19	-	-	-	-	*		
20	-	-	-	nd	nd		
21	*	*	*	*	nd	21	
22	-	-	-	-	*		
23	-	*	-	-	-		
24	-	-	-	-	*		
25	-	*	-	nd	-		
26	*	*	*	*	*	26	
28	*	*	-	-	* ^b		28
31	nd	nd	-	nd	nd		
32	-	-	-	-	*		
33	-	-	-	-	nd		
34	nd	nd	-	nd	-		
35	-	*	-	*	*		35
37	-	-	-	-	nd		
38	-	-	-	-	-		
39	nd	nd	-	-	nd		
40	-	*	*	*	-		40
41	-	-	-	nd	nd		
42	-	-	-	-	-		
43	-	-	-	-	-		
44	*	*	-	*	*	44	
46	-	-	-	-	-		
47	-	-	-	-	*		
48	nd	nd	-	-	nd		
49	-	*	*	*	nd		49
50	-	*	*	*	-		50
51/52	-	*	*	-	nd		

^a Proteins from 1M and 2M LiCl extracts^b 1M LiCl extract only

- not RNA binding

* RNA binding protein

nd protein not detected in gel

from the 39S subunits by this treatment, but a few remain associated with the RNA in core particles. These proteins (L7, L11, L13, L14, L21, L26, L40, L49, L50 and L51/52) remaining in the core particles probably bind to the RNA by strong ionic and/or hydrophobic interactions. All of these proteins were previously disclosed on the low stringency western blots; five of these (L7, L13, L14, L21 and L26) were also disclosed on the high stringency western blots and thus are very good candidates for RNA binding proteins.

In a parallel study, we used 9 M urea in moderate ionic strength buffer to find the proteins which can bind to RNA purely by ionic interactions since under these conditions hydrophobic interactions and hydrogen bonding are eliminated. We found 14 proteins which bind to RNA in the presence of 9 M urea, and significantly, six of these (L7, L13, L14, L21, L26, and L44) are among the proteins disclosed by the high stringency conditions on western blots.

The last approach took advantage of a group fractionation procedure for the preparation of soluble r-proteins from the 39S particles. This procedure uses LiCl as the only chaotropic agent and is believed to yield proteins in a "more native" conformation (17, 29-31). A subset of these proteins remains soluble when dialyzed into an RNA binding buffer of moderate ionic strength. Thus, it is possible to study the binding of these proteins to RNA in the absence of denaturants. LiCl resistant core particles contain many of the previously disclosed RNA binding proteins which are poorly represented in the soluble fraction used for this experiment. Nevertheless, we were able to detect 20 proteins which bind under ribosome reconstitution conditions (20 mM MgCl₂, 200 mM KCl). Four of these proteins (L8, L26, L28, and L44) were also identified as RNA binding proteins by high stringency western blot analysis.

Results from the four different approaches correlate very well, identifying a common set of 6 proteins (L7, L13, L14, L21, L26, and L44) which appear to be strong RNA binding proteins (Table 1). All of these proteins were scored at both low and high stringency on western blots and by at least two additional tests. A second set of 7 proteins fall into the category of weak RNA-binding proteins (Table 1). These proteins (L8, L11, L28, L35, L40, L49, and L50), were detected by 3 different tests. However, with the exception of L8 and L28, they were not disclosed on the high stringency western blots, suggesting that they bind RNA with lower affinity. It is recognized that some additional mitoribosomal proteins may also bind

to the ribosomal RNA, but via interactions which are undetectable using the above approaches.

It is informative to correlate the RNA binding properties of proteins from both groups with their topographic disposition in intact 39 S subunits and their susceptibility towards LiCl extraction. Most of the strong RNA binding proteins identified in this study appear to be buried in the ribosomal subunits, as determined by their accessibility to lactoperoxidase-catalyzed radioiodination (34). In particular, proteins L7, L26, and L44 are among the most buried within the subunit, as expected for early assembly proteins. In addition, four of the strong RNA binding proteins (L7, L13, L14 and L21) remain tightly associated with the LiCl core particles, resistant even to 4 M LiCl extraction (17). This resistance to LiCl extraction compares well to that exhibited by the strong RNA-binding proteins from other classes of ribosomes (21, 22, 36). In contrast, proteins in the "weak" RNA binding category (Table 1) are generally less buried (34) than the "strong" RNA binding proteins and possibly for this reason, are extractable by 2M LiCl from the 39S subunits, in spite of their preferential association with the core particles (17).

In conclusion, 6 proteins, L7, L13, L14, L21, L26, and L44 are good candidates for early assembly proteins since they have a high affinity for RNA, are generally found in 4 M LiCl core particles, and are among the most buried in the 39S subunit. In contrast, thirteen to seventeen strong RNA-binding proteins have been reported for the large subunit of E. coli ribosomes (36). It is not surprising to have over twice the number of RNA binding proteins in the subunits of bacterial ribosomes since their rRNA is roughly twice the size of that in mitochondrial ribosomes (37). The extra RNA binding proteins in E. coli are probably binding to RNA domains which are missing from the mitochondrial ribosome. Nevertheless, the mitochondrial strong RNA binding proteins may be homologous to a subset of the E. coli RNA binding proteins, recognizing conserved RNA domains (37).

ABBREVIATIONS

R-proteins, ribosomal proteins; 2D PAGE, two-dimensional polyacrylamide gel electrophoresis; mitoribosome, mitochondrial ribosome.

ACKNOWLEDGMENTS

This research was supported by the USPHS, National Institutes of Health Grant GM-15438.

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