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Location of single-stranded and double-stranded regions in rat liver ribosomal 5S RNA and 5.8S RNA

Indrek Toots, Andres Metspalu*, Richard Villems and Mart Saarma

Laboratory of Molecular Genetics, Institute of Chemical Physics and Biophysics, Estonian Academy of Sciences, and *Laboratory of Molecular Biology, Institute of General and Molecular Pathology, Tartu University, 14/16 Kingissepa Street, 202400 Tartu, Estonian SSR, USSR

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ABSTRACT

Rat liver 5S rRNA and 5.8S rRNA were end-labelled with ^{32}P at 5'-end or 3'-end of the polynucleotide chain and partially digested with single-strand specific S_1 nuclease and double-strand specific endonuclease from the cobra *Naja naja oxiana* venom. The parallel use of these two structure-specific enzymes in combination with rapid sequencing technique allowed the exact localization of single-stranded and double-stranded regions in 5S RNA and 5.8 S RNA. The most accessible regions to S_1 nuclease in 5S RNA are regions 33-42, 74-78, 102-103 and in 5.8 S RNA 16-20, 26-29, 34-36, 74-80 and a region around 125-130. The cobra venom endonuclease cleaves the following areas in 5S RNA: 7-8, 17-20, 28-30, 49-51, 56-57, 60-64, 69-70, 81-82, 95-97, 106-112. In 5.8S RNA the venom endonuclease cleavage sites are 4-7, 10-13, 21-22, 33-35, 43-45, 51-55, 72-74, 85-87, 98-99, 105-106, 114-115, 132-135. According to these results the tRNA binding sequences proposed by Nishikawa and Takemura [(1974) FEBS Lett. 40, 106-109], in 5S RNA are located in partly single-stranded region, but in 5.8S RNA in double-stranded region.

INTRODUCTION

5S RNA and 5.8S RNA are the components of the eukaryotic 60S ribosomal subunit [1]. One property of these small RNA-s is the binding of a specific set of ribosomal proteins [2-6]. The participation of 5S RNA and 5.8S RNA in tRNA binding [7] as well as in subunit association has been suggested [8,9]. Very recent results show the involvement of 5S RNA-protein complex in EF - 2 dependent GTPase activity of eukaryotic ribosomes [10]. Thus, it is believed that 5S RNA and 5.8S RNA are located in or near the peptidyltransferase center of eukaryotic ribosome [2,3,5,11]. In order to understand the structural background of these interactions (functions) it is of importance to know the secondary and tertiary folding of 5S RNA and 5.8S RNA, also their possible

conformational changes. An approach to reveal the single-stranded regions of these molecules has been the partial digestion with nucleases [12-15]. However, the experimental data about the double-stranded regions in these molecules are scarce, coming for 5S RNA partly from two-dimensional separation studies of partial RNase digests [15,16] and together with 5.8S RNA mostly from physical measurements, model building compilations and chemical modification studies [17-23]. Recently, Wurst et al. [24] developed a rapid method for mapping RNA structure. Partial digestion of 5'-^{32}P-RNA with single-stranded specific S_1 nuclease allowed to map the exact S_1 cleavage sites in various tRNA molecules [25,26] and also in *Drosophila melanogaster* 5.8S RNA [27].

The use of a cobra *Naja naja oxiana* endonuclease [28] which cleaves double-stranded regions without base specifity [28] in combination with S_1 nuclease digestion and rapid sequencing technique allowed to map single-stranded and double-stranded region in *E.coli* 5S RNA molecule [29] and recently in the protein L1 binding region of ribosomal 23S RNA [30]. In this paper we describe the results of the application of this strategy to rat liver ribosomal 5S RNA and 5.8S RNA.

MATERIALS AND METHODS

Materials

S_1 nuclease from *Aspergillus oryzae* which has been purified according to [31] was a gift by Dr. T.A.Avdonina. Cobra *Naja naja oxiana* venom endonuclease (CVE) (E.C. 3.1.4) and T4 RNA ligase (E.C. 6.15.3) were provided by Dr. S.K.Vasilenko. T_1 nuclease (E.C. 3.1.4.8) was purchased from Sankyo Co. Ltd., pancreatic RNase (E.C. 3.1.4.22) was the product of Serva and alkaline phosphatase (E.C. 3.1.3.1) the product of Worthington. Polynucleotide kinase was isolated from T4amN82 infected *E.coli* B cells according to [32] by Dr. M.Ustav. γ-^{32}P ATP (2000 Ci/mmole) and ^{32}P-labelled pCp (2000 Ci/mmole) were from Amersham. Rat liver ribosomal 5S RNA and 5.8S RNA were purified essentially as described earlier [3].

Preparation of 5'- and 3'-^{32}P-labelled rat liver 5S RNA and 5.8S RNA

Dephosphorylation was carried out as described [33]. 1 A_{260} unit of rat liver 5S RNA and 5.8S RNA were dephosphorylated with 0.5-1 units of alkaline phosphotase in 0.1 M Tris-HCl buffer, pH 8.0, containing 1% SDS at 45°C for 120 min in a 150 µl reaction volume. After Et-OH precipitation 100-200 pMoles of dephosphorylated rat liver 5S RNA and 100-150 pMoles of 5.8S RNA were dissolved in 10-15 µl 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM $MgCl_2$, 5 mM DTT and incubated with 1-2 µl polynucleotide kinase and 100-300 pMole of γ-^{32}P ATP (1000-2000 Ci/mM) at 37°C for 30 min. Labelled 5S RNA and 5.8S RNA were purified by polyacrylamide gel electroforesis and eluted from the gel as described [34]. Unlabelled 5S RNA and 5.8S RNA were added to the 5'-^{32}P-labelled 5S RNA and 5.8S RNA respectively as carriers for Et-OH precipitation. Finally RNA-s were dissolved in bidistilled water to give the concentration about 0.1-0.5 mg/ml. In some experiments 5'-^{32}P-labelled RNA-s were used without cold carrier RNA-s.

Untreated rat liver 5S RNA or 5.8S RNA were reacted with (5'-^{32}P)-pCp at their 3'-end of the polynucleotide chain. The reaction was performed in 50 mM Hepes/KOH, pH 7.4, 20 mM $MgCl_2$, 3 mM DTT, 80 nM ATP, 15% DMSO, 400-500 pM 5S RNA or 5.8S RNA, 500 pM of (5'-^{32}P)-pCp and 10 units of T4 RNA ligase. The reaction volume was 20 µl and the mixture was incubated for 6-8 hours at 4°C. 5S RNA and 5.8S RNA labelled at their 3'-termini were purified essentially as described above.

Sequencing and structural analysis

To determine the nuclease digestion sites, a sequence determination was performed. Two base specific ribonucleases were used in sequencing reactions - T_1 RNase and pancreatic RNase. The digestions were performed in 50 mM Tris-HCl buffer, pH 7.5, containing 7 M urea. The reaction mixture (10 µl) contained 1-5 µg of either 5S RNA or 5.8S RNA, 0.5 units of T_1 RNase or pancreatic RNase. The incubation proceeded at room temperature. RNA "ladders" were generated by 0.1 N NaOH.

For the structural investigation the following conditions

for the enzyme digestion were used:

The reaction mixture for T_1 RNase and CVE contained 50 mM Tris-HCl, pH 7.5, 200 mM NaCl and 20 mM $MgCl_2$ (buffer A) and for S_1 nuclease 30 mM NaOAc, pH 4.6, 0.1 M NaCl, 1 mM $ZnCl_2$, 1 mM $MgCl_2$, 5% glycerol (buffer B).

S_1 nuclease: 1-5 µg RNA in 10 µl buffer B was digested with 10-15 units of enzyme at 0°C for 20 min and in some experiments in buffer A with 100-1000 units of enzyme in the presence of 1 mM $ZnCl_2$. The digestion experiments of 5S RNA and 5.8S RNA with S_1 nuclease at pH 7.5 gave identical gel patterns with those, performed at pH 4.6. Therefore most of the S_1 digestions were performed at pH 4.6.

Cobra venom endonuclease: 1-5 µg RNA in 10 µl buffer A was digested with 1-5 µg of enzyme at 0°C for 20 min.

T_1 RNase: 1-5 µg RNA in 10 µl buffer A was digested with 0.5 units of enzyme at 0°C for 20 min.

The reactions were stopped either with Et-OH precipitation or with phenol treatment. The Et-OH washed samples were dissolved in dye mix, containing 50 mM Tris/borate, pH 8.3, 7 M urea, 1 mM EDTA, 0.05% bromphenol blue and 0.05% xylene cyanol, heated at 50°C for 1 min and loaded onto the gel.

The sequence gels were 20% in respect to polyacrylamide and 0.3 mm thick [35]. The mapping of 5S RNA was done by two subsequent loadings and the mapping of 5.8S RNA with three loadings. After the electrophoresis the gels were covered with Saran wrap and autoradiographed by Orwo HS-11 X-ray film. The autoradiography was performed at -45°C and sometimes by the use of Du Pont Cronex intensifying screens.

The 8% non-denaturating polyacrylamide gels were made and run in 50 mM Tris/borate, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$. The electroforesis was performed at 4°C (60 V and 40 mA) for 12 hours on 1.5 mm thick slabs. Renaturation of the RNA preparations was carried out in buffer A for 10 min at 60°C, followed by slow cooling to room temperature as described [36].

RESULTS AND DISCUSSION

RNA preparations

The existence of electrophoretically separable 5S RNA con-

formers [36,37] and 5.8S RNA multimers [38] is well established. In order to be sure that all nuclease degradation experiments would take place with the identical conformer, we controlled all these 5S RNA and 5.8S RNA samples on non-denaturing PAAG gels. Irrespective of the buffer conditions used (buffers A and B), there was no indication for conformational heterogeneity of 5S RNA. 5.8S RNA gave three bands in all buffer solutions (results not shown). The two slower moving bands which account not more that 10% of the 5.8S RNA on the gel, probably represent the dimer and multimer forms of 5.8S RNA, described by Sitz et al. [38] for Novikoff hepatoma 5.8S RNA.We do not know to what extent the minor contamination of the 5.8S RNA preparation with multimer forms influences its nuclease degradation patterns.

The strategy which we used for the detection of the secondary structure of 5S RNA and 5.8S RNA is based on the fact that single-stranded RNA regions are sensitive to S_1 nuclease and regions involved in secondary or tertiary interactions are sensitive to *Naja naja oxiana* endonuclease [28,29].

The parallel use of partial enzymatic digestion of RNA-s with S_1 nuclease and CVE in combination with rapid sequencing reactions allows exactly locate the single-stranded and double-stranded regions in 5S RNA and 5.8S RNA.

Enzymatic tests of 5S RNA structure

The partial cleavage patterns of 5'-end-labelled 5S RNA with S_1 nuclease, CVE and T_1 RNase are presented in Fig. 1.

Partial digestion of 5S RNA in denaturing conditions with T_1 RNase and pancreatic RNase generates oligonucleotides terminating in guanosine 3' monophosphates and pyrimidine 3' monophosphates, respectively. The position of guanosines and pyrimidines can be discerned from Fig. 1. Partial cleavage patterns produced by alkali and base specific ribonucleases allow the assignment of oligonucleotide lengths to fragments present in simultaneously electrophoresed S_1 nuclease and CVE digests.

The most striking feature of the S_1 nuclease cleavage pattern is the appearance of a very intensive and broad band in region 33-42 (Fig. 1), revealing that nucleotides 36-38 are the first ones, attacked in rat liver 5S RNA. The two more acces-

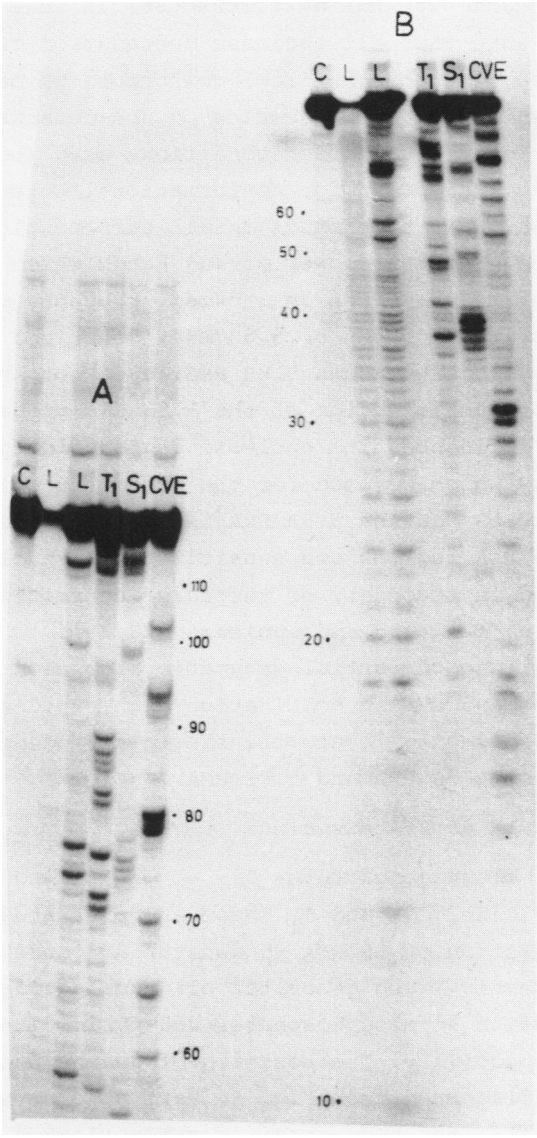

Figure 1. Electrophoretic patterns of partial enzymatic digestions of 5'-end-labelled 5S RNA. 20000 cpm (by Čerenkoff) were loaded per slot. A - first loading. B - second loading. Abbreviations: C - no enzyme, T_1 - T_1 RNase, S_1 - S_1 nuclease, P - pancreatic RNase, CVE - cobra venom endonuclease, L - "ladder". The enzyme reactions were performed at $0^{o}C$ for 20 min.

sible regions for S_1 nuclease in rat liver 5S RNA are around nucleotides 74-78 and 102-103 (Fig. 1). There are some other regions and single positions which are cleaved with S_1 nuclease - 10-13, 22-24, 84-87 and 110-111 (Fig. 1), but they are cleaved in the second step and could therefore be the reflections of the conformational changes induced by the first cleavages. Partial digestion of 5S RNA with T_1 RNase has two main regions with comparable intensity - the region around nucleotides 37 and 84-87 (Fig. 1).

Cobra venom endonuclease attacks preferentially double-stranded regions [28,29]. So far a series of different tRNA molecules and *E.coli* 5S RNA were analyzed with respect to the digestion sites of *Naja naja oxiana* endonuclease and the results show that the enzyme is specific for double-stranded regions indeed [29,39,40]. Therefore the areas attacked by CVE in conditions of partial hydrolysis are believed to locate in double-stranded regions.

Fig. 1 also shows the areas of rat 5S RNA which are cleaved by CVE. Note that S_1 nuclease as well as CVE products have the 5'-phosphate and 3'-free hydroxyl group and therefore their migration on polyacrylamide gels differs from respective oligonucleotides produced by T_1 or RNase A cleavage. The 5S RNA S_1 nuclease and CVE cleavage products move slower and this difference has been taken into account. The precise cleavage sites of rat liver 5S RNA with CVE are the following: 7-8, 17-20, 28-30, 49-51, 56-57, 60-64, 69-70, 81-82, 95-97, 106-112. Comparison of the S_1 nuclease with the CVE cleavage sites (Fig. 1) reveals that these enzyme hydrolyse, as expected, the rat liver 5S RNA at different sites. Fig. 1 shows also that there are some regions slightly hydrolysed by CVE around nucleotides attacked also by S_1 nuclease. The reason of this slight overlapping is not clear for us yet.

So far, only two sets of experiments enlight the structure of paired regions in eukaryotic 5S RNA [15,16]. Early experiments by Vigne et al. [15] demonstrated that region 38 to 39 must be strongly paired to either region 1 to 37 or region 90 to 120. More recent studies by Benhamou et al. showed that sequences 1-37, 38-86, 90-112 are held together by base pairing

and sequence 1-24 is paired to 54-120 [16]. Our results about the partial digestion of rat liver 5S RNA are to our best knowledge the first direct ones, indicating to certain exact nucleotide positions possibly involved in intramolecular base pairing. These are 7-8, 17-20, 28-30, 49-51, 56-57, 60-64, 69-70, 81-82, 95-97, 106-112. Of course, these data do not show which nucleotides of 5S RNA are hydrogen bounded with each other. A model [7] of the secondary structure of the rat liver 5S RNA molecule, with the S_1 nuclease and CVE susceptibility data, collected from about 20 different experiments, is shown in Fig. 2.

Nuclease S_1 has been employed as a probe to study the conformation of wheat embryo 5S RNA [12] *Saccharomyces cerevisiae* 5S RNA [13], but not of mammalian 5S RNA. In wheat embryo 5S RNA the most susceptible parts of the molecule are the regions between residues 8-18, and 32-40. In yeast 5S RNA S_1 nuclease cleaves preferentially nucleotides around positions 12, 40, 57, 110 [13]. In general, our cleavage data agree with those of Barber and Nichols [12] and Nishikawa and Takemura [13]. We never detected the cleavage of 5S RNA with S_1 nuclease at positions around 57, accessible in the yeast 5S RNA [13]. In

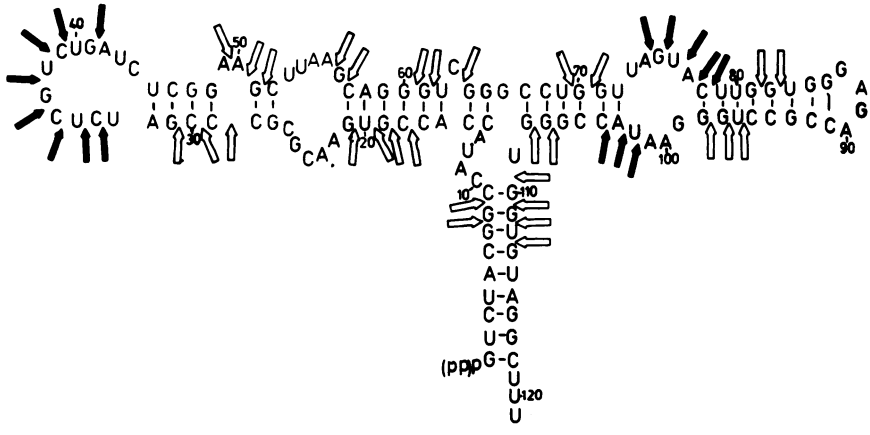

Figure 2. A summary of S_1 nuclease and cobra venom endonuclease (CVE) cleavage sites in rat liver 5S ribosomal RNA. The secondary structure model is redrawn from the publication of Nishikawa and Takemura [7]. The solid arrows indicate the S_1 nuclease cleavage and open arrows CVE cleavage, respectively.

contrast, CVE attacks preferentially positions 56-57 and therefore we suppose that this part of the molecule is in double--stranded structure.

Enzymatic tests of 5.8S RNA structure

Fig. 3 depicts the precise location of the S_1 nuclease and

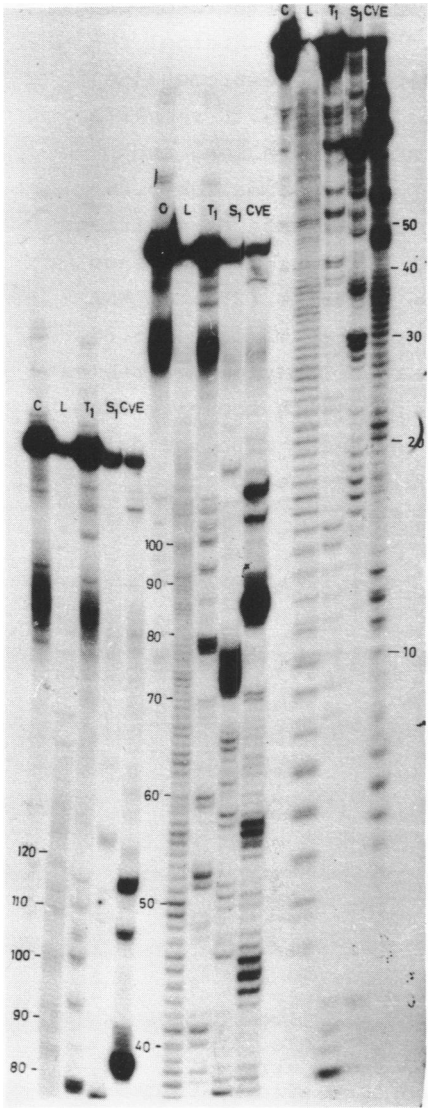

Figure 3. Electrophoretic patterns of partial enzymatic digestions of 5'-end-labelled 5.8S RNA. 20000 cpm (by Čerenkoff) were loaded per slot. A. First loading. B. Second loading. C. Third loading. The abbreviations as in Fig.1.

CVE cleavage sites on 5.8S RNA gels. S_1 nuclease cleaves 5.8S RNA preferentially at positions 16-20, 26-29, 34-36, 74-80 and in the region around 125-130. Slight cleavage is visible at positions 46-47, 51-53, 56-61 and 100-105. CVE cleavage sites could be identified in Fig. 3 as 4-7, 10-13, 21-22, 33-35, 43-45, 51-55, 72-74, 85-87, 98-99, 105-106, 114-115 and 132-135. All the cleavage data are collected in Fig. 4.

Khan and Maden used S_1 nuclease as a conformational probe for Hela cell 5.8S RNA and they found that two internal regions (nucleotides 73-82 and 125-129) and the extreme 3'-end of 5.8S RNA are susceptible to S_1 nuclease [14]. *Drosophila melanogaster* 5.8S RNA, which is 130 nucleotides long and probably represents the 5'-terminal part of mammalian 5.8S RNA shows preferential cleavage with S_1 nuclease at positions 6-8, 39-41, 49-52 and 102-104. Slight cleavage was obtained at positions 1-5, 13-17, 42-43, 75-76, 107-110 [27]. 2S RNA which probably corresponds to the 3'-part of mammalian 5.8S RNA was readily hydrolysed [27]. The reasonably good agreement of our 5.8S RNA-S_1 nuclease cleavage data with those by Khan and Maden is evident. There is less agreement with the data of Pavlakis et al. [27], but this could be due to the differ-

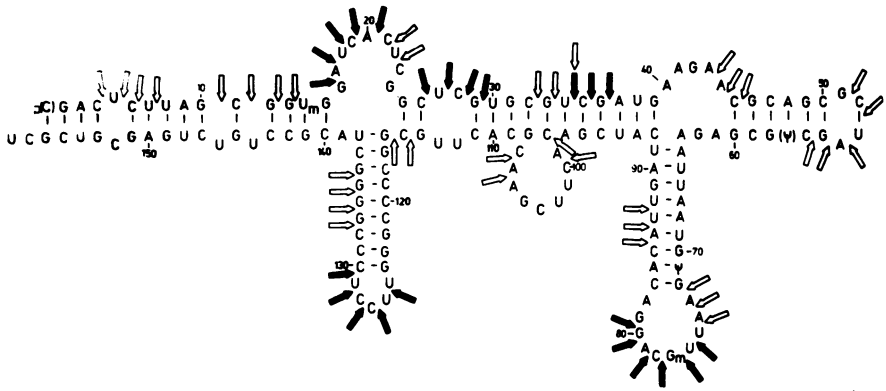

Figure 4. Secondary structure model of rat liver 5.8S RNA [21] showing the susceptibilities of nucleotides to S_1 nuclease and CVE digestion. The solid arrows indicate the S_1 nuclease cleavage and open arrows CVE cleavage, respectively.

ence of the overall structure of *Drosophila sp.* 130 nucleotides long 5.8S RNA and rat liver 5.8S RNA.

Khan and Maden have also studied the reactivity of cytidine residues and uridine plus guanosine residues in Hela cell 5.8S RNA with bisulphite and carbodiimide [23]. The available chemical reactivity data agree with our nuclease digestion results. The major differences are that the nucleotides constituting loop I (nucleotides 16-26), loop III (nucleotides 49-56) and loop V (nucleotides 98-107) in the Nazar et al. model [21] shown to be reactive with carbodiimide and bisulphite are not cleavable with S_1 nuclease in the first step. Since CVE cleaves sequences 21-22, 43-45, 51-52, and 105-106, we propose that the arrangement of nucleotides in these regions could be substantially different from that proposed in Nazar et al. model [21]. It is possible to speculate that loop III is involved in tertiary interaction, since it is first cleaved with CVE, but with S_1 nuclease only after the very first cleavages have been occurred.

Several years ago Nishikawa and Takemura proposed the involvement of 5S RNA and 5.8S RNA in the tRNA binding to ribosomes via complementary sequences [7]. According to the present results the tRNA binding sequences proposed by Nishikawa and Takemura in 5S RNA are located in partly single-stranded regions and in 5.8S RNA in double-stranded regions, and therefore, do not support this hypothesis.

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