N-terminal domain, residues 1–91, of ribosomal protein TL5 from *Thermus thermophilus* binds specifically and strongly to the region of 5S rRNA containing loop E

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Abstract In this work we show for the first time that the overproduced N-terminal fragment (residues 1–91) of ribosomal protein TL5 binds specifically to 5S rRNA and that the region of this fragment containing residues 80–91 is a necessity for its RNA-binding activity. The fragment of *Escherichia coli* 5S rRNA protected by TL5 against RNase A hydrolysis was isolated and sequenced. This 39 nucleotides fragment contains loop E and helices IV and V of 5S rRNA. The isolated RNA fragment forms stable complexes with TL5 and its N-terminal domain. Crystals of TL5 in complex with the RNA fragment diffracting to 2.75 Å resolution were obtained.

Key words: Ribosomal protein TL5; 5S rRNA-protein complex; Loop E-helix IV domain of 5SrRNA; Crystallization; *Thermus thermophilus*

1. Introduction

5S rRNA is an integral part of the ribosome. This rather short RNA, about 120 nucleotides (nt), is associated specifically with one to three proteins in ribosomes of different organisms. In *Escherichia coli*, three proteins are bound to 5S rRNA: L5, L18 and L25 [1,2]. The binding sites of these proteins on 5S rRNA were investigated [3–6]. The complex of 5S rRNA with certain ribosomal proteins is a sufficiently autonomous structural domain of the large ribosomal subunit [7–9]. There is some experimental evidence that the 5S rRNA-protein complex is involved in the formation of the ribosome peptidyltransferase center [10,11].

Structural studies of the 5S rRNA-protein complex have the potential to provide information about the structure of the intact ribosomal domain and could contribute to our understanding of RNA-protein interaction principles. Many efforts were made to crystallize and study the structure of the 5S rRNA-protein complex and its components. Crystallization of intact 5S rRNA from *T. thermophilus* and *Thermus flavus* and ribosomal protein L25 in complex with fragment 1 of *E. coli* 5S rRNA (nt 1–11, 69–87, 90–120) were reported [12–14], however these crystals were unsuitable for X-ray studies. Recently the structures of two short fragments corresponding to helix I [15] and helix IV-loop D [16] of 5S rRNA from *T. flavus*, and fragment 1 of *E. coli* 5S rRNA [17] have been solved. Fragment 1 contains an internal loop E, a unique double helix with several non-canonical base pairs, putative recognition elements for the binding of ribosomal protein L25 [17,18]. Recently the spatial solution structure of *E. coli* ribosomal protein L25, free and bound to the 5S rRNA fragment, containing the loop E region, has been determined by NMR spectroscopy [19]. A putative RNA-binding surface of L25 includes residues highly conserved in related proteins, among them the unusual acidic 5S rRNA-binding ribosomal protein TL5 from *T. thermophilus*.

In our previous work, it was shown that the N-terminal region of TL5 was protected by 5S rRNA binding against proteolysis [20]. This fact and moderate sequence homology between L25 and the N-terminal region of TL5 [21] allowed us to suggest that the RNA-binding ability of TL5 belongs to its N-terminal domain. In the present work, the 5S rRNA-binding domain of TL5 was identified and a specific TL5-binding fragment (39 nt) of *E. coli* 5S rRNA was isolated and sequenced. Complexes of intact TL5 and its isolated N-terminal domain with this RNA fragment were crystallized.

2. Materials and methods

All chemicals were from Sigma and Merck. Ribonuclease T_1, PhyM, U_2 and RNase from *Bacillus cereus* were from Pharmacia Biotech and RNase A was from Sigma. Heparin-Sepharose CL6B, CM-Sepharose CL6B, Sephadex G-75 and G-100 were from Pharmacia Biotech, and Butyl-Toyopearl 650S was purchased from Toyo-Soda (Japan).

2.1. Overexpression of the TL5 gene fragment, encoding the N-terminal region of the protein, in E. coli cells

The fragment of the gene for TL5 that corresponds to the amino acid sequence 1–91 was amplified by PCR from chromosomal DNA of the *T. thermophilus* strain VK1. The PCR primers were: start 5′-GTTTAACTTTAAGAAGGAGATATACCATGGAGTACCGTTT-GAAAGCG-3′; stop 5′-CCCCAGTGAAAGTGAAACTGAAGCT-TCTAGAGGACGAAGAAGTCCACGTGC-3′. The PCR fragment was inserted into the expression plasmid pACA [22] downstream the T7 polymerase promoter using the *sticky-feet* mutagenesis protocol [23]. The resulting plasmid, called pTL5-Nterm, was transformed into the *E. coli* strain BL21(DE3)pLysS for efficient protein production. For large scale production of the N-terminal fragment of TL5, individual colonies were picked from fresh plates and inoculated into LB medium supplemented with 0.1 mg/ml ampicillin and 0.02 mg/ml chloramphenicol. The bacterial culture was grown at 37°C to an $A_{600} = 0.6–0.7$. Biosynthesis of the N-terminal fragment of TL5 was

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induced by addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside and the cell growth was continued for 3 h under the same conditions.

2.2. Purification of protein TL5 and its N-terminal fragments

The original and overproduced ribosomal protein TL5 and its tryptic N-terminal fragment were purified as described in [20,24]. For the isolation of the overproduced 1–91 residue fragment ($TL5fr_{91}$), cells of the overproducing strain were lysed using a Sonic Dismembrator (Fisher Scientific, USA) in a buffer containing 50 mM Tris-HCl, pH 7.5, 1 M NaCl, 20 mM EDTA, 5 mM β-mercaptoethanol (5–6 times for 30 s). The cell debris was removed by low-speed centrifugation and ribosomes were removed by high-speed centrifugation for 6–7 h at $100\,000 \times g$. The lysate components were fractionated by column chromatography on Butyl-Toyopearl 650S (50 mM Tris-HCl, pH 7.5, 5 mM β-mercaptoethanol, 1.5 M $(NH_4)_2SO_4$ and linear gradient of $(NH_4)_2SO_4$, from 1.5 M to 0.0 M) and then on Heparin-Sepharose CL6B (50 mM Tris-HCl, pH 8.0, 5 mM β-mercaptoethanol and linear gradient of NaCl, from 0.2 M to 0.7 M). The protein purity was assessed by SDS-PAGE [25].

2.3. Isolation of 5S rRNA and its fragments

Ribosomal RNA was isolated from *E. coli* (or *T. thermophilus*) ribosomes as described in [26] and was separated by gel filtration on Sephadex G-100. Fragments were prepared from intact *E. coli* and *T. thermophilus* 5S rRNA by limited digestion with ribonuclease A. Fragment 1 of *E. coli* 5S rRNA was obtained as described in [27]. Smaller fragments from *E. coli* and *T. thermophilus* 5S rRNA were prepared as described in [28] with some modifications: RNase A was added to the complex of 5S rRNA with TL5 (the procedure of the complex preparation is given below) in 30 mM sodium cacodylate buffer, pH 7.4, containing 10 mM $MgCl_2$, 100 mM NaCl, 0.5 mM EDTA (the RNA:enzyme ratio was 2:1, w/w). Digestion proceeded at 25°C for 1 h and RNA fragments were fractionated by chromatography on Sephadex G-75 as in [27].

2.4. RNA sequencing

For sequencing, the stable small fragment of *E. coli* 5S rRNA was radioactively labelled at the 5′-termini by T4 polynucleotide kinase [29]. The labelled chains of the fragment were separated by PAGE (20% polyacrylamide/8 M urea) and eluted from the gel. Partial digestion with nuclease T1, nuclease PhyM and nuclease from *B. cereus* was performed in 20 mM sodium citrate buffer, pH 4.5, 7 M urea. Partial digestion with nuclease U_2 was performed in 20 mM sodium citrate buffer, pH 3.5, 7 M urea. 100 000 c.p.m. of each labelled RNA chain and 1 μg of total tRNA were preheated in 9 μl reaction mixture at 50°C for 10 min, then 1 μl of the enzyme at an appropriate concentration was added and incubation was continued for an additional 15 min. After the reaction, the RNA fragments were analyzed on 20% polyacrylamide/8 M urea gel. Alkaline ladder prepared as described by Donnis-Keller [30] was run in parallel.

2.5. Reconstitution and crystallization of RNA-protein complexes

All RNA-protein complexes were reconstituted according to [24]. Concentrated preparations of TL5 (or $TL5fr_{91}$) and 5S rRNA fragments were dialyzed against 10 mM sodium cacodylate buffer, pH 7.0, containing 4 mM $MgCl_2$, and 100 mM KCl (200 mM KCl in case of $TL5fr_{91}$). RNA-protein complexes were obtained by mixing equimolar amounts of both components and incubation of the mixture at 40°C for 15–20 min. The presence of stable complexes was determined as in [24] by the gel-shift method.

Crystallization conditions were screened utilizing the hanging drop technique at 18–20°C. Finally, 5 μl of the preparations were mixed with 5 μl of the following precipitant solutions: 10 mM sodium cacodylate, pH 7.0, 100 mM $MgCl_2$, and 7% 2-methylpentane-2,4-diol (MPD) in the case of intact TL5-RNA complex crystallization, and 10 mM sodium cacodylate, pH 7.0, 100 mM $MgCl_2$, 200 mM KCl, 150 mM NH_4Cl and 1% MPD in the case of $TL5fr_{91}$-RNA. The concentration of complexes in drops was 1–3 mg/ml. The drops were equilibrated against 1 ml of the precipitant solutions, containing 0.2 M KCl and 15% MPD for the complexes with intact TL5, and 0.2 M KCl and 1% MPD for the complex with $TL5fr_{91}$.

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                10         20         30         40
TL5fr91 MEYRLKAYYREGEKPSALRR...AGKLPGLMYNRHL.NRKVYVDLVEF 44
          : : I  I  :   I II   I I I ::I       : :I
L25      MFTINAEVRKEQGKGASRRLRAANKFPAIIYGGKEAPLAIELDHDKV 47

                50         60         70         80         90
TL5fr91 DKV.FRQASIHHVIVLELPDGQSLPTLVRQVNLDKRRRRPEHVDFFVL 91
          :  :      I::: : II  : :   :I:    : : :I:II
L25     MNMQAKAEFYSEVLTIVV.DGKEIKVKAQDVQRHPYKPKLQHIDFVRA 94
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Fig. 1. Alignment of sequences of the 5S rRNA-binding TL5 fragment ($TL5fr_{91}$) and ribosomal protein L25 from *E. coli*. Vertical lines and colons indicate identical and similar amino acid residues, respectively.

3. Results

The main goal of this work was the dissection of a rather large ribosomal protein TL5 in domains and determination of the region of the protein possessing its RNA-binding ability.

It was possible to split the protein in two large fragments by proteolysis, but the isolated N-terminal and C-terminal domains were both inactive in the RNA binding. The region of TL5 homologous to 5S rRNA-binding ribosomal protein L25 from *E. coli* contains residues 1–91 (Fig. 1). In this work, the gene for $TL5fr_{91}$ was prepared by a standard technique (see Section 2) and the fragment was purified and tested for the binding to 5S rRNA. It was found that $TL5fr_{91}$ binds with high affinity to 5S rRNA from *T. thermophilus* (data not shown) and from *E. coli* (Fig. 2A, lane 3). Moreover $TL5fr_{91}$ displaced intact TL5 from the 5S rRNA-TL5 complex (Fig. 2A, lanes 6–9). Thus we showed that the isolated $TL5fr_{91}$ represents the functionally active RNA-binding domain of TL5. Smaller N-terminal fragments of TL5 (residues 1–79

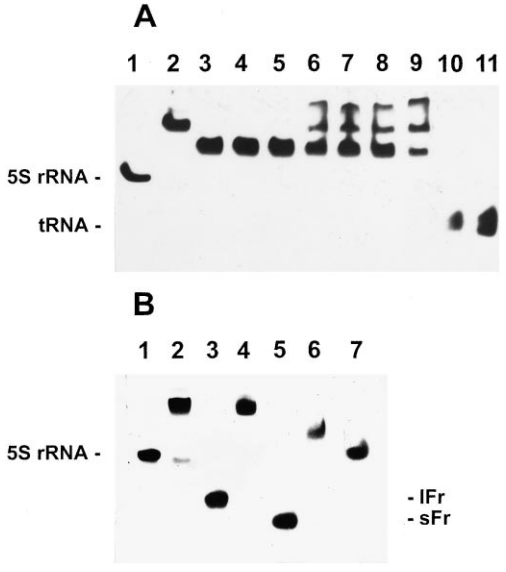

Fig. 2. Interaction of TL5 and $TL5fr_{91}$ with *E. coli* 5S rRNA (or its fragments). The samples were analyzed by PAGE in non-denaturing conditions (12% gel) as described in Section 2. A: Lane 1, 5S rRNA; lane 2, 5S rRNA+TL5 (RNA:protein molar ratio was 1:1 in the incubation mixture); lane 3, 5S rRNA+$TL5fr_{91}$ (1:1); lane 4, 5S rRNA+$TL5fr_{91}$ (1:2); lane 5, 5S rRNA+$TL5fr_{91}$ (1:3); lane 6, 5S rRNA+TL5+$TL5fr_{91}$ (1:1:1); lane 7, 5S rRNA+$TL5fr_{91}$+TL5 (1:1:1); lane 8, TL5+$TL5fr_{91}$+5S rRNA (1:1:1); lane 9, 5S rRNA+TL5+$TL5fr_{91}$ (1:3:1); lane 10, tRNA; lane 11, tRNA+$TL5fr_{91}$ (1:3). Components are given in the order of their addition to the mixture. B: Lane 1, 5S rRNA; lane 2, 5S rRNA+TL5 (1:1); lane 3, fragment 1 of 5S rRNA (lFr); lane 4, fragment 1+TL5 (1:1); lane 5, small fragment (39 nt) of 5S rRNA (sFr); lane 6, small fragment+TL5 (1:1); lane 7, small fragment+$TL5fr_{91}$ (1:1).

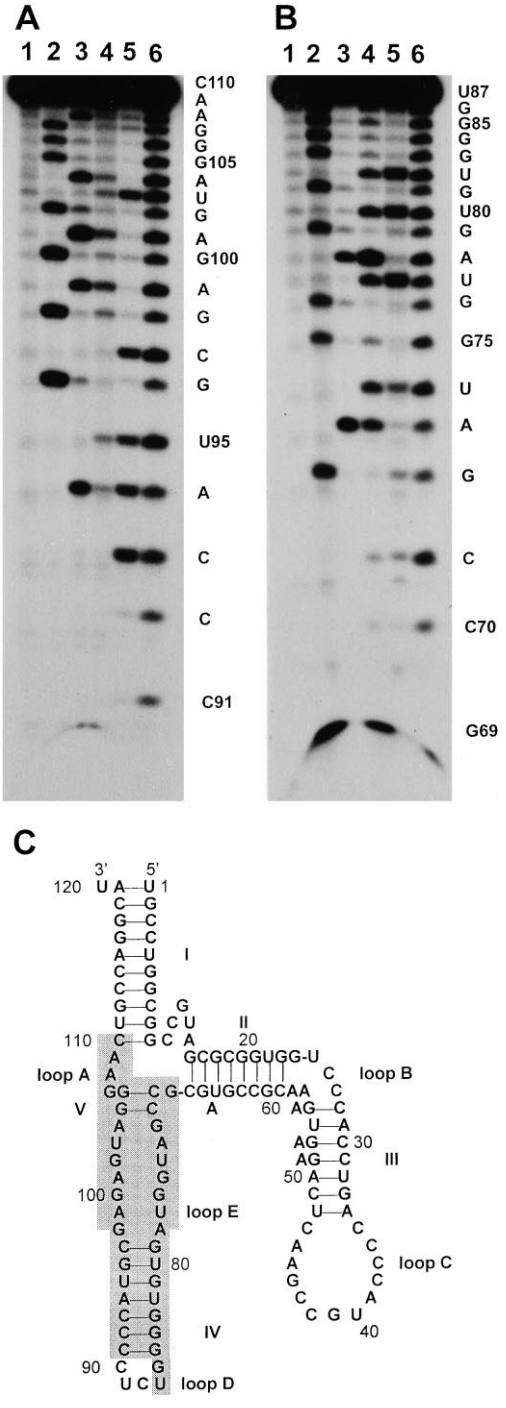

Fig. 3. RNA sequencing gel of 5′-[^{32}P]-labelled chains of the small fragment of *E. coli* 5S rRNA, protected by TL5 against Rnase A digestion, and a scheme of the proposed secondary structure of *E. coli* 5S rRNA. A: Long strand. B: Short strand. C: A scheme of the proposed secondary structure of *E. coli* 5S rRNA (the small 5S rRNA fragment protected by TL5 against RNase A digestion is included in the shaded section). Lane 1, control RNA; lane 2, RNase T1 digest (specific for G); lane 3, RNase U2 digest (specific for A > G > C > U); lane 4, RNase PhyM digest (specific for A+U); lane 5, RNase from *B. cereus* digest (specific for C+U), and lane 6, alkaline ladder.

and residues 1–80), prepared by trypsinolysis or by gene engineering, were inactive in the 5S rRNA binding. These data reveal the importance of residues 80–91 of TL5 for the formation of the RNA-binding domain of the protein.

One of the goals of this work was determination of the minimal fragment of 5S rRNA which could form a stable complex with TL5 and its RNA-binding domain. Earlier we found that a nuclease resistant 62 nt fragment 1 (1–11, 69–87, 89–120) of *E. coli* 5S rRNA, carrying the binding site for *E. coli* ribosomal protein L25, is able to bind TL5 strongly and specifically [24]. In this work, we obtained a smaller fragment of *E. coli* 5S rRNA (Fig. 2B, lane 5) protected by TL5 against digestion by RNase A. The fragment was purified by gel filtration and analysed by PAGE. It was shown that the fragment consists of two chains: 19 and 20 nt (data not shown), and these chains were sequenced (Fig. 3A,B). The chains contain nucleotides 69–87 and 91–110, hence the small TL5-binding fragment of *E. coli* 5S rRNA is limited by loop E and helices IV and V (Fig. 3C). A similar small TL5-binding fragment (about 40 nt) of *T. thermophilus* 5S rRNA was also prepared, but not sequenced.

Several RNA-protein complexes were prepared and used for crystallization: fragment 1 (62 nt) of *E. coli* 5S rRNA-TL5 (Fig. 2B, lane 4); the small fragment (39 nt) of *E. coli* 5S rRNA-TL5 (Fig. 2B, lane 6); the small fragment of *E. coli* 5S rRNA-TL5fr$_{91}$ (Fig. 2B, lane 7). Large tetragonal bipyramids of the complex of *E. coli* 5S rRNA fragment 1-TL5 (Fig. 4A) diffracted only to 10 Å resolution similarly to crystals of the analogous complex with *E. coli* L25 obtained 15 years ago [14]. TL5 in complex with the small fragment of *E. coli* 5S rRNA crystallized as hexagonal lenses (Fig. 4B) diffracting to 2.75 Å resolution. These crystals are sensitive to changes of magnesium concentration, and the best crystals have been obtained at 50 mM $MgCl_2$. Preliminary studies showed that the crystals belong to the space group $P3_112$ with unit cell dimensions $a = b = 111.63$ Å, $c = 140.22$ Å. At present the search for isomorphous heavy atom derivatives of these crystals is in progress. Crystals of TL5fr$_{91}$ in complex with the same small fragment of *E. coli* 5S rRNA diffracting to about 3 Å resolution have been also obtained recently (Fig. 4C).

It is interesting to note that in contrast to our success in crystallization of the hybrid complex of TL5 with the small 39 nt fragment of *E. coli* 5S rRNA, the non-hybrid complex of TL5 with the similar small RNA fragment of *T. thermophilus* 5S rRNA was not crystallizable in our trials.

4. Discussion

The unusual acidic ribosomal protein TL5 from *T. thermophilus* can replace ribosomal protein L25 in the native *E. coli* 5S rRNA-protein complex [31]. The whole sequence of TL5 (206 residues) does not reveal a sufficient homology with any known ribosomal protein, except some moderate homology (18% identity) between the N-terminal region of TL5 and *E. coli* ribosomal protein L25 [21]. These data supported the surmise that the N-terminal region of TL5 forms a domain that may play the role of protein L25 in *T. thermophilus* ribosomes. The results obtained in this work fully validate this suggestion: the fragment of TL5 containing residues 1–91 possesses the RNA-binding property of the whole protein. Our finding that the fragment of TL5 containing residues 1–80 is inactive in the RNA binding is well compatible with the conclusion of Stoldt et al. [19] that the C-terminal region of L25 (residues 75, 76, 85, 88, 90 and 92) is possibly involved in its RNA-binding surface.

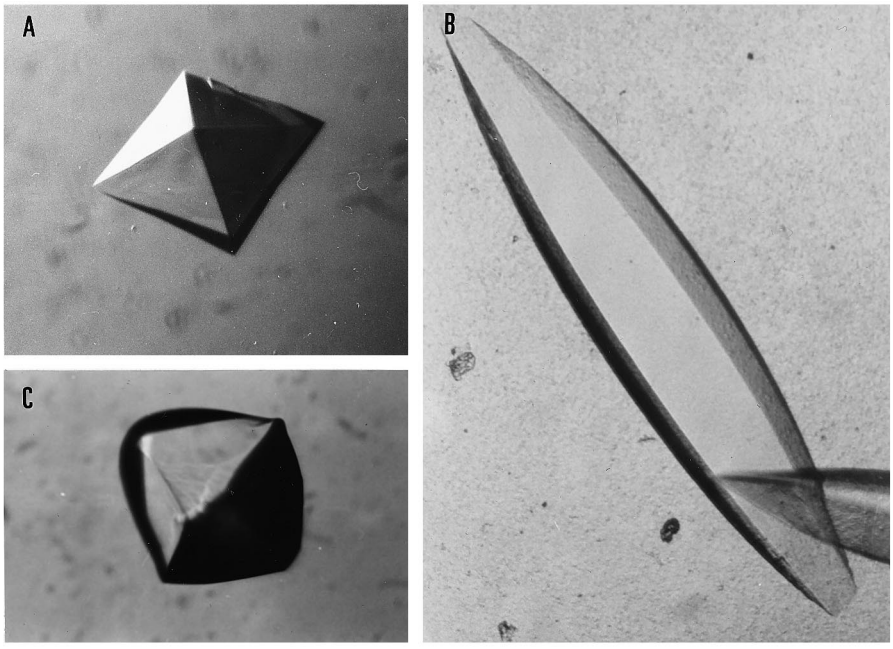

Fig. 4. Crystals of the complexes. A: The fragment 1 of *E. coli* 5S rRNA-TL5 complex. B: The small fragment of *E. coli* 5S rRNA-TL5 complex. C: The small fragment of *E. coli* 5S rRNA-TL5fr$_{91}$ complex.

A peculiarity of ribosomal protein TL5 is a significant homology of this protein with a general stress protein CTC from *Bacillus subtilis* (33% identical residues and 53% similar residues) [21]. It seems that TL5 and CTC are structurally homologous and may function similarly. A possible extraribosomal function of TL5 as well as a possible role of CTC in ribosomes was not yet studied. New data on the domain organization of TL5 can be approximated to CTC. It is highly probable that the N-terminal domain of the shock protein CTC may recognize a double-stranded RNA bulge motif related to the loop E region of 5S rRNA.

Determination of minimal rRNA fragments binding specifically ribosomal proteins is an important approach in structural studies of the ribosome. Such studies open a real possibility to determine the structure of ribosomal protein-RNA complexes, which are practically native ribosomal components. Our result in obtaining X-ray quality crystals of the TL5-RNA complex is one of the first successful steps in this direction.

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