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The structure of helix 89 of 23S rRNA is important for peptidyl transferase function of *Escherichia coli* ribosome

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ABSTRACT

Helix 89 of the 23S rRNA connects ribosomal peptidyltransferase center and elongation factor binding site. Secondary structure of helix 89 determined by X-ray structural analysis involves less base pairs then could be drawn for the helix of the same primary structure. It can be that alternative secondary structure might be realized at some stage of translation. Here by means of site-directed mutagenesis we stabilized either the "X-ray" structure or the structure with largest number of paired nucleotides. Mutation UU2492-3C which aimed to provide maximal pairing of the helix 89 of the 23S rRNA was lethal. Mutant ribosomes were unable to catalyze peptide transfer independently either with aminoacyl-tRNA or puromycin.

1. Introduction

Ribosomal RNA region composing peptidyl transferase center, elongation factor binging center and connecting those sites is one of the most conserved RNA structure. Helix 89 of the 23S rRNA extends from the peptidyl transferase loop, while the end of the helix interacts with sarcin–ricin loop via the loop of the helix 91. Mutations in the loop region of the helix 89 influence initiation of protein synthesis [1], which is in agreement with earlier observation that this site is involved in IF2 binding [2]. Region connecting helices 89 and 91 compose a binding site for antibiotics evernimicin and avilamicin [3]. The U-rich bulged region around nucleotide 2492 was suggested to be important for the fidelity of translation [4], possibly acting as the element, regulating the rate of aa-tRNA accommodation which allows more efficient dissociation of near-cognate aa-tRNA [5]. Recent experiments on mutagenesis of other 23S rRNA structural elements which were suggested to be important for fidelity of translation via influence on aa-tRNA accommodation [5] in *Escherichia coli* [6] and *Saccharomyces cerevisiae* [7] revealed only moderate effect of these elements on translation fidelity.

Not only loop region of the helix 89 is involved in several ribosomal functions. The lower part of helix 89 stem was identified as a region, essential for maintaining translation fidelity [4]. Later, the computer simulation of aminoacyl-tRNA accommodation in the ribosomal A site showed that interaction of the sugar-phosphate backbone of 23S rRNA helix 89 with aminoacyl-tRNA during accommodation is important for this process [5].

As revealed by X-ray structural analysis, secondary structure of the helix 89 includes many non-canonical pairs and triples (Fig. 1A). For example, G2458 and U2491 are not involved in any pairing [8], while U2492 forms a triple with U2460–G2490 [9]. Potentially, one can draw an alternative variant of secondary structure of helix 89, which involves more base pairs (Fig. 1B). Potential pair G2458–U2492 gains little support from RNA covariation analysis. Nucleotide 2492 shows nearly universal conservation, being uridine. At the same time, 23S rRNA nucleotide 2458 is not conservative and can be A, G or U at nearly the same probability. Potential pairing of A2459 with U2491 is somewhat more probable. A–U, G–U, G–C, C–G and U–A combinations at those positions could be found in 95.8% sequences of the 23S rRNA-like molecules. On the basis of covariation analysis Cannone et al. propose these base pairs to be probable, but with rather low statistical support [8].

It is not clear whether both secondary structures A and B (Fig. 1) are formed during protein synthesis. In this work we decided to check the importance of conformational flexibility of helix 89

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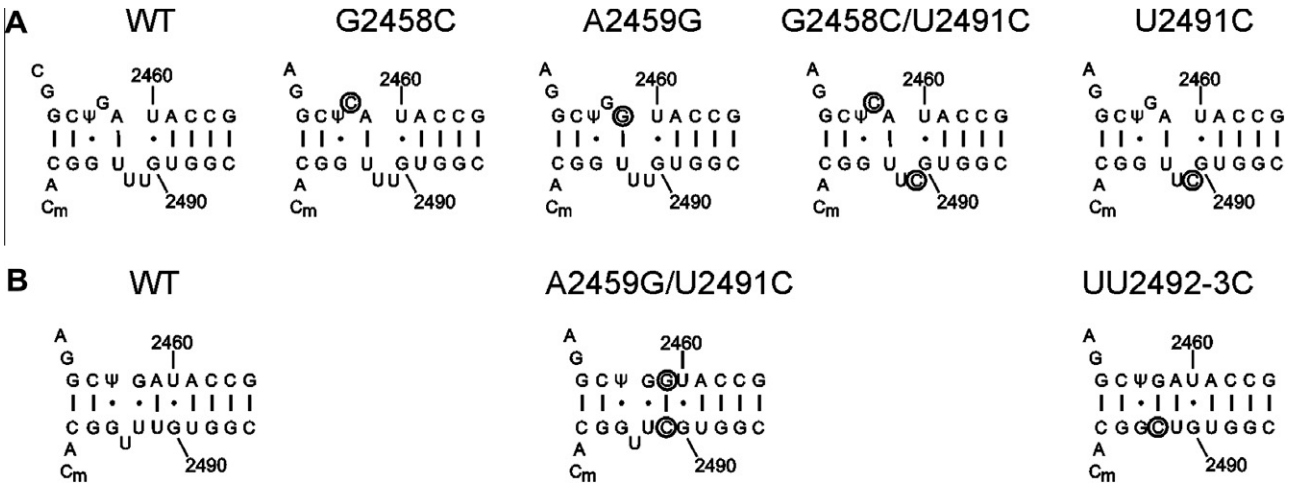

Fig. 1. Two secondary structures of helix 89: conformation A is observed in X-ray structure of ribosome (Ref), conformation B is drawn to have maximal number of paired nucleotides. Mutations that stabilize structure A or B are encircled.

stabilizing one or another secondary structure variant of the helix 89 by site-directed mutagenesis. Mutations G2458C, A2459G, G2458C/U2491C and U2491C should make the formation of structure A more favorable. On the other hand mutations A2459G/U2491C and UU2492-3C should stabilize another conformation of helix 89 – structure B (Fig. 1).

2. Materials and methods

2.1. Buffers

Buffer A: 50 mM Tris–HCl (pH 7.5), 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$. *Buffer B:* 20 mM Hepes–KOH (pH 7.5), 200 mM NH_4OAc, 1 mM $Mg(OAc)_2$, 4 mM β-mercaptoethanol. All experiments were carried out at 37 °C.

2.2. Mutagenesis and ribosome preparation

All mutations were made by standard site-directed mutagenesis procedures in a SphI–BamHI fragment of the *rrnB* operon cloned into M13 mp18 phage [10]. Subsequently, the mutated fragment was subcloned back into the pLK1192U plasmid containing a streptavidin-binding aptamer inserted at the position of helix 25 (vector pStr25) [11]. The plasmid contains the *rrnB* operon of rDNA under control of the phage λ PL promoter. Transformation of the AVS69009 strain [12], plasmid substitutions, and checking the purity of mutant rRNA in the cells were carried out as described [13]. For expression of the UU2492-3C mutant ribosomes *E. coli* strain POP2136 was used, carrying the temperature-sensitive λ repressor.

Standard preparations of ribosomes were made according to Rodnina and Wintermeyer [14]. Preparation of ribosomes with lethal mutation UU2492-3C was made using combination of two methods – prezonal isolation was done according to Rodnina and Wintermeyer [14], and then 50S subunits carrying lethal mutation were purified by affinity chromatography as described [11].

2.3. Preparation of components and ribosomal complexes

EF-Tu, initiation factors, f[^{3}H]Met-tRNA^{fMet} and [^{14}C]Phe-tRNA^{Phe} from *E. coli* were prepared as described [14–16]. f[^{3}H]Met-tRNA^{fMet} and [^{14}C]Phe-tRNA^{Phe} were purified to homogeneity by HPLC [17]. mRNA (5′-GGCAAGGAGGUAAAUAAUGUUCACGAUU-3′, first two codons underlined) was purchased from Dharmacon (USA). Initiation complexes were formed by incubation of 30S and 50S ribosomal subunits (1 μM each) with mRNA (1.5 μM), f[^{3}H]Met-tRNA^{fMet} (1.5 μM), IF1, IF2 and IF3 (1.5 μM each), DTT (1 mM) and GTP (1 mM) in buffer A at 37 °C for 45 min. The amount of f[^{3}H]Met-tRNA^{fMet} bound in the P site was determined by nitrocellulose (NC) filtration assay. The ternary complex EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe} was prepared by incubation of EF-Tu (3 μM) with GTP (1 mM), phosphoenol pyruvate (3 mM) and pyruvate kinase (0.1 mg/ml) in buffer A at 37 °C for 15 min, followed by the addition of purified [^{14}C]Phe-tRNA^{Phe} (2 μM).

2.4. Dipeptide formation assay

Initiation complex (0.4 μM) was mixed with ternary complex (0.6 μM) in buffer A and incubated for 2 min at 37 °C. Reaction was stopped with 0.5 M KOH, samples were hydrolyzed for 30 min at 37 °C, neutralized with acetic acid, and dipeptide formed was analyzed by RP-HPLC [18].

A-site binding assay: Initiation complex (0.3 μM) was mixed with ternary complex (0.45 μM) and incubated for 2 min at 37 °C. The amount of [^{14}C]Phe-tRNA^{Phe} bound in the A site was determined by nitrocellulose (NC) filtration assay.

2.5. Puromycin assay

Initiation complex (0.4 μM) was mixed with puromycin (2 mM) and incubated for 1 min at 37 °C. Reaction was stopped with 0.5 M KOH, hydrolized for 30 min at 37 °C, neutralized with acetic acid, and analyzed by RP-HPLC [18].

2.6. Chemical probing

Chemical probing of 50S subunits was done in buffer B. Modification of 50S subunits with nucleotide-specific reagents DMS, kethoxal and CMCT was performed according to Stern et al. [19] with the exception that modification were done for 10 min at 37 °C. DNA primers were complementary to the following regions of 23S rRNA: 2730–2749 and 2591–2611.

3. Results

3.1. Site-directed mutagenesis and in vivo effects of mutations

Mutations were introduced to the 23S rRNA coding part of *rrnB* ribosomal operon cloned to M13 phage by site-directed

mutagenesis. Later, DNA fragments containing mutations were cloned to the pStr25 expression vector [11]. In this plasmid, ribosomal *rrnB* operon is under control of phage lambda PL promoter and contains streptavidine-binding aptamer in the helix 25. To check the phenotype of cells with mutations in helix 89 we transformed AVS69009 strain, that lacked all seven chromosomal rRNA operons [12], with plasmids, carrying the mutations and selected against the plasmid, carrying wild-type rDNA. We found that all mutant ribosomes but containing UU2492-3C mutation could support cell life in the absence of wild-type ribosomes (Fig. 2).

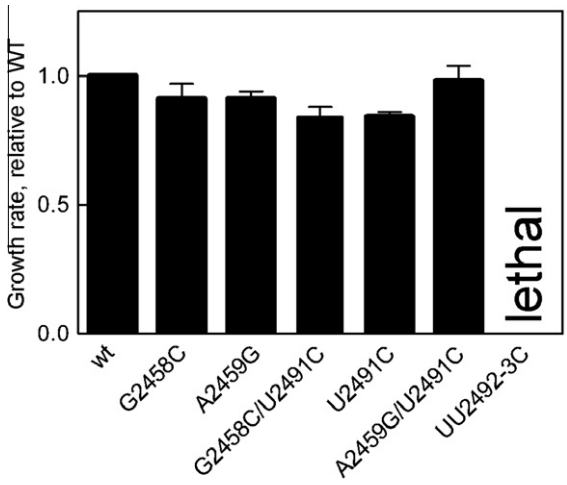

Fig. 2. Influence of mutations in 23S rRNA on the cell growth. For this experiment strain AVS69009 was used, in which protein synthesis was performed only by mutant ribosomes.

The UU2492-3C mutation caused recessively lethal phenotype and could be expressed only from a regulated promoter, with the cell life being supported by the expression of the wild-type ribosomal operons. To find out which stage of translation was abolished by the mutation we decided to perform in vitro experiments to check how mutation influenced basic ribosomal functions and structure of 23S rRNA.

3.2. Initiation complex formation and dipeptide synthesis in vitro

For in vitro experiments we performed large-scale isolation of wild-type ribosomes and ribosomes carrying the mutation UU2492-3C. Ribosomes were purified by affinity chromatography on streptavidine sepharose as described by Leonov et al. [11]. Homogeneity of the ribosome preparations was confirmed by primer extension method (direct RT-analysis technique) [13].

Efficiency of the initiation complex formation by the wild-type and mutant ribosomes was monitored by filtration through the nitrocellulose. Mutation UU2492-3C did not have significant influence on the IFs-dependent binding of initiator fMet-tRNA^{fMet} to the P site of mRNA-programmed ribosomes (Fig. 3A), which is the first step of prokaryotic translation.

Next, ternary complex EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe} was added to the initiation complexes and the synthesis of dipeptide fMet-Phe was measured. We found, that almost 75% of initiatory complex formed by wild-type ribosomes was active in dipeptide synthesis although in the case of ribosomes with mutation UU2492-3C dipeptide formation was completely abolished. There were two possible explanations of why mutant ribosomes cannot perform the peptidyl transferase reaction. On the one hand, mutation could affect initial binding of aminoacyl-tRNA to the A site or its accommodation to the A site so that aminoacylated CCA-end of tRNA could not reach the peptidyl transferase center of 50S subunit and take part in

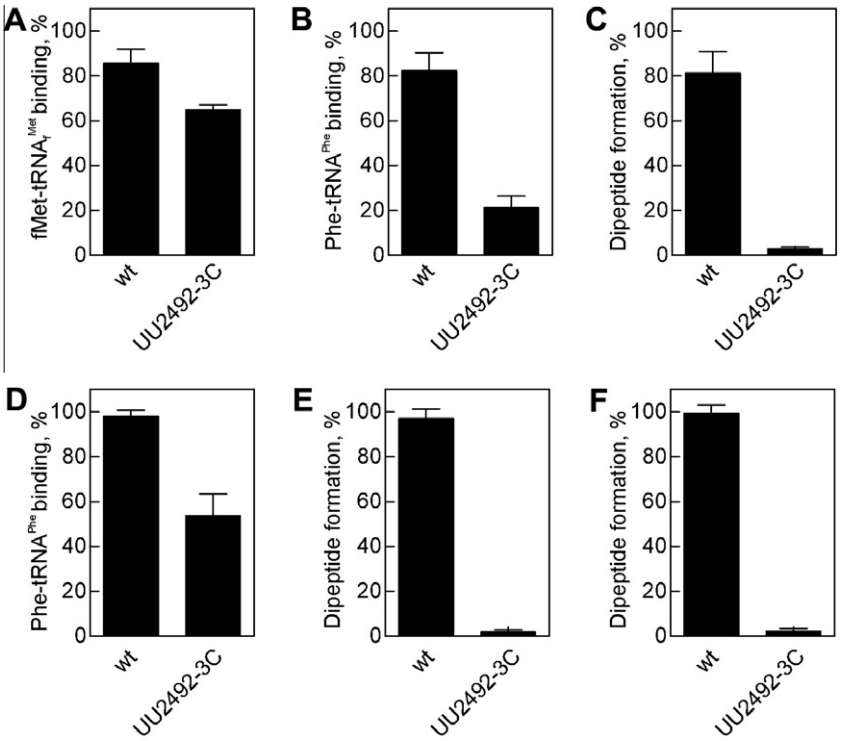

Fig. 3. In vitro study of ribosomes carrying the mutation UU2492-3C. (A) Binding of initiator fMet-tRNA^{fMet} to the P site of 70S ribosomes. (B) Binding of Phe-tRNA^{Phe} to the A site of initiated 70S ribosomes in the buffer containing 7 mM Mg^{2+}. (C) Dipeptide fMetPhe formation in the experiment (B). (D) Binding of Phe-tRNA^{Phe} to the A site of initiated 70S ribosomes in the buffer containing 14 mM Mg^{2+}. (E) Dipeptide fMetPhe formation in the experiment (D). (F) Dipeptide fMetPuro, as in experiment (B), but puromycin was used as A site substrate instead of intact Phe-tRNA^{Phe}.

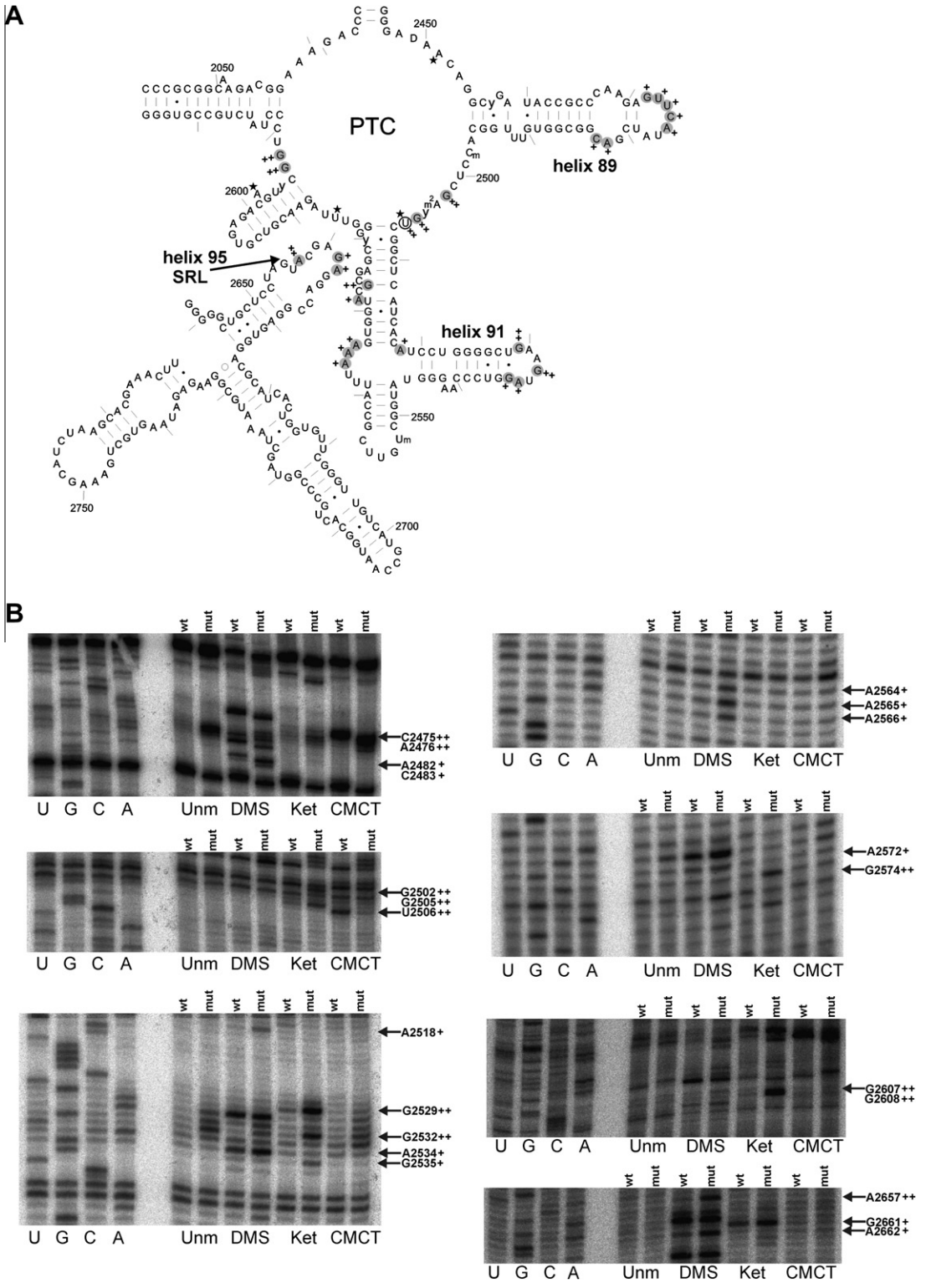

Fig. 4. Chemical probing of the 23S rRNA in large ribosomal subunits of ribosomes with the mutation UU2492-3C. (A) Nucleotides that changed their reactivity to chemical agents on the secondary structure of 23S rRNA. Nucleotides that increased their chemical reactivity are marked with gray circles, nucleotides, that decreased their chemical reactivity, marked with open circles, nucleotides that form active site of peptidyl transferase center are indicated by the stars. (B) Primer extension analysis of rRNA extracted from the large ribosomal subunits modified with chemical reagents. Fragments of sequencing gel are shown. U, G, C and A correspond to sequencing lanes; Unm, unmodified rRNA; DMS, modification with DMS; Ket, modification with kethoxal; CMCT, modification with CMCT. Wt and mut at the top of each gel fragment mark lanes that correspond to wild type ribosomes or ribosomes with the mutation UU2492-3C. Nucleotides that changed their reactivity are shown by black arrows. "+" sign shows the strength of effect: "+" – moderate effect, "++" – strong effect.

aminoacid transfer. On the other hand, it could be that Phe-tRNA^{Phe} binds to the A site and accommodates correctly, but ribosome is unable to catalyze the peptidyl transferase reaction. To find out, which of two hypothesises is correct we decided to check the influence of mutation UU2492-3C on the binding of aminoacyl-tRNA to the A site and peptidyl transferase reaction.

3.3. Binding of aminoacyl-tRNA to the A site

The efficiency of Phe-tRNA^{Phe} binding of to the ribosomal A site was measured by filtration of the ribosomal complex through nitrocellulose membrane. In parallel, the dipeptide formation was measured. We found, that binding of Phe-tRNA^{Phe} to the A site of ribosomes with mutation UU2492-3C was much lower comparing to wild-type ribosomes (27% and 77%, respectively) (Fig. 3B). At the same time almost all wild-type ribosomes were able to synthesize dipeptide, but in the case of mutant ribosomes dipeptide formation was almost undetectable (Fig. 3C). It is well known, that increase of Mg^{2+} concentration facilitates the tRNA binding to the ribosome in vitro. Raising magnesium ions concentration from 7 to 14 mM resulted in significant increase of binding efficiency of Phe-tRNA^{Phe} to the A site of mutant and wild type ribosomes (61% and 100%, respectively) (Fig. 3D) and slightly increased dipeptide formation in the case of wild type ribosomes but not in the case of UU2492-3C ribosomes (Fig. 3E). The fact that amount of aa-tRNA bound in the A site does not have any influence on the yield of dipeptide favors the hypothesis that mutation UU2492-3C affects the function of ribosomal peptidyl transferase center. To check this idea we decided to use puromycin instead of aa-tRNA as A site substrate.

3.4. Puromycin test

Puromycin is a small analog of acceptor end of aa-tRNA. It binds directly to peptidyl transferase center that allows to exclude the possible effects of the conformation of A site on the rate of dipeptide formation. We added puromycin to the ribosomal initiation complexes and measured fMet transfer from the fMet-tRNA^{fMet} to the puromycin. Although wild-type ribosomes were almost quantitatively active in this assay, mutant ribosomes were not able to catalyze peptidyl transfer reaction with puromycin as the A-site substrate (Fig. 3F). This allows us to suggest, that UU2492-3C mutation most likely affects the function of peptidyl transferase center. To have an insight on the structural changes of 23S rRNA in the vicinity of peptidyl transferase center caused by mutation UU2492-3C we decided to make a chemical probing of mutant 50S subunits.

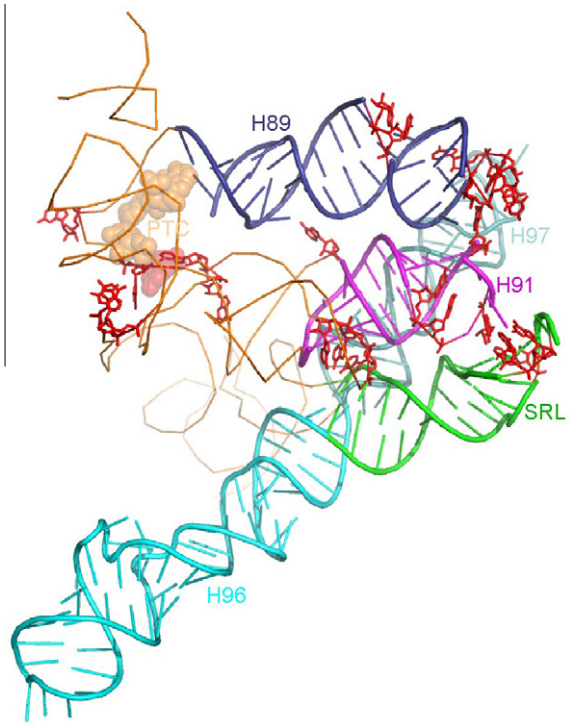

Fig. 5. Chemical probing of the 23S rRNA in 50S subunits with the mutation UU2492-3C on tertiary structure of 23S rRNA. Nucleotides that changed their chemical reactivity are red, nucleotides of "inner shell" of peptidyl transferase center are bulky. Helix 89 is blue, helix 91 is magenta, helix 95 (sarcin–ricin loop) is green, helices 96 and 97 are marked with different hues of cyan.

3.5. Chemical probing of ribosomes carrying mutation UU2492-3C

To investigate the effect of mutation UU2492-3C on the structure of helix 89 and peptidyl transferase center of the 23S rRNA we performed chemical probing using base-specific reagents DMS, kethoxal and CMCT [19]. The most prominent difference in chemical reactivity was found for the nucleotide bases involved in the interactions between the tips of the helices 89, 91, and 95 (SRL): 2472–2476, 2482–2483, 2518, 2529, 2532, 2534, 2535, 2657, 2661, 2662 (Figs. 4 and 5). Another set of nucleotides which changed their chemical reactivity includes bases 2505–2506, 2607–2608 which are located in the vicinity of nucleotides that form the "inner shell" of peptidyl transferase center [20] (Figs. 4 and 5). Nucleotides 2502, 2564–2566, 2572 and 2574 increased their reactivity as well (Figs. 4 and 5).

4. Discussion

Helix 89 of the 23S rRNA connects functionally important regions of the large ribosomal subunit, peptidyl transferase center and elongation factor binding site. Secondary structure of the helix 89 determined by X-ray structure analysis has several unpaired nucleotides, as well as non-canonical pairs and triples. Based on the primary structure one can intuitively draw a variant of the helix 89 secondary structure which involves more canonical base pairs and has less amount of bulged nucleotides.

To investigate the role of two possible conformations of helix 89 for the functioning of ribosome we artificially stabilized them by introducing mutations that favored one or another pattern of nucleotide pairing. One of the mutations, UU2492-3C, which modeled the maximal pairing structure of helix 89, resulted in lethal phenotype in vivo, whereas other mutations did not have significant effects on the cell growth comparing to the wild-type. This result is in agreement with the phenotype of partially overlapping set of mutations in *S. cerevisiae* 25S rRNA [7]. In *S. cerevisiae* deletion of a nucleotide, equivalent to U2492 in *E. coli* was shown to be lethal, although exact cause of lethality was not determined.

Detailed investigation of ribosomes with the mutation UU2492-3C revealed that this mutation slightly decreased the initiator fMet-tRNA^{fMet} binding to the P site and completely abolished peptidyl transferase function of ribosome regardless of which A site substrate was used, puromycin or intact aminoacyl-tRNA. The active site of peptidyl transferase center is formed by 23S rRNA nucleotides A2451, U2506, U2585 and A2602 [20]. Mutations of these nucleotides diminished peptidyl transferase reaction if puromycin was used as A site substrate, but could not completely inactivate the catalysis of peptide transfer if normal aa-tRNA was used [21]. Surprisingly, mutation UU2492-3C which is located in the helix 89 of 23S rRNA but not in the active site of peptidyl transferase center, abolished peptidyl transferase reaction completely for aminoacyl-tRNA as well as for puromycin. Chemical probing revealed that in ribosomes with mutation UU2492-3C nucleotide U2506 significantly changed its reactivity. Another conformational change in 23S rRNA which could affect peptidyl transferase function was localized in the vicinity of nucleotides G2607 and G2608 which also greatly changed their accessibility for chemical agents. Interestingly, that despite such dramatic influence of mutation UU2492-3C on the peptidyl transferase reaction, binding of initiatory tRNA in the P site and binding of aa-tRNA in the A site was not abolished completely. Moreover, centrifugation of mutant 50S subunits in

the sucrose gradient did not reveal any differences between wild-type and mutant 50S subunits profiles (data not shown).

Tertiary contacts of helixes 89, 91 and 95 (SRL) are very important for the functioning of ribosome. There are several examples when lethal phenotype was caused or accompanied by the disruption of contacts of these helices. For instance, this occurs when additional base pair is inserted in the helix 42 of 23S rRNA at positions C1030/G1124. Such mutation causes the movement of so-called GTPase associated center of the large ribosomal subunit towards the tip of helix 89, that, among other changes, leads to disruption of the set of contacts between loops of helices 89, 91 and SRL [22]. Also, mutations A2662C, A2662U, and A2531G, which disrupt the contact between SRL and helix 91, have a dominant lethal phenotype [23]. Lethal mutations in the P-loop of the 23S rRNA (nucleotides G2251 and G2252) which is located in the peptidyl transferase center of ribosomes, but not directly in the peptidyl transferase loop, also affected the contacts between the loops of helix 91 and SRL [24]. Freezing the maximal base paired conformation of the helix 89 studied in this paper leads to the same effect of disruption of contacts between loops of helices 89, 91 and SRL (Fig. 5).

Not only mutations in rRNA can affect the structural organization of helixes 89, 91, 95 and adjacent to them but also the deletion of protein L36 which in the crystal structure of *Deinococcus radiodurans* [25] 50S subunit is surrounded by several highly conserved functional regions of 23S rRNA, including helices 89, 91 and SRL. Deletion of ribosomal protein L36 reduces the growth rate of the bacteria almost twice. Chemical probing revealed, that contacts of helixes 89–91–95 are also disrupted in the ribosomes that miss L36 [26].

As a conclusion we can state that secondary structure variant of helix 89 determined by X-ray structure analysis is important for peptidyl transferase activity of ribosomes. Alternative secondary structure of helix 89 which can be modeled on the basis of maximal base pairing is non-functional and probably is never formed during translation.

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