HSP70 and ribosomal protein L2: novel 5S rRNA binding proteins in *Escherichia coli*

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Abstract A Northwestern analysis of *Escherichia coli* total proteins with radiolabeled 5S rRNA identified two novel 5S rRNA interacting proteins, a 70 kDa and a 37 kDa protein, and three ribosomal proteins reported on already. The N-terminal sequencing of the 70 kDa protein separated by SDS–PAGE from the high-salt-washed fraction of crude ribosome led to the discovery of a polypeptide identical in its first 10 amino acid residues to *E. coli* heat shock protein 70. The N-terminal eight amino acid sequence of the 37 kDa protein extracted from the high-salt-washed ribosome is identical to that of the ribosomal protein L2. In addition, the interaction of these proteins with 5S rRNA has been confirmed with gel mobility shift assays.

Key words: 5S rRNA; RNA binding protein; Ribosomal protein L2; Heat shock protein; HSP70; *Escherichia coli*

1. Introduction

Prokaryotic ribosomes contain over 50 distinct proteins along with ribosomal RNA. There appears to be little sequence homology between the various proteins [1]. Given the large number of steps involved in protein synthesis, early workers in the field operated under the assumption that the various proteins catalyzed specific and distinct steps in the translational process. Many, if not all, ribosomal proteins interact with ribosomal RNA. Protein–RNA interactions are known to play an important role in a variety of biological processes, yet little is known about them in comparison with DNA–protein interactions. The well-characterized prokaryotic ribosome is an ideal system in which to study and dissect these interactions. 5S rRNA is the smallest ribosomal RNA and at the same time an integral part of the large ribosomal subunit. It has been localized together with its bound proteins in the neighborhood of the peptidyltransferase center in eubacterial ribosomes [2]. Some fundamental constituents of both prokaryotic and eukaryotic ribosomes include ribonucleoprotein complexes between 5S rRNA and ribosomal proteins of approximately equal molecular weight. These complexes can be released from the large subunits under mild conditions with EDTA. 5S rRNA species interact with some ribosomal proteins, e.g. *Escherichia coli* L5, L18 and L25, *Bacillus stearothermophilus* BL5 and BL22 [3], and rat liver ribosomal protein L5 [4].

Furthermore, a study of various structures and functions of 5S rRNA and their protein complexes demonstrated that 5S rRNA is a very important cellular ribonucleic acid and that its function is not only limited to its activity in the large ribosomal subunits during protein biosynthesis [5].

In order to obtain a better understanding of the 5S rRNA–protein interaction mechanism in ribosomes, we used Northwestern analyses and gel mobility shift assays to examine the interactions of 5S rRNA and proteins, and identified a specific interaction between the heat shock protein HSP70 and ribosomal protein L2 for novel 5S rRNA interacting proteins.

2. Materials and methods

2.1. Preparation of cell extracts and ribosomal proteins from E. coli cells

E. coli JM109 cells growing in L-broth containing 0.1% glucose were harvested in midlogarithmic phase, and quick frozen. The cells were ground with alumina and suspended in buffer A (50 mM Tris–HCl, pH 7.4, 30 mM NH_4Cl, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol). Cell debris was removed by centrifugation ($38\,000\times g$, 20 min), the supernatant (S38) was centrifuged at $380\,000\times g$ 1 h to pellet the crude ribosome, and the supernatant (S380 fraction) was saved. The crude ribosome was suspended in buffer A and the high-salt-washed ribosome was pelleted through an equal volume cushion of 25% sucrose in 50 mM Tris–HCl (pH 7.4), 1 M NH_4Cl, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol ($380\,000\times g$, 1 h). The washed-out proteins from crude ribosome with the high-salt were dialyzed against buffer A and then fractionated stepwise by ammonium sulfate precipitation. The salt-washed ribosome pellet was dissolved in buffer A. The ribosomal proteins were extracted from the ribosomes by 67% acetic acid in the presence of 100 mM magnesium acetate (0°C, 1 h).

2.2. In vitro synthesis of ^{32}P-labeled 5S rRNA of E. coli

A 120 bp DNA fragment, encoding mature *E. coli* 5S rRNA, was amplified with the oligo-nucleotides, 5′-CCGGAATTCTGCCTGGCGGCCGTAGCGCG-3′ and 5′-CCCAAGCTTATGCCTGGCAGTTCCCTACTC-3′. A truncated 69 bp DNA fragment, corresponding to positions 1–69 of mature *E. coli* 5S rRNA, was amplified with oligonucleotides, 5′-CCGGAATTCTGCCTGGCGGCCGTAGCGCG-3′ and 5′-CCCAAGCTTCGCTACGGCGTTTCACTTC-3′. These primers were designed to create *Eco*RI or *Hin*dIII sites at the 5′ or 3′ ends of the resulting PCR products, respectively. The purified products were inserted into pGEM-3Z digested with both *Eco*RI and *Hin*dIII, then radiolabeled RNA was produced in the presence of ^{32}P-CTP (Amersham; specific activity 800 Ci/mol) using the in vitro run off transcription kit supplied by Ambicon Co., Ltd. as described previously [6].

2.3. Northwestern blot analysis

The crude extract or ribosomal proteins of *E. coli* JM109 cells were separated by 15% SDS–PAGE. Transfer of proteins from slab gels

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into a PVDF-membrane and Northwestern analysis were performed as described previously [7].

2.4. Gel mobility shift assays

Purified ^{32}P-labeled *E. coli* 5S rRNA was resolved in TKM-buffer (50 mM Tris–HCl, pH 7.6, 300 mM KCl, 10 mM $MgCl_2$). Prior to use, RNA was renatured by incubating for 15 min at 65°C followed by slow cooling to room temperature. The labeled RNA was incubated with various amounts of *E. coli* extracts in 20 μl of reaction mixtures containing TKM-buffer, 5% glycerol, 1 μg BSA, 1 μg tRNA, and 32 units of RNase inhibitor (Amersham Pharmacia). After incubation for 15 min at 37°C, samples were loaded onto a 5% (acrylamide: bis, 40:1) non-denaturing polyacrylamide gel containing 45 mM Tris, 1 mM EDTA, 45 mM boric acid and 5% glycerol. Dried gels were exposed to X-ray films (Fuji Co.) at −80°C, typically for 16 h.

2.5. Determination of N-terminal amino acid sequence

Blotted PVDF filters were incubated for 3 min at room temperature in a BS buffer containing 20 mM boric acid–NaOH (pH 8.0), 25 mM NaCl and then in H_2O. The electrotransferred protein bands were visualized by staining for 5 min with Coomassie brilliant blue R 250 (0.1%) in 40% methanol and 17% acetic acid. After destaining with 90% methanol, the corresponding protein bands were excised from the PVDF filters. Edman degradation was done in a gas-phase sequencer.

3. Results and discussion

3.1. Separation of 5S rRNA binding proteins by the SDS–PAGE system

In order to obtain a better understanding of the 5S rRNA–protein interaction mechanism in *E. coli*, we used a Northwestern procedure which allows the detection of RNA binding proteins by blotting electrophoretically separated total cell proteins including ribosomal proteins on PVDF filters and probing them with radiolabeled RNA by in vitro transcription (Fig. 1). Hybridization with ^{32}P-labeled 5S rRNA showed the five signals at the positions of ~70, ~37, ~22, ~14, and ~12 kDa protein in whole cell proteins and also in the crude-ribosomal fraction (Fig. 1 lanes 1 and 3). Also shown in Fig. 1 (lanes 4 and 5), the 70 kDa RNA-binding protein was washed out from crude ribosome with 1 M ammonium chloride,

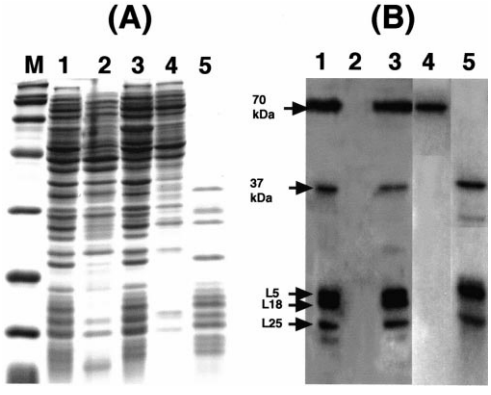

Fig. 1. Characterization of various *E. coli* cytoplasmic protein-fractions which bind to 5S rRNA by Northwestern blot analysis. Proteins extracted from *E. coli* whole cell (lane 1), the supernatant of crude ribosome (S380, lane 2), crude ribosome (lane 3), salt-washed fraction of crude ribosome (lane 4) and washed ribosome (lane 5) prepared as described in Section 2 were separated on a 15% SDS–PAGE gel, transferred to a PVDF membrane, and incubated with ^{32}P-labeled 5S rRNA. (A) is an image of the Coomassie-blue-stained gel, and (B) is from an autoradiography of the gel. The position of migration for several molecular weight markers is indicated (A, lane M). The molecular weight estimates for the RNA-binding proteins (in kDa) are designated on the left.

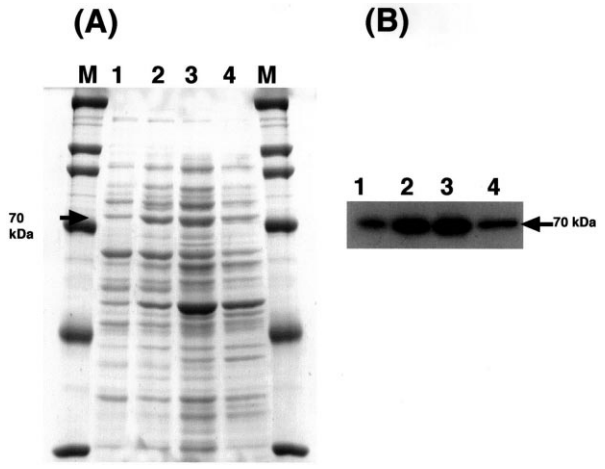

Fig. 2. Separation of 70 kDa 5S rRNA binding protein from the high-salt-washed fraction of crude ribosome by step-wise ammonium sulfate precipitation. Proteins from the 30% ammonium sulfate precipitate (lane 1) and 30–50% (lane 2), 50–70% (lane 3), and 70–80% (lane 4) ammonium sulfate fractions as described in Section 2 were separated on a 10% SDS–PAGE gel, transferred to a PVDF membrane, and incubated with ^{32}P-labeled 5S rRNA. (A) is an image of the Coomassie-blue-stained gel, and (B) is from an autoradiography of the gel.

whereas the 37, 22, 14, and 12 kDa RNA-binding proteins were not being tightly complexed to ribosome. When the washing experiment of the hybridization was made in the presence of 300 mM NaCl, only ~37, ~22, ~14, and ~12 kDa protein remained strongly bound to the 5S rRNA (not shown), suggesting the weak binding of ~70 kDa protein to 5S rRNA compared to ~37, ~22, ~14, and ~12 kDa protein. The molecular weights of 22, 14, and 12 kDa RNA-binding proteins are consistent with those of ribosomal protein L5, L18, and L25, which constitute the 5S rRNA–protein complex isolated with EDTA treatment from the ribosome [3].

These data demonstrated that *E. coli* cells express at least five 5S rRNA-binding proteins and two of them (37 kDa and 70 kDa) are novel binding proteins. Moreover, the 37 kDa protein was ribosomal protein, whereas the 70 kDa protein was non-ribosomal protein although it probably interacts with the ribosome during protein synthesis directed by 5S rRNA.

3.2. Determination of the N-terminal sequence of the novel 5S rRNA binding proteins

To identify the 37 kDa 5S rRNA binding protein, we scaled up the SDS–PAGE system and the protein was excised as a single long-band separated from the high-salt-washed ribosome (Fig. 1A, lane 5). The N-terminal sequence was determined by Edman degradation by the sequencer. Analysis of the 37 kDa protein yielded the following N-terminal sequence: Ala-Val-Val-Lys-X-Lys-Pro-Thr-Ser-Pro. When compared with the non-redundant protein database of EMBL using the BLAST search program, a perfect match of the 37 kDa protein to N-terminal sequence of a ribosomal protein L2 of *E. coli* although the fifth residue was not determined was obtained. The molecular weight of L2 protein predicted from the sequence (273 amino acids) is 31 500 [8]. This discrepancy between molecular weight determined from the sequence and observed in SDS–PAGE is not unusual.

In examining the N-terminal sequence of the 70 kDa pro-

tein, there appears to have been more bands near the 70 kDa band in the 15% gel compared to the 37 kDa protein band (Fig. 1A, lane 4). Therefore, washed-out proteins from the crude ribosome were fractionated by ammonium sulfate precipitation. By using the step-wise precipitation method with saturated ammonium sulfate, the desired 70 kDa protein was concentrated in 50–70% saturated ammonium sulfate. When the proteins of the 50–70% saturated ammonium sulfate fraction were resolved by SDS–10% PAGE, the stained gel revealed a single predominant protein with an apparent molecular mass of about 70 kDa (Fig. 2A, lane 3). When the N-terminal region of the 70 kDa protein was sequenced, only the following was obtained, Gly-Lys-Ile-Ile-Gly-Ile-Asp-Leu-Gly-Thr. A search of the protein database revealed that this 70 kDa protein was the same as the heat shock protein HSP70 in *E. coli*. The molecular weight of HSP70 protein predicted from the sequence (638 amino acids) is 68 983 [9].

These data demonstrated that ribosomal protein L2 and HSP70 are novel 5S rRNA-binding proteins. Protein L2 is the largest protein component of the *E. coli* 50S subunit and binds specifically to 23S rRNA. From affinity labeling and reconstitution studies, L2 is considered to be important for the peptidyltransferase activity of the 50S subunit [10]. Therefore, it is proposed that for the binding of 5S RNA to 23S rRNA in the 50S ribosome assembly protein L2 plays an essential bridging role between two rRNAs and constitutes the peptidyltransferase center.

3.3. The proteins binding to 5S rRNA via a band shift assay

To confirm that the salt washed proteins from the ribosome including HSP70 protein indeed interact with 5S rRNA, an RNA mobility shift assay was conducted. Incubating the washed-out proteins including HSP70 with ^{32}P-labeled 5S rRNA, synthesized in vitro, led to the appearance of complex I that could be followed by non-denaturing PAGE (Fig. 3A). When the ribosomal proteins extracted from high-salt-washed 70S ribosome were used as a 5S-rRNA binding protein fraction, the formation of large complex II was observed (Fig. 3B). In a test for interaction between ribosomal proteins and the washed-out proteins in 5S rRNA binding, RNA binding assays of ribosomal proteins were performed in the presence of the proteins including HSP70 (Fig. 3C, lane 2) compared to the control. The amount of large complex II was significantly reduced (about 20% compared to control in density scanning) by adding the washed-out proteins, suggesting that ribosomal proteins and HSP70 recognize the localized structure of 5S rRNA. However, we could not completely exclude the possibility that other proteins in the fraction are responsible for the band shift, because many other proteins are included in binding reactions besides binding ribosomal proteins and HSP70.

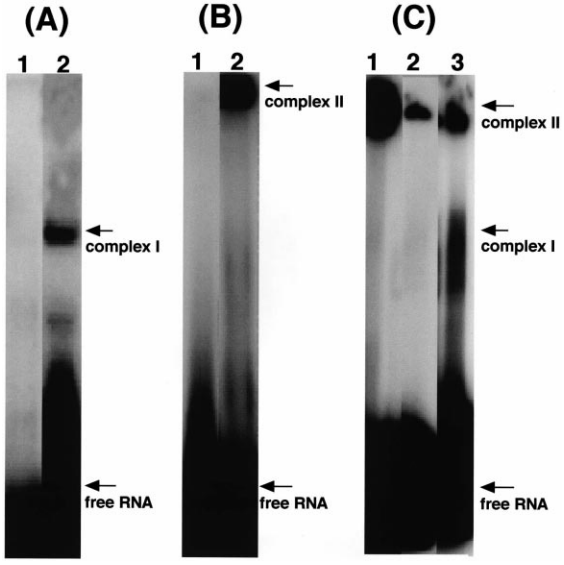

Fig. 3. Gel-mobility shift of 5S rRNA by *E. coli* cytoplasmic proteins. A: radiolabeled 5S rRNA was incubated for 15 min in the absence (lane 1) or presence of 10 μg of high-salt-washed proteins from crude ribosome (lane 2) and analyzed by non-denaturing PAGE. B: radiolabeled 5S rRNA was incubated for 15 min in the absence (lane 1) or presence of 10 μg of ribosomal proteins (lane 2). C: the formation of radiolabeled 5S rRNA-ribosomal proteins complex II in the absence (lane 1) or presence of 10 μg of high-salt washed proteins from crude ribosome (lane 2 and 3). Dried gels were exposed to X-ray films at −80°C, for 5 h (lane 1 and 2) or for 24 h (lane 3). Two RNA–protein mobility shift complexes (complex I and II) are indicated.

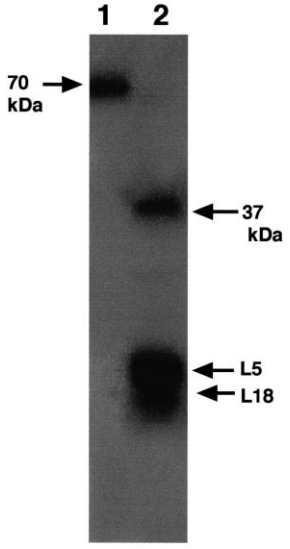

Fig. 4. Northwestern analyses of RNA binding protein activity by the truncated 5S rRNA fragment. Proteins extracted from *E. coli* salt-washed fraction of crude ribosome (lane 1) and the washed ribosome (lane 2) were separated on a 10% SDS–PAGE gel, transferred to a PVDF membrane, and incubated with ^{32}P-labeled truncated 69 nucleotide RNA (position 1–69 nt).

3.4. Localization of an HSP70 binding region within 5S rRNA

To localize the protein binding site, we synthesized ^{32}P-labeled RNA representing the truncated 69 nucleotide form, position 1–69 nt of the entire 5S rRNA. The ability to bind protein was assessed by Northwestern blot analysis of the washed-out protein fraction from the crude ribosome or ribosomal protein fraction. Binding activity was detected in HSP70 and three ribosomal proteins (L2, L5 and L18) although no binding of the 12 kDa (L25) protein could be detected (Fig. 4). It appears that the binding of HSP70 and the three ribosomal proteins is specific to a relatively small region, the 5′ half of 5S rRNA (position 1–69), and L25 ribosomal protein is specific to the 3′ half of the molecule. The binding sites of *E. coli* ribosomal proteins have been characterized by footprinting experiments. Our results confirm the previous data that *E. coli* L18 protects the central helix of

5S rRNA and residues 38–42 from digestion by α-sarcin, whereas *E. coli* L25 protects residues 72–109 from digestion by α-sarcin [11,12].

The results presented here indicate that *E. coli* contains at least five 5S rRNA binding proteins, HSP70 and ribosomal protein L2, L5, L18 and L25. HSP70 interacts with position 1–69 nt of the entire 5S rRNA to make an unstable ribonucleoprotein that dissociates with high salt from the ribosome, mimicking the interaction of several translating factors in the translation cycle. Moreover, it is suggested that 5S rRNA involves association with 23S rRNA through L2 ribosomal protein.

Studies using heterologous and homologous in vitro translation systems have demonstrated that *E. coli* chaperones DnaJ and DnaK (HSP70) are associated with nascent polypeptides [13]. Temperature-sensitive mutations in HSP70 result in ribosome assembly defects indicating that HSP70 may also play a role in ribosomal assembly [14]. Furthermore, several reports indicate that DnaJ and HSP70 may be directly associated with ribosomes even in the absence of a nascent polypeptide [15]. The 5S rRNA binding property of HSP70 reported here raises the possibility that HSP70 does associate with ribosomes and may promote correct folding of a nascent polypeptide. This possibility is consistent with published data, showing certain shock proteins are found to be associated with the ribosome under stress conditions. From the sequence similarity with the heat shock protein and the 5S rRNA binding property of ribosomal protein TL5 from *Thermus thermophilus*, it was suggested that TL5 might function as a protector of a cell from heat stress or even an RNA chaperone on ribosomes [16].

We surmise that HSP70 acts transiently at a ribosome rather than being a stable component of a 5S rRNA binding small subset in ribosomes and that HSP70 is associated with a ribosome following initiation but prior to departure of nascent polypeptides. Taken together with the data presented here and other observations, we propose a model in which HSP70 is concerned with the dissociation of nascent polypeptide from ribosomes directed by 5S rRNA and promotes the release of nascent polypeptide from ribosomes.

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