Effect of point mutations at position 89 of the *E. coli* 5S rRNA on the assembly and activity of the large ribosomal subunit

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Abstract Nucleotide residue U89 in the D loop of *Escherichia coli* 5S rRNA is adjacent to two domains of 23S rRNA in the large ribosomal subunit [Dokudovskaya et al., RNA 2 (1996) 146–152]. 50S ribosomal subunits were reconstituted containing U89(C, G or A) mutants of 5S rRNAs and the activities of the corresponding 70S ribosomes were studied. The U89C mutant behaves similarly to the wild-type 5S rRNA. Replacement of the pyrimidine base at position U89 by more bulky purine bases impairs the incorporation of 5S rRNA into 50S subunits, whereas the particles formed showed full activities in poly(U)-dependent poly(Phe) synthesis in the presence of either U89G or U89A 5S rRNA mutants. The activity of the reconstituted particles depends on the incorporation of 5S rRNA in agreement with early observations.

Key words: 5S ribosomal RNA; Mutation; Ribosomal reconstitution; Ribosomal activity; Ribosomal structure

1. Introduction

5S RNA is a small ribosomal RNA. It is an essential constituent of the large ribosomal subunit. Ribosomes lacking 5S rRNA have only residual biological activity; this rRNA is responsible for an activation step late in the reconstitution of the 50S subunit [2]. 5S rRNA has been assumed to be responsible for different activities of the ribosome, but its exact role in ribosome functioning is still unknown (for reviews see [3,4]).

5S rRNA can be incorporated into the large ribosomal subunit as a complex with three specific proteins in the case of prokaryotes and with one protein in the case of eukaryotes. The 5S rRNA-protein complex forms a separate domain in the ribosome. Incorporation of the 5S rRNA-protein complex (at least in the case of eukaryotic ribosomes) is a late step in the assembly in vivo of the large ribosomal subunit [2,5]. The 5S rRNA-protein complex can be easily extracted from 50S or 60S ribosomal subunits without significant changes in their physical properties [6].

According to IEM data the 5S rRNA-protein complex is situated in the central protuberance of the large ribosomal subunit [7,8]. A cross-linking study of the contacts between 5S rRNA and 23S rRNA has shown that nucleotide U89 of 5S rRNA could be cross-linked to both nucleotide A960 in helix 39 and nucleotide C2475 in helix 89 in the 23S rRNA [1]. The cross-linked sites are located close to the EF-G binding domain and the 'peptidyltransferase ring', respectively, thus U89 was proposed to connect these functional domains in the large ribosomal subunit and to play an important role in the communication between the GTPase-associated center and the peptidyltransferase center during translation.

Site-directed mutagenesis is a very powerful method to examine the functional and structural importance of individual nucleotide residues in RNA molecules. In this work we have introduced point mutations into *Escherichia coli* 5S rRNA at position 89 and investigated the behavior of these mutants in the reconstitution and the activities of the 50S subunit in vitro.

2. Materials and methods

Ultrapure rNTPs were from Pharmacia; RNasin, Taq DNA polymerase, alkaline phosphatase and T4 polynucleotide kinase were from Boehringer Mannheim; inorganic pyrophosphatase and pyruvate kinase were from Sigma; γ-[^{32}P]ATP and [^{14}C]Phe were from Amersham. The T7 RNA polymerase was isolated from *E. coli* strain BL21 containing plasmid pAR1219 according to a published procedure [9]. S-100 extract, 50S and 30S ribosomal subunits were obtained as described [10].

2.1. Preparation of mutant 5S rRNA

The DNA template containing the *E. coli* 5S rDNA sequence linked to a T7 promoter was prepared as described [11] and transcribed by T7 polymerase using the procedure of [12]. In order to obtain mutant 5S rDNAs, amplification of DNA by PCR was carried out in the presence of the following oligodeoxynucleotides, complementary to the 3′ part of the 5S rDNA molecule: (1) 5′-GGCCTGGCAGTTCCCTACTCTCGCATGGGGTGACCCC-3′, (2) 5′-GGCCTGGCAGTTCCCTACTCTCGCATGGGGCGACCCC-3′, (3) 5′-GGCCTGGCAGTTCCCTACTCTCGCATGGGGGGACCCC-3′, carrying the mutated nucleotide (underlined).

5S rRNA was purified by electrophoresis in 4% PAGE with 7 M urea [13]. Radioactive labeling at the 5′ end was carried out using γ-[^{32}P]ATP and T4 polynucleotide kinase [13]. 5S rRNA was used for the reconstitution of 50S ribosomal subunits after the second purification in 4% PAGE with 7 M urea.

2.2. Reconstitution of 50S ribosomal subunits

23S rRNA was isolated from crude 70S ribosomes as described [14]. Total protein of 50S ribosomal subunit (TP50) was isolated using the procedure of [14]. A two-fold molar excess of the ^{32}P-labeled 5S rRNA was mixed with 23S rRNA and TP50 and reconstituted as described [2]. Half of the reconstitution mixture was used for the measurement of the activity of the obtained particles in the poly(U)-dependent poly(Phe) synthesis as described [14]. The rest of the reconstitution mixture was applied to a 10–30% sucrose gradient in 20 mM HEPES-KOH, pH 7.6, 20 mM magnesium acetate, 400 mM NH_4Cl, 0.2 mM EDTA, 4 mM 2-mercaptoethanol and centrifuged for 18 h at 4°C at 20 000 rpm in an SW 40 Ti rotor (Beckman). After fractionation (the fraction size was about 0.5 ml), the radioactivity

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Abbreviations: IEM, immunoelectron microscopy; EF-G, ribosomal elongation factor G; PTC, peptidyltransferase center

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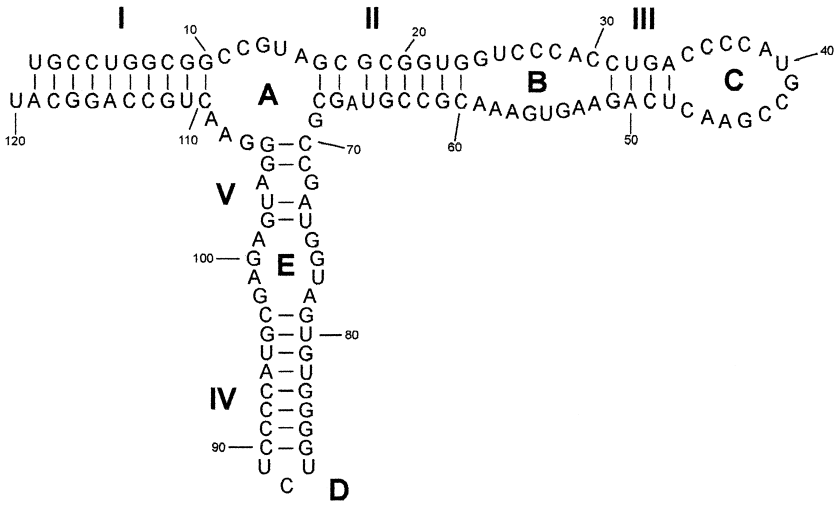

Fig. 1. Secondary structure of 5S rRNA from *E. coli*.

and optical density were determined and the fractions that contained the 50S ribosomal subunit were collected.

3. Results and discussion

Nucleotide U89 is situated in the D loop of *E. coli* 5S rRNA (Fig. 1).

As emphasized above, the location of nucleotide U89 in close proximity to major ribosomal functional sites – the peptidyltransferase center and the EF-G binding domain – could indicate its significant role in the formation of active ribosomes. In order to check this possibility we tested the effect of point mutations at position 89 in the *E. coli* 5S rRNA molecule on the in vitro assembly and activity of the ribosome in poly(U)-dependent poly(Phe) synthesis [14].

DNA templates encoding 5S RNA molecules with the point mutations were obtained by amplification of 5S rDNA by PCR in the presence of oligodeoxynucleotides containing substitutions at positions corresponding to U89. After purification via PAGE, 5S rRNA molecules were used for the reconstitution of the 50S ribosomal subunits in vitro.

The poly(Phe) synthesis activity of the 50S subunits reconstituted in the presence of the wild-type 5S rRNA transcript was similar to that of the subunits reconstituted in the presence of native 5S rRNA molecules and amounted to $30 \pm 6\%$ of the activity of native 50S ribosomal subunit. The residual activity of the particles obtained in the absence of 5S rRNA was about $6 \pm 3\%$ of the activity of native 50S ribosomal subunits.

The activity of the ribosomal subunits reconstituted in the presence of U89A and U89G mutant 5S rRNA molecules in the poly(U)-dependent system of poly(Phe) synthesis was reduced in comparison to the wild-type 5S rRNA (Table 1). A reduced activity of $65 \pm 7\%$ was observed in the case of the U89A mutant, and an activity of $75 \pm 10\%$ was found in the case of U89G. The activity of the 50S subunit reconstituted in the presence of U89C mutant 5S rRNA was equal to that of 50S subunit reconstituted with wild-type 5S rRNA ($99 \pm 6\%$).

Two factors could cause the reduction in the activity of ribosomes. On the one hand, mutations in the 5S rRNA molecule can directly influence the activity of the obtained particles. On the other hand, reduced activity could be the result of an impaired incorporation and thus a decrease in the fraction of 50S subunits in the reaction mixture containing 5S rRNA.

In order to discriminate between these two possibilities reconstituted 50S ribosomal subunits were isolated via sucrose gradient (Fig. 2) and the amounts of 50S subunits and 5S rRNA were determined. First, the optical density of fractions containing 50S subunits was measured and the amount of the ribosomes was calculated. These results were normalized to the amount of the 50S ribosomal subunits containing the wild-type 5S rRNA. Second, radioactive 5S rRNA molecules were used for reconstitution and the amount of 5S rRNA in the reconstituted particles was calculated from the radioactivity of the 50S subunit peak. These results were also normalized to the amount of the 50S ribosomal subunits containing the wild-type 5S rRNA.

In the case of U89A and U89G mutants incorporation of 5S rRNA into the ribosome was reduced (Table 1). $57 \pm 6\%$ of the U89A mutant 5S rRNA was incorporated into the ribosome. In the case of U89G mutant an incorporation of $79 \pm 7\%$ was found. The U89C mutation did not influence the incorporation of 5S rRNA into the large ribosomal sub-

Table 1
Effect of mutations at position 89 of the 5S rRNA molecule on 5S RNA incorporation into 50S ribosomal subunits and the activity of reconstituted particles in the poly(U)-dependent system of poly(Phe) synthesis

Mutant	Incorporation of 5S rRNA (%)	Activity of the reconstituted 50S ribosomal subunits (%)
Wild-type	100	100
U89C	97 ± 4	99 ± 6
U89G	79 ± 7	75 ± 10
U89A	57 ± 6	65 ± 7

The deviations from averaged values are given.

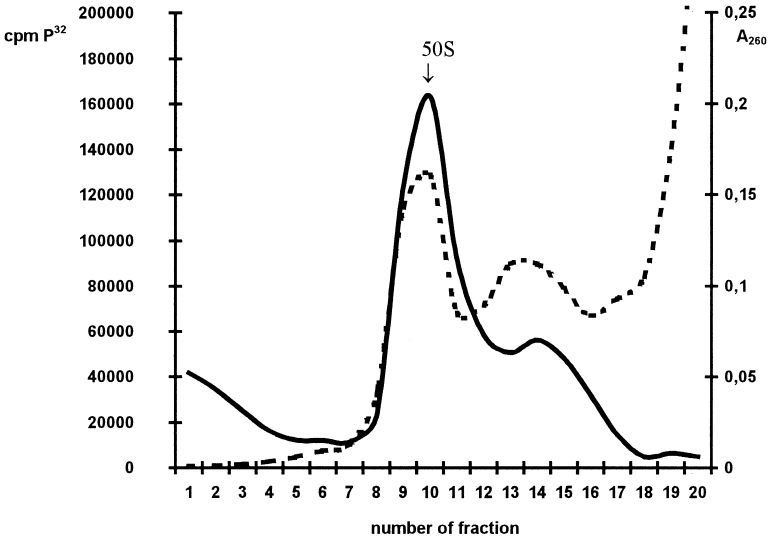

Fig. 2. Sucrose density gradient profiles of the reconstituted 50S ribosomal subunits containing ^{32}P-labeled 5S rRNA. Solid line, optical density; broken line, radioactivity.

unit. The incorporation level correlates well with the observed corresponding activity (Table 1). It follows that the U89A and U89G mutations in 5S rRNA disturb mainly its incorporation into the 50S ribosomal subunit, but after incorporation the resulting 50S subunit is fully active in poly(Phe) synthesis. The base at this position does therefore not form a functionally important base pair, and the base character is not involved in an important function. We note that in agreement with these observations the nucleotide sequences of the D loop (including position 89) of the structure of prokaryotic 5S rRNA are not conserved. Moreover, all eukaryotic 5S rRNAs have G at the position equivalent to U89 in *E. coli* 5S rRNA [4]. Nevertheless, this residue could be involved in the other type of interaction.

It has previously been shown that the U89 residue of 5S rRNA in biologically active ribosomes of *E. coli* is in direct contact with two highly conserved nucleotides A960 and C2475 of 23S rRNA [1]. The hairpin loop containing C2475 is an element of the universal core of the 23S rRNA secondary structure and is located in close proximity to the ribosome peptidyltransferase center. The hairpin loop containing A960 is also a very conserved element of rRNA of the large subunit and is located not far from the ribosomal EF-G binding domain. Thus it was suggested that U89 is involved in the organization of long distance tertiary interactions between the two functional domains of the ribosome. A possibility is that by analogy with the photochemical mechanism of cross-linking of thio-U8 to C13 in tRNA [15] the U89 base of 5S rRNA in the ribosome partially overlaps both the A960 and C2475 bases of 23S rRNA and that possibly all three bases form a stacked structure.

It is known that base-base stacking interactions are not specific. One would therefore expect that if the proposed scheme of interaction between 5S rRNA and 23S rRNA is correct, the ribosome with 5S rRNA mutated at position U89 might retain its activity as observed. Such types of interaction might be important both for ribosome function and for insertion of 5S rRNA into and retention in the large ribosomal subunit.

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