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Mapping contacts of the S12–S7 intercistronic region of str operon mRNA with ribosomal protein S7 of *E. coli*

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Abstract In *E. coli*, S7 initiates 30S ribosome assembly by binding to 16S rRNA. It also regulates translation of the S12 and S7 cistrons of the 'streptomycin' operon transcript by binding to the S12–S7 intercistronic region. Here, we describe the contacts of N-terminally His_6-tagged S7 with this region as mapped by UV-induced cross-linking. The cross-links are located at U(−34), U(−35), quite distant from the start codons of the two cistrons. In order to explain the mechanism of translational repression of S12–S7, we consider a possible conformational rearrangement of the intercistronic RNA structure induced by S7 binding.

Keywords: Ribosome biogenesis; RNA–protein interactions; Protein S7; Streptomycin operon; Translational regulation; *E. coli*

1. Introduction

For fast growth, prokaryotic cells utilize large amount of proteins synthesized by ribosomes, giant supra-molecular RNA–protein complexes [1]. Therefore, a high level of protein synthesis is dependent on a large amount of ribosomes, their number being well correlated with growth rate. Ribosomal biogenesis itself is an energy-demanding process and needs to be highly regulated [2,3].

The genes for rRNA and ribosomal proteins genes are not physically linked in the prokaryotic genome, for example in *E. coli* the rrnB operon is located at 4164682–4166223 and ribosomal protein S7 (rpsG) at 3471564–3472103 (GenBank Accession No. U00093). The rRNA transcription responds to changes in growth conditions [2,4], and the level of ribosomal proteins is adjusted to the level of rRNA synthesis by a translational feed-back mechanism [2]. Ribosomal protein genes are grouped into operons and transcribed as polycistronic mRNA, translation of the grouped cistrons being coupled. One protein plays a regulatory role, and usually it is a key protein for ribosomal assembly. Under unfavorable growth conditions, rRNA synthesis ceases, the key protein is then no longer recruited for ribosomal assembly and starts functioning as a translation repressor. Thus, the regulation mechanism of ribosomal biogenesis is based on the ability of regulatory ribosomal proteins to recognize either rRNA or mRNA.

Nowadays, the study of ribosomal biogenesis has gained new interest. A massive usage of antibiotics has led to the wide-spread occurrence of multi-resistant pathogenic bacteria, and the search for new antibacterial drugs is a novel challenge. Most antibiotics in the class of translational inhibitors block ribosomal functioning [5], but inhibitors interfering with ribosomal biogenesis are considered to be more promising for drug design [6,7].

Prokaryotic protein S7 plays a key role in the biogenesis of ribosomal small subunits. In addition to protein S4, protein S7 initiates the small subunit assembly [8]. A minimal 16S rRNA binding site for S7 has been identified as a segment of about 200 nucleotides, being the core of the major 3′-end domain [9–11]. In addition, S7 is the regulatory protein of the streptomycin (str) operon. In *E. coli*, this operon contains four genes, encoding S12, S7, and the elongation factors G and Tu, respectively (Fig. 1) [2]. The chromosomal str resistance phenotype of certain bacteria, pathogens included, appears to be due to mutations in S12. This has stimulated a more detailed studying of the str operon [12–15].

A unique feature of the *E. coli* str operon is the extended S12–S7 intercistronic region of about 100 nucleotides. According to our bioinformatics search, other bacteria like *Salmonella*, *Shigella* and *Yersinia*, also have such an extended intercistronic region. Despite this long region, the translation of S7 is coupled to that of S12 [16,17]. S7 represses the translation of both by binding to the intercistronic region. Saito and Nomura [17] have shown, that S7 can bind in vitro to a str mRNA fragment of 400 nucleotides containing the intercistronic region. Our deletion analysis revealed a minimal S7 binding site of about 100 nucleotides [18,19] with a putative secondary structure shown in Fig. 2 [19].

Several attempts to map this binding site in more detail have thus far led to controversial results. Firstly, a search for identity elements in the sequence of both S7 targets: 16S rRNA and str mRNA, has revealed two different sets of putative binding sites [3,11,17]. Secondly, chemical and enzymatic probing data suggest, that S7 binds at the branching region of helices III, IV

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Abbreviations: r6hEcoS7, recombinant His_6-tagged protein S7 of *E. coli*; RNA143, S12–S7 intercistronic region of str operon transcript of *E. coli*; PAGE, polyacrylamide gel electrophoresis; str, streptomycin

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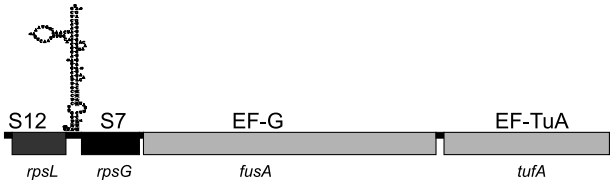

Fig. 1. Schematic presentation of the *E. coli* str operon [2] with names for proteins (above) and genes (below). The S12–S7 intercistronic region is shown as a detailed structure in Fig. 2.

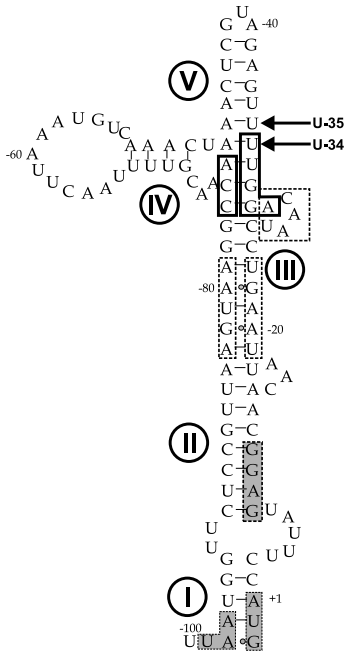

Fig. 2. Putative secondary structure of EcoStr143 [19] except its 40 downstream nucleotides. The first A of S7 initiation codon AUG is taken as +1. Stop codon of S12, SD and initiation codon of S7 are in shaded boxes. Nucleotides identical to 16S rRNA sequences are in boxes with drawn lines [17] and dotted lines [11]. Helices are numbered according to Saito and Nomura [17]. Arrows show residues modified in the complex with S7.

and V of the intercistronic region [16,17]. Unfortunately, this RNA probing provided rather ambiguous results, possibly due to conformational changes of RNA upon protein binding.

This work describes a mapping of contacts between recombinant S7 and the intercistronic region of *E. coli* str mRNA by means of direct UV-induced RNA-protein cross-linking.

2. Materials and methods

Buffer A: 50 mM Tris–HCl, pH 9.5, 2 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 1 mM dithiotreitol (DTT), 0.005% NP-40, 5% dimethylsulfoxide (DMSO), 1 mM betain. Buffer B: 50 mM HEPES–KOH, pH 7.0, 100 mM KCl. Buffer C: 50 mM Tris–HCl, pH 8.5, 10 mM $MgCl_2$, 60 mM KCl, 10 mM DTT, 0.5 mM dNTP. Buffer D: 20 mM Tris–Ac, pH 7.8, 7 mM $MgAc_2$, 300 mM NH_4Cl, 0.2% BSA.

2.1. Isolation of recombinant E. coli S7 protein (r6hEcoS7) from overproducing E. coli cells

r6hEcoS7 is S7 with N-terminal His_6-tag and 10 extra aminoacids [10], it was isolated according to QIAGEN protocols as described [10,28]. Cells were disrupted by lysozyme and sonication in 10% glycerol, 0.5 mM PMSF and 1% Triton X-100 (Merck, Germany). After adding urea to 8 M, the lysate was applied to a Ni-NTA column, urea was washed out, and protein was eluted with 0–0.5 M imidazole gradient. The S7 fraction was dialyzed against 20 mM HEPES–KOH, pH 7.5, 100 mM NaCl, 0.2 mM DTT; 5% glycerol, 0.5 mM PMSF, and stored at −70°. For RNA complex formation, the protein was first dialyzed against 20 mM Tris–HCl, pH 7.6, 4 mM $MgAc_2$, 400 mM NH_4Cl, 0.2 mM EDTA, 4 mM mercaptoethanol.

2.2. Transcription of the S12–S7 intercistronic region (EcoStr143)

The template for synthesis of EcoStr143 (Fig. 2) was pDD146, a derivative of pDD157/pGEM3Z already described [18,19]. The plasmid insert has three extra nucleotides comparing to EcoStr143 DNA. For PCR, as left primer GACGAATTCTAATACGACTCACTATAGTTAATGGTTCTCCGTTAAG (with T7 RNA polymerase promoter sequence underlined) was used and as right primer CCGGCAGAATTTTACGGCTGACC (MWG-Biotech AG, Germany). The PCR for DNA template production was in a Thermocycler (BioRad, USA) with 20–400 μl of buffer A containing 200 mM dNTP, 20 pmole primers, 50–500 ng DNA and 2–5 U of *Taq* DNA polymerase. The DNA product was isolated with 1–2% agarose gel electrophoresis, extracted with 6 M NaI, and purified with a PCR purification kit (Roche, Germany). Radioactive EcoStr143 was transcribed using α-[^{32}P] UTP (Amersham, Germany) with T7 RNA polymerase (Fermentas, Lithuania), and purified by 8% polyacrylamide gel electrophoresis (PAGE)/7 M urea by conventional procedures.

2.3. Complex of r6hEcoS7 with EcoStr143

Complex formation of r6hEcoS7 with EcoStr143 was performed as described [19]. RNA and protein were incubated in buffer D, first separately, then together, for 30 min at 37 °C in each step. Complex formation was measured by nitrocellulose filter binding (0.45 μm, Millipore HA, USA; Schleicher & Schuel BA85, Germany) with 20 pmole RNA at a flow rate of 0.5 ml/min [10,19]. Filters were counted in water (Tracor Analytic, France). Data were processed according to Scatchard [20]: $RP/P_f = (-RP + R_0)/aK_d$, with R_0 and P_0 as initial concentrations of RNA and protein, respectively, RP as complex concentration, $P_f = (P_0 - RP)$ as concentration of non-bound protein, and aKd as the apparent dissociation constant.

2.4. UV-induced RNA-protein cross-linking of complex r6hEcoS7–EcoStr143

The complex was formed in 200 μl buffer D using 150 nM EcoStr143 and 10-fold excess of r6hEcoS7, then irradiated at 0 °C with UV light (260 nm, Stratolinker, USA) at 15 cm distance for 10 min. The cross-linking yield was checked by Laemmli PAGE. The bulk of S7 was removed from the cross-linked complex with Protease K (Roche, Germany). EcoStr143 modified residues were identified by a conventional reverse transcriptase assay with the 5′-[^{32}P] labeled PCR right primer of Section 2.2. About 0.5 pmole of primer was hybridized with 2–5 pmoles of RNA in 5 μl buffer B at 95 °C for 1 min, and cooled down slowly to 43 °C. Reverse transcription was done in 9 μl buffer C with 2 U of AMV RT (Roche, Germany) at 43 °C for 1 h, and for template sequencing 70–400 μM ddNTP was used. Samples were analyzed by 8% PAGE/7 M urea.

3. Results and discussion

3.1. r6hEcoS7 binds to EcoStr143 with high affinity

As shown by Nomura et al. [16,17] for S7 isolated from ribosomes by conventional methods, the protein binds to the intercistronic region of a 398 nucleotides fragment of str mRNA. In further studies of protein S7-RNA complexes, recombinant S7 with N-terminal His_6 tag was used (6hEcoS7 [11] and r6hEcoS7 [10]), because of the simple purification. In the first case, 6hEcoS7 was able to bind a truncated str mRNA fragment of 230 nucleotides with aK_d of 150 ± 20 nM [11]. Using a slightly different protein r6hEcoS7 and a modified protocol for isolation [10], we obtained a threefold more stable complex with aK_d of 55 ± 7 nM (Fig. 3), and could narrow the S7 RNA

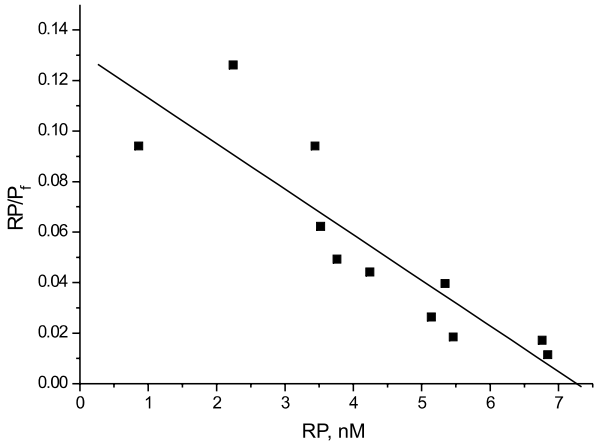

Fig. 3. Scatchard plot for r6hEcoS7 binding to EcoStr143. *RP*, complex concentration; P_0, initial protein concentration; P_f, non-bound protein concentration; RNA concentration, 20 nM.

binding site down to 143 nucleotides (Fig. 2). The influence of the His_6 tag on protein–RNA interactions has not been studied in detail yet. For example, additional His residues could destabilize tRNA–EF-Tu complexes as much as twice [21], which is just contrary to the here described S7 interaction.

3.2. Identification of contact of r6hEcoS7 with EcoStr143 by UV-induced cross-linking

To map S7 contacts with the intercistronic region of its mRNA, we applied UV-induced cross-linking. Our computer measurements of the distance from M115 of S7 to U1240 of 16S rRNA, which are cross-linked within *E. coli* 30S subunit [22,23], fits well with X-ray subunit structure of *T. thermophilus* ribosomes [24].

After 260 nm UV irradiation, the r6hEcoS7–EcoStr143 complex had become cross-linked for 20–30% (Fig. 4). Positions of resulting nucleotide modifications were determined by reverse transcription with a 5′-labeled primer (Fig. 5). By convention, it is assumed that reverse transcriptase stops one nucleotide before chemical modification.

In RNA alone (lane 1), two regions (U(−7) to C(−2), and the U stretch U(−69) to U(−66) of helix IV) have surprising

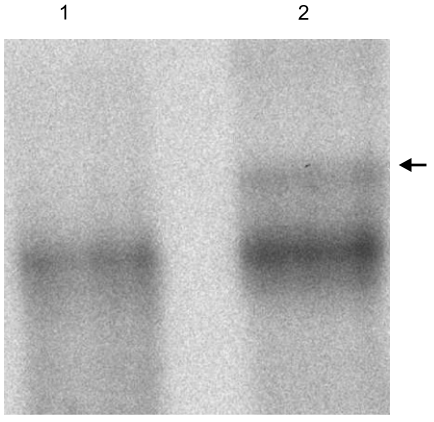

Fig. 4. Laemmli PAGE analysis of UV-induced RNA-protein cross-linking of complex EcoStr154–r6hEcoS7. Lane 1, UV-irradiated EcoStr143 alone; lane 2, UV-irradiated complex. The arrow points to complexed RNA.

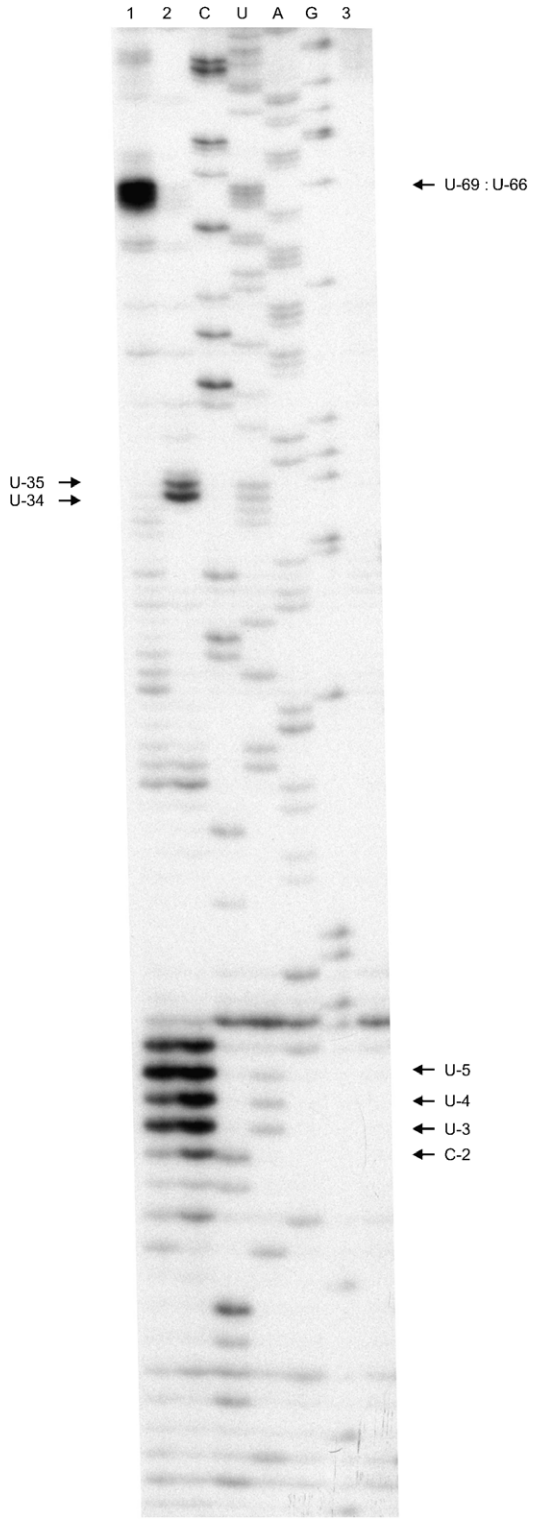

Fig. 5. 8% PAGE/7 M urea analysis of reverse-transcriptase primer extension of EcoStr143 after UV irradiation. Lane 1, irradiated RNA alone; lane 2, irradiated RNA–protein S7 complex; lanes C, U, A, G, RNA sequencing; lane 3, RNA with no irradiation. Left arrows show modified residues U(−34) and U(−35) within the complex; right arrows show modified residues for RNA alone.

properties. The first region (−5)UUUC(−2) is photosensitive both in RNA alone (lane 1) and in the complex with S7 (lane

2), and U(−7) is liable to hydrolysis in the absence of irradiation (lane 3), probably due to specific structural features (see [25,26]). The second U stretch becomes protected from UV modification when in complex with S7 (lane 2).

Besides these data, lane 2 clearly shows characteristic stop signals for RNA-S7 cross-links at nucleotides U(−34), U(−35): both are located at the branching of helices III, IV and V (Fig. 2). Though it has not be excluded that S7 binding could re-position these Us for photo-modification, the cross-linking event correlates well with the following data of Saito and Nomura [17]. Upon S7 binding, the U stretch of helix V became protected against RNAse V1 and the opposite strand (−45)CAA(−47), and neighboring residues A(−38) and C(−43) became accessible to dimethyl sulfate. S7 binding probably induces unwinding of RNA helix V and thus facilitates repression of translational coupling.

In vivo mutagenesis data for helix V [16,17] also support this suggestion. Mutation of base pair A(−47):U(−35) to G(−47):U(−35) (mutation just opposite the U(−35) cross-link) does not affect S7 inhibition, but reduces translational coupling, probably due to a weakened stability of the helix. In contrast, a double and compensatory mutation of base pair A(−47):U(−35) to U(−47):A(−35) which maintains helical structure, did not affect translational coupling but led to twofold reduced S7 inhibition. Therefore not only the helical structure, but possibly the uridine itself is important for recognition by S7.

Binding of S7 to the branching region of EcoStr143 perfectly fits with sequence identities found for both mRNA and 16S rRNA. Two neighboring sites appeared: a first set of 16S rRNA (1327)CCA(1329)/(1307)UUGGA(1331) with str mRNA (−76)CCA(−74)/(−34)UUGGA(−30) (base-paired bases are underlined) [17], and a second set of 16S rRNA (1346)AGUAA(1350)/(1372)UGAAU(1376) and (1236)ACAAU(1240) with str mRNA (−83)AGUAA(−79)/(−23)UGAAU(−19) and (−30)ACAAU(−26) [11].

For translational coupling, ribosomes should either slide or jump from the termination codon of S12 to the initiation codon of S7. Ribosomes would probably not slide to reinitiate, because intercistronic point mutations cancel the translational coupling [17]. We therefore think that a unique three-dimensional structure of the intercistronic region of str mRNA facilitates ribosomal jumping during translational coupling.

The following mechanism of inhibition of translational coupling by S7 could then be envisaged. The RNA regions under consideration (Fig. 2) fit into about two turns of RNA helix of about 25 Å high, and this size is comparable with the size of protein S7 ($30 \times 35 \times 55$ Å) [24,27]. There might be at least two scenarios of how S7 could inhibit re-initiation. If S7 does not change the RNA structure, then it could cause premature termination of S12 synthesis, meanwhile blocking initiation of S7 synthesis. On the other hand, if S7 would induce conformational changes of the RNA, then the sites of S12 termination and S7 initiation are pulled apart which would hamper ribosomal jumping. Both events can uncouple the translation and therefore more data are required to establish the proper mechanism.

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References

[1] Spirin, A. (1999) in: Ribosomes (Siekevitz, P., Ed.), Kluwer Academic/Plenum Publishers, NY.
[2] Zengel, J.M. and Lindahl, L. (1994) Diverse mechanisms for regulating ribosomal protein synthesis in Escherichia coli. Prog. Nucleic Acid Res. Mol. Biol. 47, 331–370.
[3] Nomura, M. (1999) Regulation of ribosome biosynthesis in *Escherichia coli* and *Saccharomyces cerevisiae*: diversity and common principles. J. Bacteriol. 181, 6857–6864.
[4] Asato, Y. (2005) Regulation of ribosome biosynthesis in *Escherichia coli* and *Saccharomyces cerevisiae*: diversity and common principles. Curr. Issues Mol. Biol. 7, 109–117.
[5] Poehlsgaard, J. and Douthwaite, S. (2005) The bacterial ribosome as a target for antibiotics. Nat. Rev. Microbiol. 3, 870–881.
[6] Champney, W.S. (2003) Bacterial ribosomal subunit assembly is an antibiotic target. Curr. Top. Med. Chem. 3, 929–947.
[7] Klostermeier, D., Sears, P., Wong, C.H., Millar, D.P. and Williamson, J.R. (2004) A three-fluorophore FRET assay for high-throughput screening of small-molecule inhibitors of ribosome assembly. Nucleic Acids Res. 32, 2707–2715.
[8] Nowotny, V. and Nierhaus, K.H. (1988) Assembly of the 30S subunit from *Escherichia coli* ribosomes occurs via two assembly domains which are initiated by S4 and S7. Biochemistry 27, 7051–7055.
[9] Kopylov, A.M. (2002) X-ray analysis of ribosomes: the static of the dynamic. Biochemistry (Mosc.) 67, 372–382.
[10] Rassokhin, T.I., Golovin, A.V., Petrova, E.B., Spiridonova, V.A., Karginova, O.A., Rozhdestvenskii, T.S., Brosius, J. and Kopylov, A.M. (2001) Study of the binding of the S7 protein with 16S rRNA fragment 926-986/1219-1393 as a key step in the assembly of the small subunit of prokaryotic ribosomes. Mol. Biol. (Mosk.) 35, 617–627.
[11] Robert, F. and Brakier-Gingras, L. (2001) Ribosomal protein S7 from *Escherichia coli* uses the same determinants to bind 16S ribosomal RNA and its messenger RNA. Nucleic Acids Res. 29, 677–682.
[12] Tracevska, T., Jansone, I., Nodieva, A., Marga, O., Skenders, G. and Baumanis, V. (2004) Characterisation of rpsL, rrs and embB mutations associated with streptomycin and ethambutol resistance in *Mycobacterium tuberculosis*. Res. Microbiol. 155, 830–834.
[13] Brzostek, A., Sajduda, A., Sliwinski, T., Augustynowicz-Kopec, E., Jaworski, A., Zwolska, Z. and Dziadek, J. (2004) Molecular characterisation of streptomycin-resistant *Mycobacterium tuberculosis* strains isolated in Poland. Int. J. Tuberc. Lung Dis. 8, 1032–1035.
[14] Huang, H.N., Han, J.X., Wang, J.H., Song, C.Z., Liang, H. and Zhang, Z.L. (2003) rpsL gene analysis associated with streptomycin resistance in *Mycobacterium tuberculosis*. Yi Chuan Xue Bao 30, 376–381.
[15] Allen, T.D., Watkins, T., Lindahl, L. and Zengel, J.M. (2004) Regulation of ribosomal protein synthesis in *Vibrio cholerae*. J. Bacteriol. 186, 5933–5937.
[16] Saito, K., Mattheakis, L.C. and Nomura, M. (1994) Post-transcriptional regulation of the str operon in *Escherichia coli*. Ribosomal protein S7 inhibits coupled translation of S7 but not its independent translation. J. Mol. Biol. 235, 111–124.
[17] Saito, K. and Nomura, M. (1994) Post-transcriptional regulation of the str operon in *Escherichia coli*. Structural and mutational analysis of the target site for translational repressor S7. J. Mol. Biol. 235, 125–139.
[18] Spiridonova, V.A., Golovin, A.V., Drygin, D. and Kopylov, A.M. (1998) An extremely high conservation of RNA-protein S7 interactions during prokaryotic ribosomal biogenesis. Biochem. Mol. Biol. Int. 44, 1141–1146.
[19] Spiridonova, V.A., Rozhdestvensky, T.S. and Kopylov, A.M. (1999) 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*. FEBS Lett. 460, 353–356.
[20] Hulme, E.C. and Birdsall, N.J.M. (1992) Strategy and tactics in receptor-binding studies in: Receptor – Ligand Interactions. A Practical Approach (Hulme, E.C., Ed.), pp. 63–176, Oxford University Press, Oxford, New York, Tokyo.

[21] Vorstenbosch, E.L., Potapov, A.P., de Graaf, J.M. and Kraal, B. (2000) The effect of mutations in EF-Tu on its affinity for tRNA as measured by two novel and independent methods of general applicability. J. Biochem. Biophys. Methods 42, 1–14.
[22] Zwieb, C. and Brimacombe, R. (1979) RNA-protein cross-linking in *Escherichia coli* 30S ribosomal subunits: precise localization of the nucleotide in 16S RNA which is coupled to protein S7 by ultraviolet irradiation. Nucleic Acids Res. 6, 1775–1790.
[23] Urlaub, H., Kruft, V., Bischof, O., Muller, E.C. and Wittmann-Liebold, B. (1995) Protein-rRNA binding features and their structural and functional implications in ribosomes as determined by cross-linking studies. EMBO J. 14, 4578–4588.
[24] Wimberly, B.T., Brodersen, D.E., Clemons Jr., W.M., Morgan-Warren, R.J., Carter, A.P., Vonrhein, C., Hartsch, T. and Ramakrishnan, V. (2000) Structure of the 30S ribosomal subunit. Nature 407, 327–339.
[25] Behlen, L.S., Sampson, J.R. and Uhlenbeck, O.C. (1992) An ultraviolet light-induced crosslink in yeast tRNA(Phe). Nucleic Acids Res. 20, 4055–4059.
[26] Huggins, W., Ghosh, S.K., Nanda, K. and Wollenzien, P. (2005) Internucleotide movements during formation of 16 S rRNA-rRNA photocrosslinks and their connection to the 30 S subunit conformational dynamics. J. Mol. Biol. 354, 358–374.
[27] Wimberly, B.T., White, S.W. and Ramakrishnan, V. (1997) The structure of ribosomal protein S7 at 1.9 A resolution reveals a beta-hairpin motif that binds double-stranded nucleic acids. Structure 5, 1187–1198.
[28] Karginov, A.V., Karginova, O.A., Spiridonova, V.A. and Kopylov, A.M. (1995) In vivo assembly of plasmid-expressed ribosomal protein S7 of *Thermus thermophilus* into *Escherichia coli* ribosomes and conditions of its overexpression. FEBS Lett. 369, 158–160.