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Identification of a structural motif of 23S rRNA interacting with 5S rRNA

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Abstract To identify RNA motifs interacting with 5S rRNA, a systematic evolution of ligands by exponential enrichment experiment was applied. Some of the resulting RNA aptamers contained a consensus sequence similar to the sequence in the loop region of helix 89 of 23S rRNA. We show that the synthetic helix 89 RNA motif indeed interacted with 5S rRNA and that the region around loop B of 5S rRNA was involved in this interaction. These results suggest the presence of a novel RNA–RNA interaction between 23S rRNA and 5S rRNA which may play an important role in the ribosome function.

Key words: 5S rRNA; Systematic evolution of ligands by exponential enrichment; Aptamer; 23S rRNA

1. Introduction

5S rRNA is a component of the 50S ribosomal subunit of prokaryotic ribosome. Its small size (120 nt in *Escherichia coli*), availability in the isolated state, and universal secondary structure made it a favorite substrate for investigation. Immunoelectron microscopy of specific nucleotides in the 5S rRNA showed that 5S rRNA lies within the central protuberance of the 50S ribosomal subunit, not far from the peptidyltransferase center [1–3]. Various experimental approaches, such as enzymatic cleavage, chemical modification, and sequence-alignment studies, have also unraveled the structure of 5S rRNA [4].

Although the studies on the structural aspects of 5S rRNA have been very successful, the role of 5S rRNA inside the ribosome has not yet been clearly proved. An *E. coli* 50S ribosomal subunit without 5S rRNA loses the binding ability of tRNA to the A-site, and its peptidyltransferase activity decreases in vitro [5,6]. However, the mechanism of how 5S rRNA is involved in this process is not yet understood. The uncertainty in the role of 5S rRNA results partly from the lack of information on its interaction with other rRNAs or tRNAs within the ribosome. Therefore, understanding of these RNA–RNA interactions is essential in order to demonstrate the role of 5S rRNA inside the ribosome.

The interaction of 5S rRNA with other ribosomal components has been investigated in the following ways: (1) with biophysical methods of cryoelectron microscopic [7–10] and X-ray crystallographic analyses [11] of ribosomes, and (2) with photochemical methods using photoreactive chemical linkers such as 4-thiouridine [12,13], 5-methyleneamino uridine [14], and 2′-amino 2′-deoxyuridine [14]. The biophysical studies especially produced a model of the ribosome structure with resolutions up to 5.5 Å [10]. At this level, it is, in principle, possible to fit secondary structure elements of rRNAs directly into the corresponding electron density maps. However, it is impractical to find the plausible fitting because 16S rRNA and 23S rRNA have dynamic structures. Although the photochemical studies resulted in more direct information about the interaction between 5S rRNA and other rRNAs, only information on RNA–RNA interactions involving nucleotide U89 of 5S rRNA is available. This is mainly because crosslinks from U89 of 5S rRNA were predominant over those from other sites. The high crosslinking ability of U89 could mask crosslinks from other sites of 5S rRNA in the crosslinking experiments. We developed this study to get information on RNA–RNA interactions involving sites other than U89 of 5S rRNA within the ribosome. We performed a SELEX (systematic evolution of ligands by exponential enrichment) experiment to screen RNA motifs interacting with 5S rRNA, and we used the structure of selected RNA aptamers to get information about plausible sites of rRNAs interacting with 5S rRNA. We found that helix 89 of 23S rRNA interacts with 5S rRNA in the loop B region.

2. Materials and methods

2.1. Construction of a random RNA library

The oligodeoxynucleotide library containing a randomized 48-mer region, 5′-AAGCTTGCATGCGGATCC-$(N)_{48}$-GAGCTCGAATTCACCTATAGTGAGTCGTATTA-3′, was kindly provided by Prof. F.J. Schmidt (University of Missouri-Columbia). The library of 1 μg was transcribed in vitro with T7 RNA polymerase (RiboMAX™, Promega, Madison, WI, USA) in 20 μl of the reaction buffer. In vitro transcription products were purified by gel elution of the crush and soak method [15]. The gel-purified RNA was dissolved in 0.2 ml of the binding buffer (30 mM Tris–acetate, pH 7.5, 60 mM magnesium acetate, 120 mM potassium acetate, and 120 mM ammonium acetate), incubated at 55°C for 15 min, and we slowly cooled it down to room temperature.

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Abbreviations: SELEX, systematic evolution of ligands by exponential enrichment; EF-G, elongation factor-G

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2.2. SELEX procedure

The affinity column for in vitro selection was prepared as described [16]. In brief, 5S rRNA was purified by gel elution from total *E. coli* RNA. The gel-purified 5S rRNA was oxidized at the 3′-terminal sugar with $NaIO_4$ and then coupled to Sepharose–adipic acid hydrazide resin (Amersham Pharmacia Biotech). In order to minimize the enrichment of undesirable RNA species binding to the Sepharose resin itself, we pre-selected the RNA pool on the uncoupled Sepharose–adipic acid hydrazide resin. After being passed through the pre-column of Sepharose–adipic acid hydrazide resin, we then loaded the RNA pool onto the 5S rRNA-attached affinity column. We washed the column with the binding buffer and then eluted the bound RNA with three column volumes of the elution buffer (25 mM Na–EDTA, pH 8.0). We recovered the selected RNA by ethanol precipitation and reverse-transcribed it with an AMV reverse transcriptase using a cDNA primer (5′-AAGCTTGCATGCGGATCC-3′). Then, the cDNA was amplified by PCR with the cDNA primer and a T7 primer (5′-TAATACGACTCACTATAGGTG-3′), and used for the next round of selection. After the 12th round of selection, the amplified cDNA was cloned into the pGEM-T vector (Promega), and its sequence was determined.

2.3. In vitro preparation of helix 53 and helix 89 RNA

To synthesize helix 53 and helix 89 motifs of 23S rRNA [17], we used the following DNA templates: 5′-ACACCGCCGTCGATATGAACTCTTGGGCGGTATCACCTATAGTGAGTCGTATTA-3′ (helix 89) and 5′-GTACAGGAATATTAACCTGTTTCCCTATAGTGAGTCGTATTA-3′ (helix 53). Helix 53 RNA and helix 89 RNA were prepared by in vitro transcription with T7 RNA polymerase from the DNA templates to which the T7 primer was annealed. The transcribed RNA sequences were 5′-G_{1380}GGAAACAGGUUAAUAUUCCUGUAC$_{1404}$-3′ for helix 53 RNA, and 5′-GGU$_{2457}$GAUACCGCCCAAGAGUUCAUAUCGACGGCGGUGU$_{2491}$-3′ for helix 89 RNA. The first two extra G nucleotides of helix 89 RNA were incorporated into the helix 89 motif during the in vitro transcription by T7 RNA polymerase.

2.4. Gel mobility-shift assay

Helix 53 and helix 89 RNA were internally labeled with [α-^{32}P]CTP (Amersham Pharmacia Biotech), purified by gel elution, and quantified with a liquid scintillation counter (Wallac 1409 LSC). 5S rRNA and 0.1 μM of the labeled RNA were separately heated in the binding buffer (30 mM Tris–acetate, pH 7.5, 60 mM magnesium acetate, 120 mM potassium acetate, and 120 mM ammonium acetate) at 55°C for 15 min and slowly cooled to room temperature. The labeled RNA was mixed with increasing concentrations of 5S rRNA and incubated at room temperature for 20 min. For a competition experiment, 1 nmol of a DNA oligonucleotide (5′-TATGAAC-3′) complementary to the loop region of helix 89 RNA was added to the reaction mixture and further incubated for 20 min at room temperature. The complex was electrophoresed on a 5% non-denaturing polyacrylamide gel in the binding buffer at a constant voltage of 20 V for 20 h at 4°C. Relative amounts of RNA species were estimated by analyzing the dried gel with a phosphorimager (Fuji FLA-2000).

2.5. Enzymatic protection experiment

Helix 89 RNA or 5S rRNA was labeled at 5′-end with [γ-^{32}P]ATP (Amersham Pharmacia Biotech) and T4 polynucleotide kinase (New England BioLabs). The labeled RNA of 2.5 pmol alone or mixed with the non-labeled cognate RNA of 100 pmol was used for nuclease-cleavage reactions. The reaction volume was adjusted to 10 μl with the binding buffer, and incubated for 20 min at room temperature. Then, 1 U of S1 nuclease (Sigma) or 0.0007 U of RNase V1 (Amersham Pharmacia Biotech) was added to the above mixture, and the reaction volume was adjusted to 20 μl with the binding buffer, including an additional 1 mM $ZnCl_2$ for nuclease S1 cleavage. The reaction mixture was incubated for another 20 min at room temperature. The cleavage products were recovered by ethanol precipitation and electrophoresed on 15 or 12% polyacrylamide–7 M urea gels.

3. Results and discussion

An initial pool of RNAs for SELEX was prepared by in vitro transcription using T7 RNA polymerase from the double-stranded DNA pool of about 10^{14} independent sequences. Selection was performed with a 5S rRNA-attached column which was prepared by coupling the 3′-terminal sugar of 5S rRNA to the commercial Sepharose–adipic acid hydrazide resin. The affinities of selected RNA pools for 5S rRNA increased as the number of the selection cycle increased (data not shown). After the 12th round of selection, the selected RNA aptamers were reverse-transcribed into cDNA and cloned into the pGEM-T™ vector for sequence analysis (Fig. 1). The aptamers were categorized into several groups according to the features of the consensus sequences. Since loop regions of RNA are generally accepted as the sites capable of interacting with other RNAs or proteins, we searched consensus sequences of the aptamers that were homologous to the sequences of loop regions in a secondary structure model of 23S rRNA [18]. We found a consensus sequence homologous to 'GUUAAUA' around the loop region of helix 53 and to 'GUUCAUA' in the loop region of helix 89 of 23S rRNA (Fig. 2).

In order to examine whether helix 53 and helix 89 RNA motifs of 23S rRNA could bind to 5S rRNA, the corresponding regions were synthesized as helix 53 RNA and helix 89 RNA, respectively. The interaction of 5S rRNA with helix 53 RNA, or helix 89 RNA, was examined by the gel mobility-shift assay (Fig. 3). Helix 89 RNA bound to 5S rRNA with the K_d value of about 3.0 μM. However, any interaction between helix 53 RNA and 5S rRNA was not observed under the same condition. To examine whether the interaction between helix 89 RNA and 5S rRNA occurs through Watson–Crick base-pairing, a competition assay was performed in the presence of a DNA oligonucleotide complementary to the loop of helix 89 RNA. The addition of the oligonucleotide

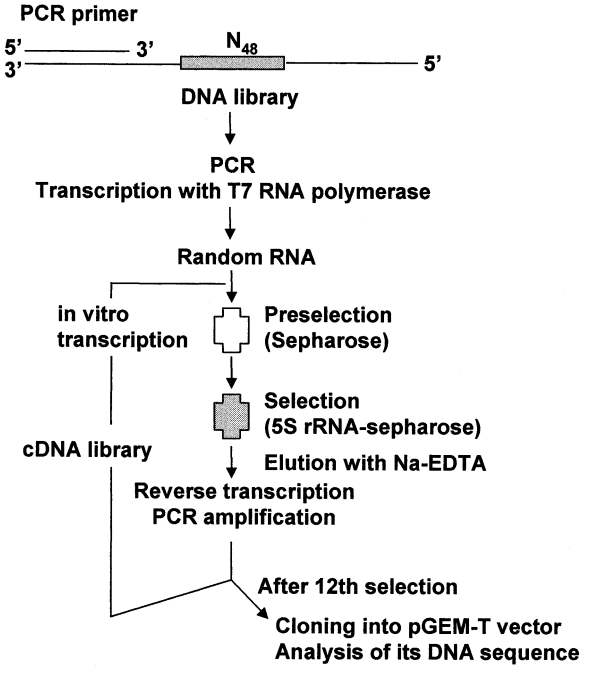

Fig. 1. Outline of the experimental strategy for SELEX. The RNA pool was prepared by in vitro transcription of the amplified DNA library carrying a randomized 48-mer DNA region. RNA aptamers binding to 5S rRNA were selected with 5S rRNA-attached affinity column chromatography. After the 12th round of selection, cDNA of each RNA aptamer was cloned into the pGEM-T™ vector, and its sequence was analyzed.

Clone number	
1	**GUU--AUA**GCUGUGAAACAGGAACUGGAAUGGGAGCCGUGAUACUGAC
2	AAA**GUUCAGA**GCUAAAUCUCAAAAGGCAUGAUCUCAACGGCGCGUUC
3	UAACAUACGGACAAGGGAGGGGGGCGGGU**GUUCACA**UAUGGGAUGUUG
4	AGUGAAAUAAGA**GUUCACA**UUGACUGACCAAGCUAUGAGAUACCGCGUG
5	UUGCA**GUUCAUG**UAAGGGUAUUUGUAGUUGCAUGCACGUGUGCCUG
6	UCGUGCUAAGAUCUUU**UUCAUG**CGCCAGACUGGAGAUGUCUAUGCCUC
7	AUGGGAGGAUGUGUAGUGCACGUAAU**GUUC--UA**CCCACUUCAGUCAUGG
8	UGGUCAAGAAGAUGGAGG**GUUCGAUA**GUAGCCAGGCAGAAGACUUUUG
Helix 53	A_{1385}CAG**GUUAAUA**UUCCUGU$_{1402}$
Helix 89	G_{2455}CUGAUACCGCCCAAGA**GUUCAUA**UCGACGGCGGUGUUUGGC$_{2496}$

Fig. 2. Sequences of the randomized region in RNA aptamers. RNA molecules containing the consensus sequence homologous to those that appeared in helix 53 and helix 89 motifs of 23S rRNA are shown. The consensus sequences are underlined in bold letters.

did not affect the binding ability of helix 89 RNA to 5S rRNA. This result proposes that a specific tertiary interaction rather than Watson–Crick base-pairing is involved in the complex formation between helix 89 RNA and 5S rRNA. This assumption may explain why helix 53 RNA, having a loop region similar to helix 89 RNA, did not bind to 5S rRNA.

Potential regions participating in the interaction between 5S rRNA and helix 89 RNA were determined by footprinting, using S1 nucleases (specific to single-stranded regions) and RNase V1 (specific to double-stranded regions). First, nucleotides of helix 89 RNA shielded by 5S rRNA from cleavages were examined (Figs. 4A and 5). Nucleotides G2470, G2472, and G2481 of helix 89 RNA were protected by 5S rRNA from S1 nuclease digestion. Since these nucleotides are conserved among many species ([18] and Fig. 4C), it has been postulated that they may play an important role in the function of 23S rRNA. Therefore, the role of helix 89 may be related with binding to 5S rRNA. Second, 5S rRNA was examined for nucleotides protected by helix 89 RNA. Three regions, U22 to G24, A29 to G33, and U55 around loop B of 5S rRNA, were protected from the nuclease attack (Figs. 4B and 5). The region of C27 to U32 in loop B of 5S rRNA was known as one of the protected sites within the 50S subunit of ribosome from iodine cleavage [19]. Our result implies that this protection may result from the interaction with 23S rRNA within the ribosome. It is not clear yet, however, whether the interaction between the loop B motif of 5S rRNA and the helix 89 motif of 23S rRNA occurs inside ribosome. It is noteworthy that the loop B motif of 5S rRNA is not covered with its counterpart ribosomal proteins, L5, L18, and L25 during the ribosome assembly [20]. This fact supports an assumption that the loop B motif would function as a site for interaction with 23S rRNA inside the ribosome.

Bogdanov et al. [4] have proposed that the signal for triggering the translocation process may be transferred from the elongation factor-G (EF-G) binding center to the peptidyltransferase center, and that U89 in loop D of 5S rRNA may play a role in this signal-triggering process. However, the helix 89 motif of 23S rRNA was not only known to be one of the sites interacting with EF-G [21], but it was also shown, in this study, to interact with the region around loop B of 5S rRNA. Therefore, the loop B motif of 5S rRNA may be able to act as a plausible functional motif of 5S rRNA involved in this signaling process through the tertiary interaction with the helix 89 motif of 23S rRNA.

For probing RNA–RNA interactions, approaches with photochemical labeling have the shortcomings of predominant and uncontrolled labeling in spite of many advantages [22]. In this study, we have been able to successfully circumvent this problem by applying SELEX to the probing of structural and functional motifs involved in the interaction between 5S rRNA and 23S rRNA. Our information on the interaction between 5S rRNA and 23S rRNA will be very important and useful for further works of constructing fine maps of RNA–RNA interactions inside the ribosome and of monitoring the dynamic changes of the interaction during the ribosome action.

It is a generally accepted view that the protoribosome early in the evolution of life was composed of only RNA molecules

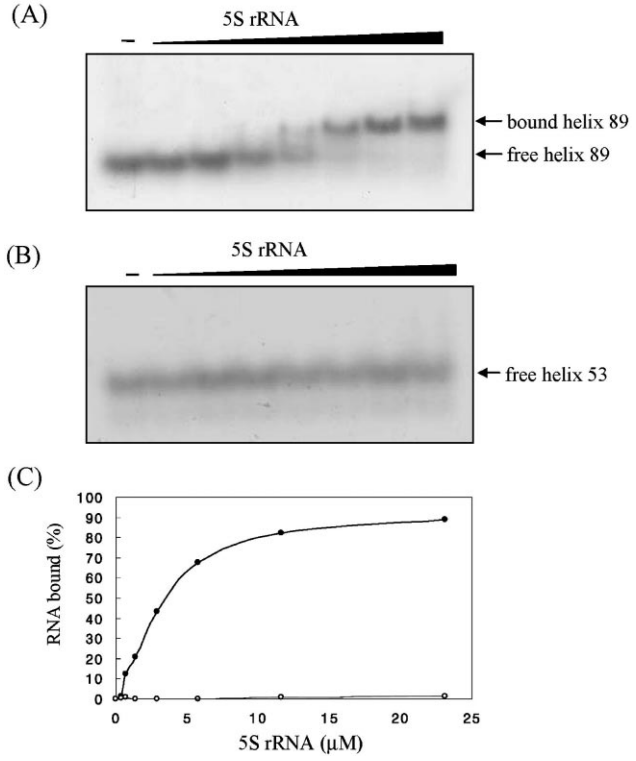

Fig. 3. Characterization of binding activities of helix 89 RNA and helix 53 RNA for 5S rRNA. A: Interaction between helix 89 RNA and 5S rRNA. The internally labeled helix 89 RNA was incubated with increasing amounts of 5S rRNA, and the reaction mixture was fractionated by 5% polyacrylamide gel electrophoresis under native conditions as described in Section 2. B: Interaction between helix 53 RNA and 5S rRNA. The same binding assay was performed as described in (A). The lanes marked as (–) indicate helix 89 RNA alone (A) or helix 53 RNA alone (B) without 5S rRNA. The amounts of 5S rRNA were increased up to 10 μg. C: A representative binding curve of helix 89 RNA (●) and helix 53 RNA (○). The K_d value of the complex of helix 89 RNA with 5S rRNA was about 3.0 μM.

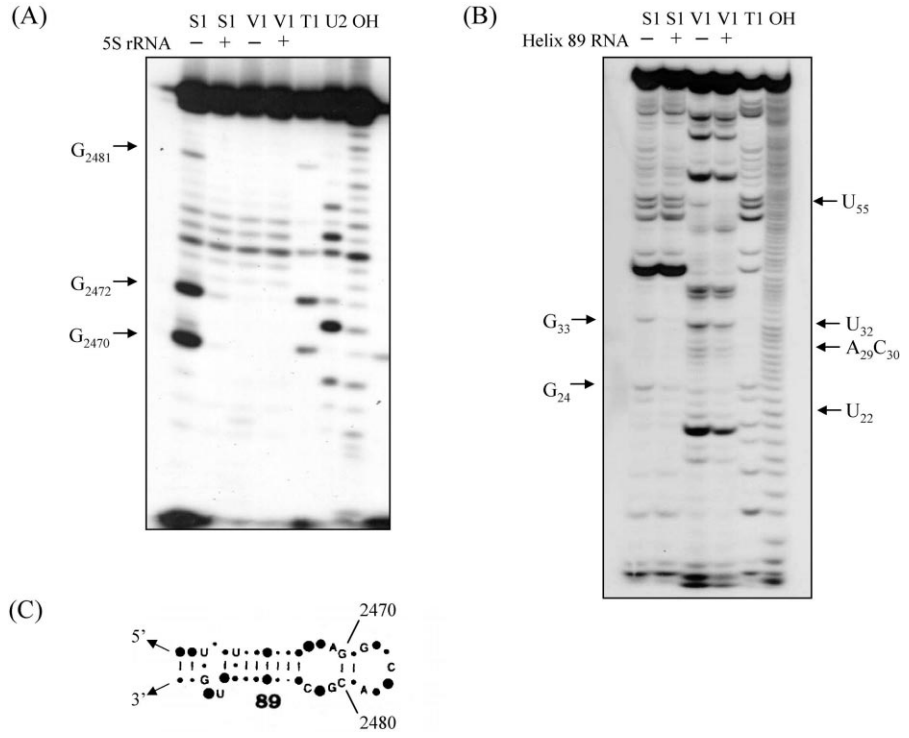

Fig. 4. Enzymatic probing of interaction between helix 89 RNA and 5S rRNA. Helix 89 RNA (A) and 5S rRNA (B) labeled at the 5′-end were partially digested with S1 nuclease or RNase V1 in the presence (+) or absence (−) of the cognate RNA. Aliquots of enzymatic digestion were loaded onto a denaturing 15% polyacrylamide gel for helix 89 RNA (A) or 12% polyacrylamide gel for 5S rRNA (B), along with the corresponding partial-alkaline hydrolysate (OH), the denaturing partial RNase T1 digest (T1), and the denaturing partial RNase U2 digest (U2). A ratio of the band intensity in the presence lane (+) to the intensity in the absence lane (−) was calculated. Bands under the value of 0.65 were regarded as indicating the protected sites. The protected nucleotides are indicated by arrows. C: A sequence-conservation plot superimposed on the helix 89 motif of 23S rRNA [18]. Bases indicated by letters are fully conserved among the 42 eukaryotic, archaebacterial, and eubacteria/chloroplast sequences examined. Dots of decreasing radius reflect decreasing degrees of base conservation from 99 to 25%.

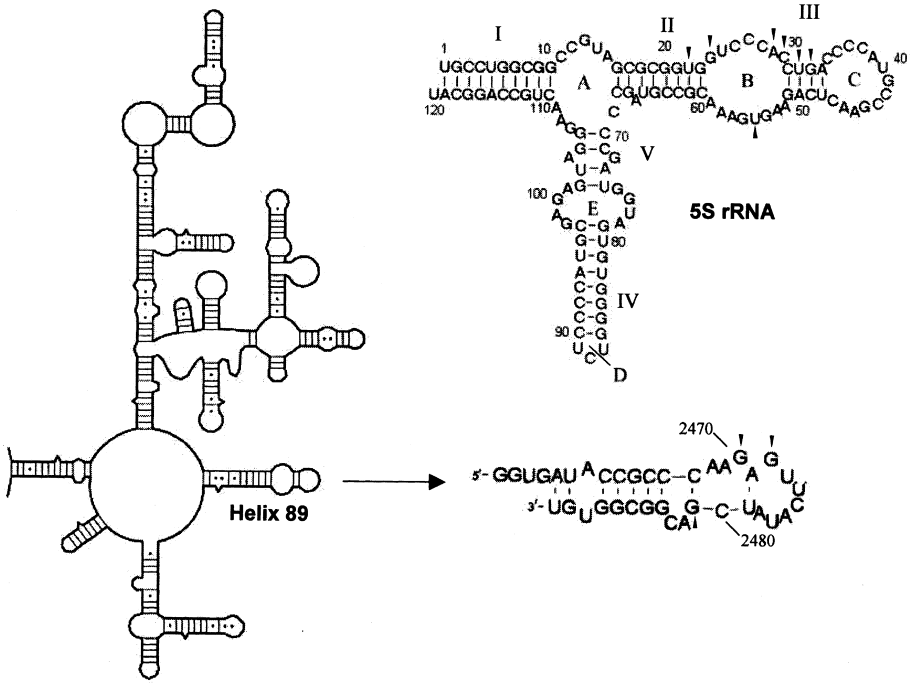

Fig. 5. Schematic representation of possible contacts between 5S rRNA and helix 89 RNA. The secondary structure of helix 89 RNA is redrawn based on the enzymatic mapping in this study. The wedges indicate the protected sites by interaction between two RNAs. Only domain V of 23S rRNA is illustrated [23].

without any protein components. In the protoribosome, 5S rRNA might have carried out an important function during the peptide synthesis through the interaction with other rRNAs. The interaction of 5S rRNA with other RNAs in the RNA world could remain in the present time as a kind of molecular fossil. The experimental approach described here provides a tool that can be used to investigate this kind of molecular fossil.

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