A study of the thermophilic ribosomal protein S7 binding to the truncated S12–S7 intercistronic region provides more insight into the mechanism of regulation of the *str* operon of *E. coli*[1]

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Received 8 September 1999

Abstract A study of the ability of His6-tagged ribosomal protein S7 of *Thermus thermophilus* to interact with the truncated S12–S7 intercistronic region of *str* mRNA of *Escherichia coli* has been described. A minimal S7 binding mRNA fragment is a part of the composite hairpin, with the termination codon of the S12 cistron on one side and the initiation codon of the next S7 cistron on the other. It has a length in the range of 63–103 nucleotides. The 63 nucleotide mRNA fragment, which corresponds to a putative S7 binding site, binds very poorly with S7. Tight RNA structure models, which behave as integral systems and link the S7 binding site with the translational regulation region of the hairpin, are suggested. This observation provides more insight into the mechanism of S7-directed autogenous control of translational coupling of *str* mRNA.

Key words: RNA-protein heterologous interaction; Ribosomal protein S7; RNA structure model; *str* operon; *Escherichia coli*; *Thermus thermophilus*

1. Introduction

Ribosomal (r) biogenesis is a very consuming process and is therefore subject to tight regulation. The synthesis of the r proteins adjusts to the synthesis of rRNA by autogenous control. One gene of the r protein operon encodes a regulatory r protein which controls both its own expression and the expression of some other proteins of the operon. During balanced cell growth newly synthesized regulatory r protein binds to the rRNA and assembles into the ribosome. If the level of rRNA synthesis is decreased dramatically an excess of the r protein turns off the synthesis of most (or all) proteins of the operon, which balances the level of the r components. This mechanism is based on the ability of the single regulatory r protein for highly specific interaction (recognition) with certain regions of both rRNA and mRNA [1].

Beside S4, a key regulatory r protein for the ribosomal small subunit biogenesis is S7. During *Escherichia coli* ribosomal assembly S7 binds to the 3′-domain of the 16S rRNA and promotes binding of the other r proteins of the domain [2,3]. Dragon and Brakier-Gingra [4] have found that a minimal 16S rRNA binding region for S7 is a rather small rRNA fragment, just 108 out of 1542 nucleotides. S7 is the regulatory protein for the *str* operon [5,6]. The autogenous control of the *str* operon of *E. coli* has several peculiarities. It has a tangible basal level of S7 synthesis not regulated by S7 [5]. The size of the regulatory S12-S7 intercistronic mRNA region is large, about 100 nucleotides [6]. It is very interesting that this regulatory mRNA region turned out to be one of the most extended regions among known cistrons translated by the mode of translational coupling [1]. By chemical and enzymatic probing Saito and Nomura [6] have mapped the binding region of S7 for the 412 nucleotide fragment of the *str* mRNA. It turned out to be a 59 nucleotide RNA segment of the S12-S7 intercistronic hairpin (−80 to −22, taking the first A in AUG of the S7 cistron as +1, Fig. 3A).

Therefore, ribosomal small subunit biogenesis regulation in *E. coli* cells is governed by two types of very specific RNA-protein interactions: S7–16S rRNA and S7–*str* mRNA. A comparative study of the structure of these two RNP complexes will lead to deciphering the mode of autogenous control of ribosomal biogenesis. More generally, it will allow us to understand the phenomenon of recognition of different RNA structures by a single protein.

A study of the ability of His6-tagged ribosomal protein S7 of *Thermus thermophilus* to interact with the small truncated S12–S7 intercistronic region of *str* mRNA of *E. coli* has been described. The 63 nucleotide mRNA fragment, which corresponds to a putative S7 RNA binding site [6], binds very poorly with S7. This incongruity between our data and those of Nomura has led us to suggest more organized RNA structure models than proposed earlier [6]. They behave as integral systems and link the S7 binding site with the functional translational region of the hairpin. This observation provides more insight into the mechanism of S7-directed autogenous control of translational coupling of *str* mRNA.

2. Materials and methods

The initial template for the synthesis of *str* mRNA fragments was pDD151, a 5′ derivative of pDD157 described earlier [7]. Transcription from authentic T7 promoter yielded a 151 nucleotide mRNA fragment. Truncated DNA templates were made by PCR. Left primers (3, 5) had the additional non-complementary sequence of T7 RNA polymerase promoter (underlined). Right primers (4, 6) determined the 3′-end of the RNA product.

1. GTAAAACGACGGCCAGT (standard forward sequencing primer)
2. CAGGAAACAGCTATGAC (standard reverse sequencing primer)
3. GACGAATTCTAATACGACTCACTATAGTTAATGGTTCTCCGTTAAG

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[1] This paper is dedicated to Mikhail A. Prokof'ev.

PII: S0014-5793(99)01351-4

4. CATGGAAATACCGTTG
5. GACGAATTCTAATACGACTCACTATAGTAAGGCC-AAACGTTTTAAC
6. *CAAGC*TTCAGGATTGTCCAAAAC

(The non-complementary sequence of primer 6 creates the restriction site, marked in italics.).

Radioactive RNA was transcribed in vitro from DNA template [8] using α-[^{32}P]UTP (Physico Energy Institute, Obninsk, Russia) with T7 RNA polymerase (MBI, Fermentas, Lithuania; or kindly provided by S. Kochetkov, IMB RAS, Russia). RNA was purified by 8% PAGE with 7 M urea. Specific radioactivity was calculated as Cerenkov counts per A_{260} (5000–50 000 cpm/pmol).

His6-tagged recombinant ribosomal protein S7 of *T. thermophilus* was isolated as described [9], combining several mini-scale preparations. Protein concentration was determined by both Laemmli PAGE and the standard Bradford method. The molecular weight of the recombinant S7 was calculated as 19.3 kDa.

The complex was formed in 200 μl of the buffer 20 mM Tris-Ac, pH 7.8, 7 mM $MgCl_2$, 300 mM NH_4Cl, 4 mM mercaptoethanol, 0.002% bovine serum albumin (MBI, Fermentas, Lithuania). RNA and protein were incubated separately, then together at 42°C for 30 min. The protein binding isotherm was measured by the nitrocellulose filter binding assay (0.45 μm, Millipore HA or Schleicher and Schuell BA85), the flow rate was 0.5 ml/min. RNA concentrations were as indicated, and the protein concentration was varied. The filters were counted by Cerenkov in water. The data were approximated from several experiments using the computer program Origin 4.00 (USA).

3. Results and discussion

The three-dimensional structures of two thermophilic S7 ribosomal proteins have recently been determined by X-ray analysis [10,11]. However, a structural study of complexes of thermophilic S7 with both rRNA and mRNA fragments is currently not available. Most of the biochemical research has been confined to the traditional object *E. coli*. Therefore, as an intermediate step in our study, it seems reasonable to use heterologous complexes of a thermophilic protein with functionally defined RNA fragments of *E. coli*. Recently Tanaka et al. [12] presented a preliminary computer model of the three-dimensional structure of the heterologous complex of *Bacillus stearothermophilus* S7 with a *E. coli* 16S rRNA fragment. For other r proteins of the small subunit, S8 and S15 [13,14], usage of heterologous complexes had also provided some advantages, in spite of all obvious potential imperfections.

From two functionally important types of the S7-RNA complexes, i.e. with the *str* mRNA [7] and with the 16S rRNA [4], the first one has been selected for an initial structural study. S7 of *T. thermophilus* is able to bind corresponding fragments of RNAs of *E. coli* in vitro. It binds both a minimal fragment of 16S rRNA (108 nucleotides) and the small regulatory S12–S7 intercistronic region of *str* mRNA of *E. coli* (157 nucleotides), as we have shown earlier [7]. The second complex is the most stable among the S7 RNP complexes known to date. The secondary structure of the mRNA intercistronic region, proposed by Saito and Nomura [6], seems to be simpler than the one corresponding to the 16S rRNA binding site of S7 [4]. Thus this mRNA fragment can be considered an attractive candidate for structural research. However, the large size of the complex makes it difficult to apply direct methods of structural analysis. A preliminary stepwise deletion analysis of the mRNA fragment was performed, and the ability of the truncated mRNA fragments to bind S7 was measured by nitrocellulose filter binding assays.

Conventional conditions for ribosomal reconstitution are commonly used to study ribosomal ribonucleoprotein complexes: 20 mM Mg^{2+}, 350 mM KCl. However, for every particular complex the conditions might have to be specific ones. As the first step of the research, conditions for complex formation were optimized for two key parameters.

For optimization of the Mg^{2+} concentration a high ionic strength, 300 mM NH_4Cl, was applied to reduce possible non-specific interactions. The binding of S7 to mRNA151/240 does not vary significantly (Fig. 1A). (Here and throughout the rest of the article the first number after 'mRNA' corresponds to the length of the sense mRNA fragment, and the second to the total RNA length). There is a slight maximum at 7–12 mM Mg^{2+}. The lowest value, 7 mM, was taken for further study.

For the ionic strength value, a very clear maximum was revealed at 200 mM NH_4Cl (Fig. 1B). For further study a slight higher concentration of the salt was selected, 300 mM, to reduce possible non-specific interactions.

The study of the interaction of S7 with three mRNA frag-

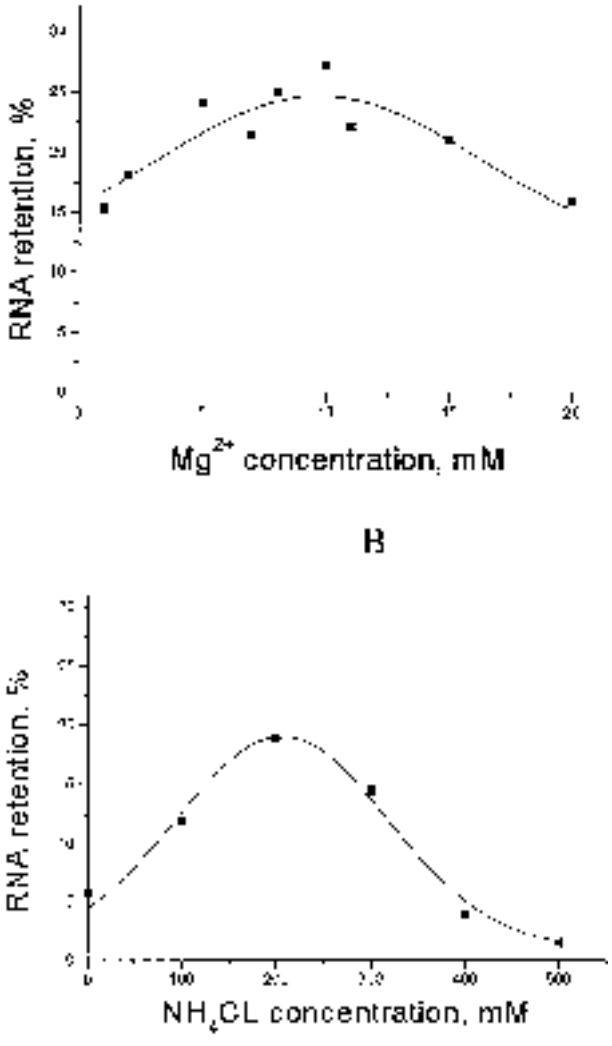

Fig. 1. A: Mg^{2+} concentration dependence of S7-RNA151/240 complex formation according to the nitrocellulose filter binding assay. 300 mM NH_4Cl. RNA-protein ratio 1:6. B: NH_4Cl concentration dependence of S7-RNA151/240 complex formation according to the nitrocellulose filter binding assay. 7 mM Mg^{2+}. RNA-protein ratio 1:6.

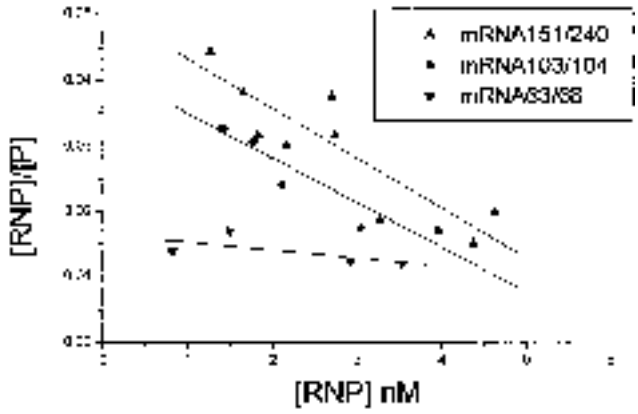

Fig. 2. Scatchard plot for S7 binding with the truncated S12–S7 intercistronic region of *str* mRNA. $[RNA]_0$ and $[P]_0$ are initial RNA and protein concentration, respectively $[RNA151/240]_0$, 15 nM; $[RNA103/104]_0$, 46 nM; $[RNA63/68]_0$, 21 nM; $[P]_0$: 0–300 nM. [RNP] = [retained RNA]; $[P] = [P]_0 - [RNP]$. K_a (μM^{-1}): RNP151/240 = 7.6 (Δ1.6), RNP103/104 = 6.9 (Δ1.5), RNP63/68 = 1.3 (Δ1.0).

ments was performed at the optimal conditions. S7 binding with the initial mRNA fragment was done with the 240 nucleotide RNA, where 151 nucleotides came from *str* mRNA, the others were transcribed as the 3′-end additional sequence from the vector (mRNA151/240). For mRNA151/240 the plateau value of the binding isotherm can be reached at a 10–14 molar excess of the protein. This value is much less than was shown for other thermophilic ribosomal proteins, S8 and S15 [13,14]. The complex is more stable than the one described earlier for conditions of integral reconstitution of the ribosome [7].

The first truncated derivative of the mRNA, mRNA103/104 (−100 to +3), corresponds exactly to the *str* mRNA intercistronic region. Saito and Nomura [6] proposed a secondary structure model of the fragment as a single composite hairpin with the termination codon of S12 on one side and the initiation codon of S7 on the other (Fig. 3A). Compared to mRNA151/240, mRNA103/104 has a major deletion of the 3′-end sequence of the coding region of the S7 cistron. The truncated mRNA103/104 derivative binds to S7 with about the same affinity as the original mRNA151/240 (Fig. 2). Interesting peculiarities of this mRNA fragment have been observed during variations of renaturing conditions. Values for complex formation vary in the range of 2–3-fold, which may indicate a possible conformational variability of this RNA fragment. It may be proposed that the deletion of the 3′-end nucleotides of the S7 cistron yields a loss of RNA structural elements which sustain a proper RNA structure.

The second truncated derivative of the intercistronic *str* mRNA region, mRNA63/68, corresponds to the S7 RNA binding site, proposed by Saito and Nomura [6]. It is the upper part (−82 to −20) of the composite hairpin. It has stem III on one side and might be considered a relatively self-contained part of the RNA, if the proposed secondary structure is correct. S7 binds very poorly with the mRNA63/68 (Fig. 2). This means that Nomura's footprinting localization of the protein binding site on the mRNA [6] revealed just the protein contact regions of mRNA, rather than the protein binding RNA fragment as it is.

Considering our data together with those of Saito and Nomura, it might be strongly suggested that either the RNA binding site of the protein is more extended or some deleted RNA structural elements, which are not directly involved in

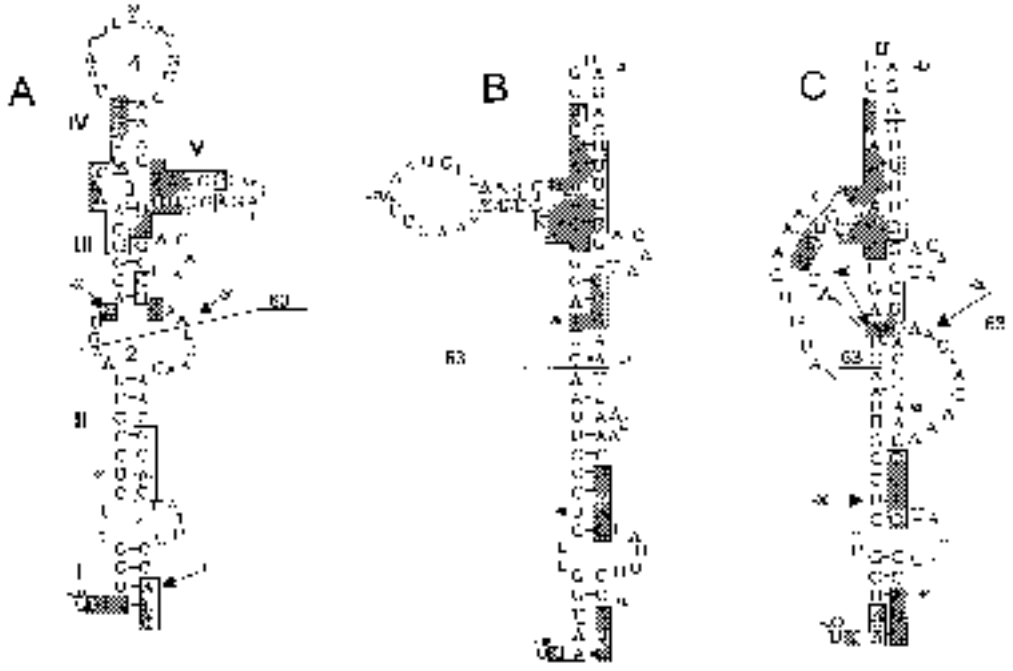

Fig. 3. Putative models of a secondary structure of the S12–S7 intercistronic region of *str* mRNA of *E. coli* (mRNA103/104). A putative RNA binding site of S7, the termination codon of S12, Shine-Dalgarno sequence and the initiation codon of S7 are in stippled boxes. The first A of S7 AUG is +1. The sense part of mRNA63/68 is marked by dissecting line with the number 63. A: Saito-Nomura model with the original numbering of the secondary structure elements [6]. B: This model combines two functionally important segments of the hairpin via double stranded formation within the interior loop 2. C: This model combines two functionally important segments of the hairpin via pseudoknot formation.

protein binding, are needed to sustain the proper conformation of the RNA. Some theoretical reasons favor the second option. We carried out a thorough analysis of all possible secondary structure elements of the initial RNA151/240 fragment. This was accomplished by the trivial dot-matrix analysis gaining to achieve eventually a most integral final structure of the mRNA fragment. A structure has to allow the status of stem V to influence the status of stem II, and vice versa. It turned out that it is possible to achieve extended inter-stem stacking interactions if stem V comes for a coaxial stacking with stem III (Fig. 3). The putative interior loop 2 can be structurally organized in two ways. First, a double stranded structure can be formed if an A-G base pair is allowed, which is not a rare element of the RNA secondary structure [15,16]. Second, an interesting feature of the intercistronic *str* mRNA fragment structure is the possibility of an alternative formation of a pseudoknot. At present, there are no solid data to prefer either one of the structures. Nevertheless, if we assume that any structure which unites stems V, IV and III with stem II exists, then a combination of our data and those of Saito and Nomura (e.g. KS18-2 mutant [6]) may bring some structural rationale into the mode of feedback regulation of the translational coupling by protein S7.

The main point is that the intercistronic mRNA hairpin has an integral structure. It allows stems III, IV, and V (putative S7 binding site) to interact with stem II (Shine-Dalgarno sequence) via extended interactions of a double stranded region formed by interior loop 2. Stem II nests a structure of an apparent S7 binding site, therefore the isolated RNA fragment, mRNA63/68, loses its ability to bind the protein. And vice versa, the binding of S7 to stems III, IV, and V can modulate the status of stem II, thus influencing translational coupling.

The AUG initiation codon of S7 belongs to stem I. It is separated from the proposed integral system by interior loop 1. Therefore its status is not affected by S7 binding and provides some independent basal level of S7 translation.

To our knowledge *E. coli* is a first bacterial example studied so far that has this versatile mode of regulation of *str* operon translation for the S7 protein [1]. *T. thermophilus str* operon does not have an extended intercistronic region, allowing a simple sliding mode for translational coupling. At the moment, we can only guess what is the selective advantage for *E. coli* to have this regulatory RNA structure. One obvious speculation is that a basal level of S7 translation creates a kind of stand-by situation. S7 is a key protein of the assembly of the ribosomal small subunit. In the case of a rapid shift-up and turning on the rRNA synthesis this specific system of translational regulation provides an initial amount of S7 for the immediate assembly of the ribosomes.

Acknowledgements: The authors are grateful to A. Karginov for the isolation of S7, E. Ekimova for participation in experiments, S. Kochetkov for providing T7 RNA polymerase, S. Kopylov for the original dot-matrix computer program, T. Rassohin and A. Golovin for discussions and help in preparation of the manuscript, V. Ramakrishnan, B. Wittmann-Liebold and A. Bogdanov for support and helpful discussions. The work is supported by Grants RFBR 98-04-49005, RFBR-NSFC 99-04-39072 and Universities of Russia 5157.

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