Probing the Functional Motifs of *Escherichia coli* 5S rRNA in Relation to 16S rRNA Using a SELEX Experiment

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The function of 5S rRNA, a constituent of a large subunit of ribosome, is not clearly known yet. To identify RNA motifs interacting with 5S rRNA, and thereby to get an insight into the function of 5S rRNA in the ribosome, a SELEX (Systematic Evolution of Ligands by Exponential Enrichment) experiment was performed. RNA molecules binding to *Escherichia coli* 5S rRNA were selected from a 48-mer random sequence library through 12 rounds of selection, cloned, and sequenced. Two groups of the selected RNA molecules had the consensus sequences GCGG and GUGAAA, respectively, which are present in the segment, G_{688} through A_{696}, of *E. coli* 16S rRNA. The gel mobility shift assay showed that 5S rRNA interacted with the 16S rRNA fragment containing the GCGG and GUGAAA sequences. The enzymatic protection experiment shows that the $A_{29}CCUGA_{34}$ and $G_{51}AAGUG_{56}$ sequences of 5S rRNA and the $C_{680}AGG_{683}$ and $G_{688}CGG_{691}$ sequences of the 16S rRNA fragment are involved in the interaction between the two RNA molecules. On the basis of this observation, we suggest that 5S rRNA and 16S rRNA play a role for the association of two ribosomal subunits.

Introduction

The ribosomes of all the organisms, procaryotic and eucaryotic, consist of two subunits, large and small. The ribosome of the eubacterium contains 16S rRNA in 30S subunit, 23S rRNA and 5S rRNA in 50S subunit. Presently, the notion that the rRNAs are directly involved in the ribosomal functions is generally accepted. There is extensive biochemical, genetic and phylogenetic evidence for the functional role of ribosomal RNAs. This includes the participation of rRNA in mRNA selection, tRNA binding (in A, P and E sites), ribosomal subunit association, proofreading, factor binding, antibiotic interaction, termination and the peptidyltransferase function.[1-3] So far, mRNA selection (interaction of Shine-Dalgarno sequence and anti-Shine-Dalgarno sequence)[1,4] and peptidyltransferase function[5-7] are firmly established as an rRNA-based mechanism by the direct experimental evidences.

For a long time, 5S rRNA was a favorite substrate for investigation of phylogenetic relation between different species,[8] resulting in a very fast accumulation of 5S rRNA sequence data. Its small size, its availability in the isolated state, and its universal secondary structure (which undoubtedly reflects the universal function in protein biosynthesis)[9,10] gave reason to hope that the role of 5S rRNA in ribosome function would be solved in the near future. However, in spite of the many works, this problem relating to a deeper understanding of the role of 5S rRNA inside the ribosome particle was proved to be very complicated. Recently, Russian ribosomologists have shown, using several photoreactive chemical linkers, that U_{89} of 5S rRNA was cross-linked to nucleotides U_{2477},[6] and U_{958}, G_{1022}, and G_{1138}[7] of 23S rRNA. From these findings, they proposed a model that loop e of 5S rRNA molecule participated in the peptidyltransferase activity and also in the GTPase activity of 23S rRNA. This is the first direct evidence ever obtained that 5S rRNA is directly involved in the ribosomal function.

Here we describe a SELEX (systematic evolution of ligands by exponential enrichment) experiment to find the RNA motifs which bind specifically to 5S rRNA of *E. coli*. SELEX is a technique for isolating nucleic acid molecules with affinities for a target molecule from a random pool with a large number of sequences by iterative rounds of affinity selection and amplification.[11] In this work, we isolated RNA motifs which were specifically binding to *E. coli* 5S rRNA from a 48-mer random RNA pool. The sequence analysis of these motifs showed that some of the selected RNA molecules contained well-conserved sequence motifs GCGG and GUGAAA. The helix 23 (the helix was numbered as by Raué *et al.*[12]) in domain of 16S rRNA of *E. coli* comprising conserved motifs GCGG and GUGAAA was prepared to study its interaction with 5S rRNA in solution. The gel mobility shift assay and the enzymatic protection experiment showed that there was a specific interaction between the two RNA molecules. From this observation, we have discussed a possibility that 5S rRNA is involved in the association of two ribosomal subunits and also in the binding of tRNA to ribosome.

Experimental Section

RNA library. The oligonucleotide library, 5'-AAGCT-

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TGCATGCGGATCC-$(N)_{48}$-GAGCTCGAATTCACCTAT-AGTGAGTCGTATTA-3', which was a gift of Professor F. J. Schmidt (Missouri-Columbia University), contained 48 randomized nucleotides franked by primer binding site at 5' side and the promoter sequences at 3' side, respectively. This library was amplified through five cycles of polymerase chain reaction (PCR). One microgram of this population was transcribed with T7 RNA polymerase (RiboMAX^{TM}, Promega) in a 20 μL reaction volume according to the manufacturer's directions. Transcripts were purified by 5% denaturing polyacrylamide gel electrophoresis. The purified RNA composed of 81 nucleotides was dissolved in 0.2 mL of the binding buffer (30 mM Tris-acetate, pH 7.5, 60 mM Mg-acetate, 120 mM K-acetate, 120 mM NH_4-acetate) and brought to 55 °C for 15 min and slowly cooled to room temperature.

Affinity column. 200 μg of purified *E. coli* 5S rRNA by a 5% denaturing polyacrylamide gel electrophoresis was dissolved in 100 μL of 0.1 M K-phosphate, pH 8.0. Then 50 μL of freshly prepared 20 mM $NaIO_4$ was added and placed on ice in the dark for 2 h. The RNA was recovered by ethanol precipitation and redissolved in 0.1 mL of 0.1 M K-acetate, pH 5.0. The oxidized RNA was coupled to 0.5 mL of Sepharose-adipic acid hydrazide resin (Pharmacia) at 4 °C for 16 h with gentle mixing.[13]

Selection protocols. In order to minimize the enrichment of undesired RNA species binding to the Sepharose resin itself, RNA pool was preselected on Sepharose-adipic acid hydrazide. 5 μg of the RNA pool was loaded into a Sepharose-adipic acid hydrazide column with a 0.5-1 mL bed volume in the binding buffer and eluted with 1.5 mL of the binding buffer. The eluted RNA was applied to the 5S rRNA-affinity column with a 0.5 mL bed volume. The affinity column was washed with eight column volumes of the binding buffer and then the bound RNA population was eluted with three column volumes of 25 mM Na-EDTA, pH 8.0, as a selected RNA pool. The complementary DNA (cDNA) of the selected RNA was produced by primer extension with the cDNA primer (5'-AAGCTTGCATGCGGA-TCC-3'). The cDNA was amplified by PCR with the cDNA primer and the T7 primer (5'-TAATACGACTCACTATAG-GTG-3'). The amplified DNA was purified by 5% polyacrylamide gel electrophoresis. The DNA was used as the template for *in vitro* transcription to generate an RNA pool for the next rounds of selection. Multiple rounds of successive selection were carried out to enrich RNA molecules with high binding affinity to 5S rRNA. After the 12th selection, the amplified DNA was ligated into pGEM-T (Promega) and introduced into *E. coli* DH5α. The individual insert DNA sequences were determined by the chain-termination method using Sequenase version 2.0 (United States Biochemical).

Preparation of the 16S rRNA fragment. Template DNA for 16S rRNA fragment (helix 23) was purchased from Bioneer, Korea. The 16S rRNA fragment was synthesized by *in vitro* transcription with T7 RNA polymerase from the template 5'-CTCCAGATCTCTACGCATTTCACCGCTACAC-CTGGAACACCTATAGTGAGTCGTATTA-3' to which the T7 primer 5'-TAATACGACTCACTATAGGTG-3' containing the T7 promoter was annealed. The sequence of the resulting 16S rRNA fragment was $GGUGU_{677}UCCAGGU-GUAGCGGUGAAAUGCGUAGAGAUCUGGAG_{713}$, the sequence from 688 to 696 of 16S rRNA is underlined (Experimentally determined secondary structure of 16S rRNA fragment is presented in Figure 5).

Gel mobility shift assay. RNA was internally labeled by *in vitro* transcription with [α-^{32}P] CTP. The labeled RNA and 5S rRNA were separately heated in the binding buffer at 55 °C for 15 min and slowly cooled to room temperature. The labeled RNA was mixed with 5S rRNA at different concentrations in a final volume of 10 μL and incubated at room temperature for 20 min. The samples were applied to a 5% non-denaturing polyacrylamide gel and electrophoresed in the binding buffer at a constant voltage of 20 V for 20 hr at 4 °C. Relative amounts of the free labeled 16S rRNA fragment and the 16S rRNA fragment-5S rRNA complex were estimated with Fuji FLA-2000 Phosphorimager.

Enzymatic protection experiment. The 16S rRNA fragment or 5S rRNA was labeled at the 5' end using [α-^{32}P]ATP and T4 polynucleotide kinase. The non-radiolabeled 16S rRNA fragment or 5S rRNA (100 pmol in case of 16S rRNA fragment and 116 pmol in case of 5S rRNA) was added to the labeled cognate RNA (*ca.* 2.5 pmol) to make the final reaction volume to 10 μL of binding buffer, and incubated for 20 minutes at room temperature. Then 1 unit of nuclease S1 or 0.0007 unit of RNase VI was added to the above mixture and the reaction volume was adjusted to 20 μL. Then the reaction mixture was incubated for 20 minutes at room temperature. The cleavage products were recovered by ethanol precipitation and analyzed on a 15% or 12% polyacrylamide gel containing 7 M urea.

Results

Selection of RNA molecules binding to 5S rRNA. RNA molecules binding to 5S rRNA were selected from a pool of about 10^{14} RNA sequences containing 48 randomized nucleotides. For this purpose, the *E. coli* 5S rRNA was oxidized at its 3'-end and covalently fixed to a Sepharose matrix by hydrazone formation to prepare a 5S rRNA affinity column and then the RNA library was passed through the affinity column. RNA species bound to the column were eluted by reducing the ionic strength and chelating the Mg^{2+} with EDTA. The selected RNAs were recovered, reverse-transcribed, and then the transcribed cDNAs were amplified by polymerase chain reaction (PCR). A new pool of RNA, enriched in 5S RNA-binding motifs, was prepared by transcription from the PCR-amplified DNA. This new RNA pool was used for the next round of selection and amplification. The affinities of selected RNA pools for 5S rRNA increased as the number of selection cycle increased (data not shown). After the 12th rounds of selection, the individual RNAs were cloned from the pool and sequenced. The determined sequences were classified into six groups according to the features of consensus sequences (not shown here).

Group I
1 A**GUGAAA**UAAGAGUUCACAUUGACUGACCAAGCUAUGAGAUACCGCGUG
2 ACUCUGUUCUUUCUA**GUGAA**UUCUAAUUUUAUCCGAUCGCUCCUAUUGGG
3 GGUCAAGAUAAAAUUCGGGAAGGAUCGUAGU**GUGAAA**GUGUAAUAGU
4 **GUGUAAAA**GGUGUGCUGAUUAACAUUCGAGUAACGCAGGAACUGUGGU
5 U**GUGUAAA**UACAAAGUAACGAGGCUACGUUUUAGUGUUCCAAUGGAGUUG
6 ACUAUAG**GUGAA**UCGAGUUCANNNAGGGGGGUAAUUUGUUACUGCGGUCG
7 ACUAUAG**GUGAA**UUCGAGCUCGGUCAUCGAAGCUUUCAGGACACGAUCGC
8 UAGCUAGUUUAUUACUGCA**GUAAAA**AGACAAAAGGGAGUNACCCAGUC
9 ACUCUGUUCUUCUAA**GUGAA**UUCUAAUUUAUCCGAUCGUCUCUAUUGG

Group II
1 UUGACAUGGCAUGCUCAAUACUGCAGGAACACCUUCGCAGUAAC**GCGG**
2 UAACAUACGGACAAGGGAGGGGG**GCGG**GUGUUCACAUAUGGGAUGUUG
3 GUGCGCUUUG**GCGA**GNNNGGNNUAUAGUGAGUGACCGAUGGCAUAC
4 CGAGGUGACGGCAACGAAUGAGUCCCCAAAUACGGCCCGUUCA**GCGG**
5 GAAUUGAUAAAACCUA**GUGG**GAUAGUGAGGUAGGGGAAAAGGCUGAGC
6 GUGUAAAAGGUGUGCUGAUUAACAUUCGAGUAAC**GCAG**GAACUGUGGA
7 UGGUCAAGAAGAUGGAGGGUUCGAUAGUAGCCAG**GCAG**AAGACUUUUG
8 ACUAUAGGUGAAUCGAGUUCANNNAGGGGGGUAAUUUGUUACU**GCGG**UCG
9 AUAGAGAAACGACUAGAAGUAAGUAAGUCUGAAUUCGGCAU**GCAG**UCC
10 UAACAAAUCUCACUUAUAUUGGGGCAAUA**GCGA**AUUAUGAUCACAGCCU
11 AGG**GCGA**UAUCUCAAGGCCUAUUGUAUGAGUCCCCGGAUGCGUGAUGGG
12 AGAGGAUUAGAAGGAUCAGCAUGACAAAGUCCCAAAC**GCGA**GGUCCGGG
13 UAGCUAGUUUAUUACU**GCAG**UAAAAAGACAAAAGGGAGUNACCCAGUC
14 UAGCUAGUUUAUUACU**GCAG**UAAAAGACAAAGGGAGUAGCACCCGUGC
15 ACAAAGAGCCGAUGU**GUGG**GGUCUAGCAGUAUUAGAACGGGGGUACAU

Figure 1. Sequences of randomized regions of the selected RNA molecules. RNA molecules of group I contain the consensus sequences corresponding to the segment G_{691}UGAAA$_{696}$ of 16S rRNA, whereas those of group II contain consensus sequences corresponding to segment G_{688}CGG$_{691}$ of 16S rRNA. The consensus sequences are underlined and bolded

Two groups of them had the consensus sequences GCGG and GUGAAA, respectively (Figure 1), which also appear in the sequence from 688 to 696 in helix 23 of *E. coli* 16S rRNA.

Binding of the 16S rRNA fragment to 5S rRNA. The 16S rRNA fragment containing the sequence from 688 to 696 of 16S rRNA was synthesized by *in vitro* transcription. To determine the dissociation constant (K_d) of the 16S rRNA fragment-5S rRNA complex, a gel mobility shift assay was performed (Figure 2). The band intensities of free and retarded RNAs were determined by phosphorimager FLA-2000 and used for calculation of the K_d. Figure 2 shows that K_d values vary biphasically with the increasing amount of 5S rRNA, about 2.8 μM of 5S rRNA being the transition point of K_d values varying from 6.2 μM to 12.4 μM. The dual values of K_d may reflect two different modes of the binding of 5S rRNA at multiple sites on 16S rRNA fragment or vice versa (See below). We do not know yet the exact reason for the duality of K_d values.

Enzymatic protection of the 16S rRNA fragment-5S rRNA complex. To determine the potential regions for interaction between the 5S rRNA and the 16S rRNA fragment, the 5S rRNA-16S rRNA fragment complex was digested with single strand-specific nuclease S1 and double strand-specific RNase V1. Two regions, G_{688}CGG$_{691}$ and C_{680}AGG$_{683}$ in 16S rRNA fragment, were protected from nuclease S1 and RNase V1 digestion by 5S rRNA (Figure 3). The regions A_{29}CCUGA$_{34}$ of stem C and G_{51}AAGUG$_{56}$ of loop b in 5S rRNA were protected from nuclease S1 by the 16S rRNA fragment (Figure 4). On the other hand, residues at G_{41} of loop c and A_{57} of loop b in 5S rRNA became

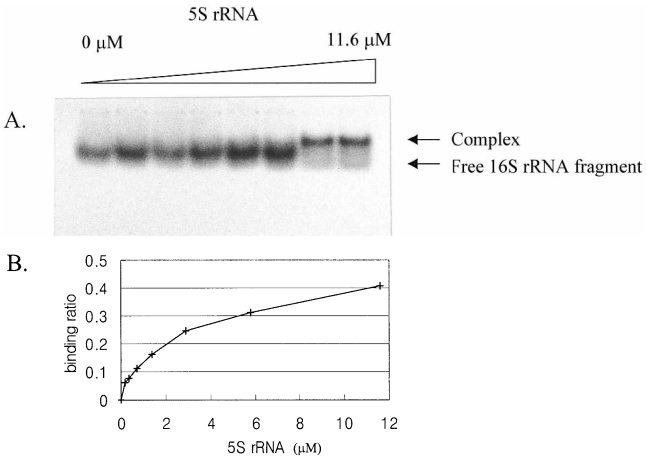

Figure 2. Gel mobility shift assay of 5S rRNA-16S rRNA fragment complex. A. Increasing amounts of 5S rRNA (0 to 11.6 μM) were added stepwise to 1 μM of the internally labeled 16S rRNA fragment, and the reaction mixtures were subjected to gel mobility shift assay, as described in Experiments. B. Complex formation in A is represented graphically. The complex formation occurs biphasically. The *Kd* value of the complex is 6.2 μM in the lower concentration range of 5S rRNA whereas it is 12.4 μM in the higher concentration range of 5S rRNA.

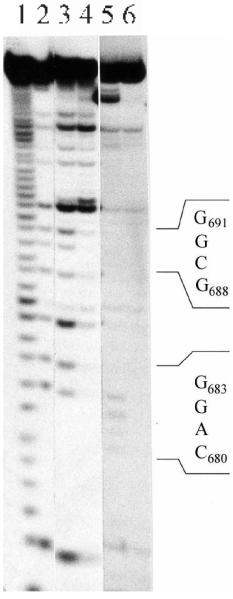

Figure 3. Protected sequences of 16S rRNA fragment from enzymatic hydrolysis by forming a complex with 5S rRNA. Sequence G_{688}CGG$_{691}$ is protected from nuclease S1, while sequence C_{680}AGG$_{683}$ is protected partly (C_{680}AG_{682}) from RNase V1 and also protected partly ($G_{682}G_{683}$) from nuclease S1 (protected nucleotides are represented in Figure 5). Lane 1, alkaline ladders; lane 2, the denatured 16S rRNA fragment treated with RNase T1; lanes 3 and 4, the 16S rRNA fragment and the 16S rRNA fragment-5S rRNA complex treated with 1 unit of nuclease S1 at room temperature for 20 min, respectively; lanes 5 and 6, the same as in lanes 3 and 4 except that 0.0007 unit of RNase V1 was used instead of nuclease S1. The hydrolysates were electrophoresed on a 15% polyacrylamide gel

more susceptible to nuclease S1 in the presence of the 16S rRNA fragment than in the presence of 5S rRNA alone, sug-

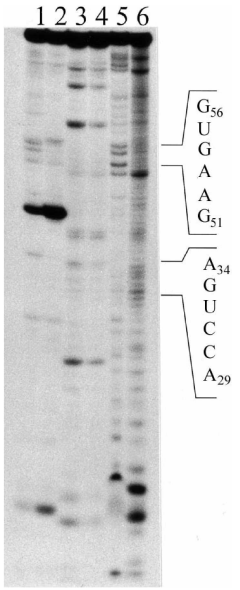

Figure 4. Protected sequences of 5S rRNA from enzymatic hydrolysis by forming a complex with 16S rRNA fragment. Sequence $G_{51}AAGUG_{56}$ is protected from nuclease S1, while sequence $A_{29}CCUGA_{34}$ is protected mostly ($A_{29}CCUG_{33}$) from RNase V1 and is protected partly (nucleotide A_{34}) also from nuclease S1. Lanes 1 and 2. 5S rRNA and the 16S rRNA fragment-5S rRNA complex treated with 1 unit of nuclease S1 at room temperature for 20 min, respectively; lanes 3 and 4. 5S rRNA and the 16S rRNA fragment-5S rRNA complex treated with 0.0007 unit of RNase V1 at room temperature and for 20 min, respectively; lane 5. denatured 5S rRNA treated with 1 unit of RNase T1; lane 6. alkaline ladders. The hydrolysates were electrophoresed on a 12% polyacrylamide gel.

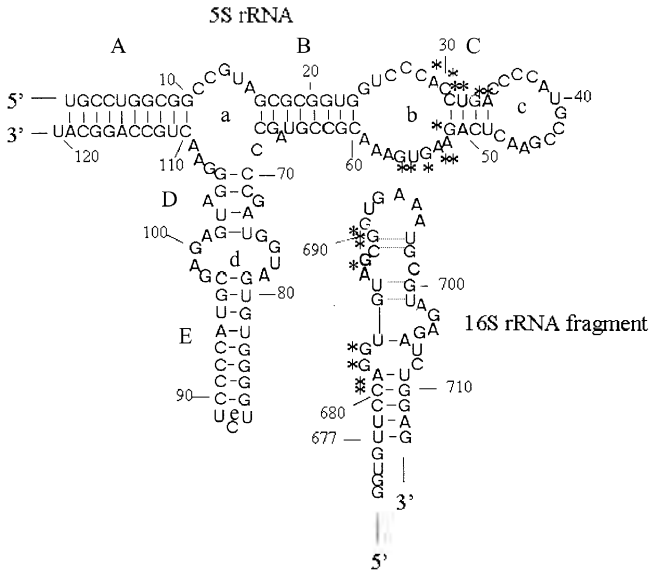

Figure 5. The scheme illustrates the contacts between 5S rRNA and the 16S rRNA fragment (helix 23 of 16S rRNA). Asterisks (*) indicate the protected nucleotides. Dotted lines in the stem regions of the 16S rRNA fragment indicate the unstable base pairing. Stems A, B, C, D, and E as well as loops a, b, c, d, and e are marked in the secondary structure of the 16S rRNA fragment.

gesting that these nucleotides were liberated from intramolecular interaction and became more accessible to nuclease S1 in solution on forming a complex with the 16S rRNA fragment.

Discussion

Combinatorial selection experiments of SELEX have been employed to isolate synthetic RNA species capable of recognizing a variety of molecules, including proteins,[14,15] amino acids,[16,17] nucleotides[18,19] and antibiotics.[20,21] In the present work, we have extended the scheme of the combinatorial RNA selection to RNA-RNA interaction to get information about the interaction between *E. coli* 5S rRNA and its cognate RNAs. After the 12th round of the selection, two conserved sequence motifs GCGG and GUGAAA were found among the selected RNA molecules (See Figure 1). These two motifs are found in the sequence from 688 to 696 of *E. coli* 16S rRNA. It was shown by many workers that helix 23 comprising the segment $G_{688}CGGUGAAA_{696}$ of 16S rRNA is one of the helices involved in the association of ribosomal subunits.[1] It is also known that G_{693} of helix 23 of 16S rRNA is intimately involved in the binding of tRNA to P site.[22]

Supposing that 5S rRNA and helix 23 might make direct contact inside ribosome leading to the above-mentioned biochemical functions, we have chosen the 16S rRNA fragment containing the segment from 688 to 696 of 16S rRNA and 5S rRNA in order to probe the possible interaction. By using a gel mobility shift assay and an enzymatic protection experiment, we have shown that the 16S rRNA fragment specifically interacts with 5S rRNA at two specific sites. The enzymatic protection experiment indicated that this interaction involved $A_{29}CCUGA_{34}$ of stem C and $G_{51}AAGUG_{56}$ of loop b in 5S rRNA. On the basis of these findings, we argue that 5S rRNA is involved in the association of ribosomal subunits and also in the binding of tRNA to P site. We do not understand much of the nature of the bonds between the sequences $A_{29}CCUGA_{34}$ and $G_{51}AAGUG_{56}$ of 5S rRNA and the sequences $C_{680}AGG_{683}$ and $G_{688}CGG_{691}$ of the 16S rRNA fragment, nor it is clear that which specific region of the two sequences in 5S rRNA is coupled to which particular region of the two sequences in the 16S rRNA fragment. Since most of these regions are located in double stranded areas except for $G_{54}AAGUG_{56}$, it would not possible for them to form the complementary Watson-Crick base pairing. It seems that the bonding between 5S rRNA and 16S rRNA fragment are probably formed in a sort of non-Watson-Crick tertiary interaction. This is a problem still to be studied.

Recently, Joseph and coworkers probed the structure of rRNA with hydroxyl radicals generated locally from iron(II) tethered to the 5' ends of anticodon stem-loop analogs (ASLs) of transfer RNA. They showed that bases of the 690 and 790 of 16S rRNA and two regions around bases from 35 to 41 and from 49 to 53 of 5S rRNA were cleaved by P site-bound ASLs.[23] This result definitely shows that the parts of helix 23 of 16S rRNA and stem C and loop c of 5S rRNA are located very closely inside the ribosome. In this context, it is interesting to note that the sequence $G_{688}CGG_{691}$ of 16S

rRNA fragment was protected by 5S rRNA and that the sequence $G_{51}AAGUG_{56}$ of 5S rRNA was protected by 16S rRNA fragment. Under this situation, it is possible that the sequence $U_{55}G_{56}$ in $G_{51}AAGUG_{56}$ of 5S rRNA may interact with $C_{680}A_{681}$ in $C_{679}CAGG_{683}$ of 16S rRNA in a manner of Watson-Crick base-pairing. Furthermore, Shpanchenko and coworkers showed that $G_{54}UG_{56}$ of 5S rRNA was protected from the iodine-induced cleavage after the association of 50S subunit with 30S subunit to form 70S subunit.[24] This result further supports our suggestion that the sequence containing $G_{54}UG_{56}$ of 5S rRNA is directly involved in the association of two subunits. If we compare the pattern of reciprocal protection between 5S rRNA and helix 23 of 16S rRNA in solution with the pattern of cleavage by ASLs on both 5S rRNA and 16S rRNA inside the ribosome, a significant speculation can be made that stem B-loop b region and loop c of 5S rRNA and helix 23 of 16S rRNA have kept their well-conserved structures and functions along the evolution. It is a generally accepted view that the original ribosome prior to the advent of the living cell was composed solely of RNAs and evolved to the present ribosome composed of RNAs and proteins. Specifically, it is assumed that the functional domains of the protoribosome was constituted by the ribozyme-like RNA modules in ancient time, and they have been conserved with little mutational variations along the evolution (reviewed by Noller).[2] Following this reasoning, we imagine for sure that the experimental approach described here with the selection of specific RNA molecules binding to a particular target rRNA molecule (5S rRNA in this study) from a random RNA population is useful for and can be applied to the probing of structural and functional motifs of ribosomal RNAs.

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