This paper is dedicated to the memory of Professor Hans Gunter Willmann of the Max Planck Institut für Molekulare Genetik, Berlin, who died on March 31 1990.

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TERTIARY STRUCTURE AND COMPUTER MODELING OF PLANT 5S RIBOSOMAL RNA

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A new model of secondary and tertiary structure of higher plant 5S rRNA is proposed. It consists of three domains. Domain $\alpha$ includes stem I and loop A; domain $\beta$ contains stems II and III and loops B and C; domain $\gamma$ consists of stems IV and V and loops D and E. We propose that the domains $\beta$ and $\gamma$ adopt RNA-A like structure due to irregularities caused by the different in size internal loops B and E and the bulges occurring in the model. A suggested bending of RNA could bring single stranded fragments of domains beta and gamma close enough to each other to allow tertiary interactions. The new model of plant 5S rRNA differs from those suggested previously for eukaryotic 5S rRNA, by arrangement of the domains $\beta$ and $\gamma$ and the base pairing scheme of domain $\gamma$. The model is based on our results of partial digestion obtained with single and double strand specific nuclease. The experimental results were confirmed by computer aided secondary structure prediction analysis of all higher plant 5S rRNAs and computer modeling using energy minimization approach. Further support of our model have been provided by experiments including alpha sarcin, ribonuclease H and chemical modifications.

Ribosomal 5S RNA (5S rRNA) is a component of the large ribosomal subunit and takes part in a number of interactions during protein biosynthesis. Its role is not completely understood. Recently, it has been suggested that

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ribosomal protein L5-SS rRNA complex is the recognition site for elongation factor 2 [1]. 5S rRNA interacts with transcriptional factor III A (TFIIIA), a protein essential for polymerase (Pol III) dependent 5S rRNA gene transcription, and forms 7S and 42S particles in *Xenopus laevis* oocytes [2-4]. Interaction of 5S rRNA with TyC(G) sequence of transfer RNA proposed earlier has not been substantiated [5]. All specific functions and interactions of 5S rRNA known to date are summarized in Fig. 1. The crystals of 5S rRNA from *Thermus thermophilus* have been grown, but so far they are unsuitable for structural studies [6]. More recently crystals of the nucleolytic fragment of *E. coli* 5S rRNA and cocrystals of this fragment with L25 ribosomal protein have been obtained [7]. Parallely 5S rRNA has been subjected to high resolution

Fig. 1. Summary of activities of ribosomal 5S RNA
n.m.r. analysis, but because of its size and complex architecture this produced only very limited structural information [8, 9].

At present over 500 nucleotide sequences of 5S rRNA are known [10]. Their analysis suggests that 5S rRNA was very much conserved during evolution [11], similarly to tRNA. Based on sequence homology and numerous experimental data a few models of its secondary structure have been proposed [12, 13]. Up to now 18 nucleotide sequences of cytoplasmic 5S rRNA from higher plants are known [10]. Eight of them have been recently sequenced in our laboratory ([14] and unpublished results). On the basis of biochemical experiments and free energy calculations we propose a new model of secondary structure and tertiary interactions of higher plant 5S rRNAs. We will discuss in detail the differences between this and the other models proposed for eukaryotic 5S rRNA [12, 13]. Using computer modeling we constructed and tentatively refined a three-dimensional model of plant 5S rRNA.

MATERIALS AND METHODS

Ribosomal 5S RNA from lupin seeds and wheat germ were purified from crude RNA preparations on Sephadex G-75 column as described previously [9, 14]. To obtain a large amount of pure 5S rRNA the Sephadex G-75 column fraction, containing this RNA, was further separated on benzoylated DEAE-cellulose and Sepharose 4B columns. All enzymes used in these studies were obtained from commercial sources. For mung bean nuclease, conditions were as described earlier [15]. Enzyme concentrations for limited digestions were as follows: S₁ and mung bean (M.b.) nucleases 10⁻²-10⁻⁴ units/μg RNA, cobra venom (V) nuclease 10⁻¹-10⁻³ U/μg RNA, T₁ RNase 10⁻⁴-10⁻⁵ U/μg RNA.

RNA samples were labeled with [γ-³²P]ATP and T4 kinase or [³²P]pCp and T4 RNA ligase and renatured. Unlabeled 5S rRNA, of the same origin was used as a carrier.

For RNA denaturation, the buffer was devoid or magnesium. These conditions were similar to those described earlier [16]. Enzymatic RNA sequencing has been done according to a procedure described in Ref. [14]. Computer calculations were done using the programme with new thermodynamic parameters obtained by others [17]. Computer modeling was done on VAX 750 using programme FRODO [18] and the model was further refined using the energy minimalization method with molecular dynamics programme package XPLOR [19].

RESULTS

It has been observed that in Papilionaceae there is a nucleotide sequence heterogeneity on 5S rRNA and 5S DNA level [20, 21]. For yellow lupin seeds (Lupinus luteus), the heterogeneity has been observed on the gene level but not
on RNA level [21]. Alignment of all nucleotide sequences of *Papilionaceae* and other higher plant 5S rRNAs to a general model of the secondary structure revealed common and varied nucleotides (Fig. 2, Table 1).

We decided to investigate the possible effect of these varied nucleotides on the structure of 5S rRNA in solution. Specific nucleases cleave nucleic acid chain only either in single or double stranded regions [22, 23]. Wheat germ and lupin seeds 5S rRNAs differ in seven nucleotide positions (Table 1). Both RNAs were subjected to limited hydrolysis with the mung bean, *S*, and cobra

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**Table 1**

*Positions in plant 5S rRNAs at which different nucleotides are found*

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venom nucleases. The digestion patterns were almost identical for both 5S rRNA species (Fig. 3, data for wheat germ 5S rRNA are not shown). The cleavage sites in plant 5S rRNA are distributed over the entire molecule, however, some parts of the RNA molecule are more susceptible to digestion than the others (Fig. 3). It was possible to observe stretches of single stranded regions cut by single strand specific nucleases: mung bean and S1, as well as double stranded or structured regions cleaved by cobra venom nuclease [22]. Interestingly, loop C, which is proposed to be exposed in the model, was not digested evenly. The same concerns loop D and other single stranded regions. Based on the digestion data (Figs. 3 and 4) we propose a new model of secondary structure of plant 5S rRNA (Fig. 6). A C105 bulge, similar to that of U63, and a new arrangement of loop D and stem IV are proposed. All the differences between the model for lupin seeds 5S rRNA and other eukaryotic 5S rRNAs are localized in domain γ. The digestion patterns for wheat germ 5S rRNA and lupin 5S rRNA are virtually identical; therefore, despite nucleotide
Fig. 4. The secondary structure model of lupin 5S rRNA with the summary of the results of specific nuclease digestion patterns.

Fig. 3. Limited hydrolysis of 5' end label lupin 5S RNA with specific nucleases: The lines are labeled from left to right: panel A: 1, no enzyme (– E); 2, T₁ at 5×10⁻⁴ U/μg denatured RNA; 3, ladder generated with H₂O; 4, 6, T₁ at 1×10⁻⁴ U/μg native RNA; 5, 7, T₁ at 5×10⁻⁵ U/μg native RNA; 8, 10, S₁ at 2×10⁻³ U/μg native RNA; 9, 11, S₁ at 4×10⁻⁴ U/μg RNA; 12, 14, V₁ at 2×10⁻² U/μg RNA; 13, 15, V₁ at 4×10⁻³ U/μg RNA; in lines 4, 5, 8, 9, 12 and 13, the reaction was terminated with formamide containing 0.1 M EDTA. In lines 6, 7, 10, 11, 14 and 15, the reaction was terminated with glycerol. Panel B: 1, control, no enzyme (– E); 2, U₂ digestion as additional marker of the sequence; T₁, hydrolysis was carried out in 15 mM Tris/HCl buffer pH = 7.5, 10 mM MgCl₂, 200 mM NaCl; 3, partial hydrolysis of RNA with T₁ at 10⁻³ U/μg under denaturing conditions [21]; 4, conditions like line 3 except T₁ at 5×10⁻⁴ U/μg; 5, ladder generated with H₂O; 6, T₁ at 5×10⁻⁴ U/μg native RNA; 7, T₁ at 1×10⁻⁴ U/μg native RNA; 8, T₁ at 1×10⁻⁵ U/μg native RNA; 9, S₁ at 2×10⁻² U/μg RNA; 10, S₁ at 2×10⁻³ U/μg RNA; 11, S₁ at 4×10⁻⁴ U/μg RNA. V, hydrolysis was carried out in the same buffer as for T₁ nuclease. 12, V at 1×10⁻¹ U/μg RNA; 13, V at 2×10⁻² U/μg RNA and 14, V at 4×10⁻³ U/μg; electrophoresis was done on 20% (A) and 6% (B) polyacrylamide gels.
sequence differences, the structure of domains α and β are the same for both RNAs. It was intriguing to note heavy digestions by S₁ and mung bean nucleases in loop B, but not in presumably more exposed loops C and D. Our interpretation of this result is that the conformation of terminal loops C and D is affected by constraints in the structure whereas that of loop B is not. A similar interpretation can be applied for the analysis of S₁ digestion pattern of initiator and elongator tRNA anticodon loops [24]. All initiator tRNAs give two cuts in the anticodon loop but elongator tRNAs have always more than two [24]. In the course of analysis of crude 5S rRNA isolated from various plants, we detected on polyacrylamide gel a new RNA band (Fig. 5A). The RNA was isolated from the gel, and sequenced (Fig. 5). The lupin RNA sequence was identical to the fragment 65-108 of lupin 5S rRNA corresponding to domain γ (Fig. 4). We attempted to determine its secondary structure and obtained partial nuclease digestion patterns. These data were compared with those for the intact 5S rRNA molecule (Fig. 5). The fragment is only digested by single strand specific enzymes but not with the V enzyme. It is clear that the conformation of the RNA fragment depends on whether it is free in solution, or is a part of an intact 5S rRNA molecule (Fig. 6), [25].

In order to understand conformational flexibility of 5S rRNA, we carried out calculations of the free energy of a lupin 5S rRNA molecule, its point “mutants”, separate domains and other plant 5S rRNA molecules, using ZUCKER computer programme [26]. The free energy (ΔG) of different 5S rRNAs calculated using new free energy parameters for base pairing, stacking, loops, bulges and dangling ends [17] varies from −25.8 to −33 kcal/mol. These values correspond quite well to experimentally determined free energies for 5S rRNAs from wheat germ [27] and lupin seeds [28]. Unfortunately, the calculated models of secondary structure for both these molecules are quite different. The lupin 5S rRNA model is rather elongated in shape and is not compatible with the nuclease digestion pattern. Wheat germ 5S rRNA model resembles very much that proposed by Nishikawa & Takemura [29], and is almost identical with our new model proposed for lupin 5S rRNA [25]. Since the calculated models are based only on the information about the nucleotide sequence, they do not allow to determine tertiary interactions and their energy contributions. Interestingly, both loops C and D have uridine in the middle of the loop: U38 and U88. This nucleotide is known to form the so called U-turn in tRNA. Another source of perturbation of the helicity of the two domains β and γ could be a different number of nucleotides in 5’ and 3’ portions of the loops. Loop B has 6 and 5 and loop E has 6 and 4 nucleotides, respectively. The unusual structural features can cause these domains to bend, twist or kink and enable interactions between loop C and D and/or B and E for example A100-U53, C34-G85, C35-G86, C36-G87 and A37-U88 (Fig. 6).

A computer model of higher plant 5S rRNA molecule was built using the knowledge based on the secondary structure from solution data (limited
nuclease digestion) and proposed tertiary interactions. Standard A-RNA helices were used for the base paired regions [30]. Each fragment was assembled separately using programme FRODO and interactive graphic system [18]. The fragments were subjected to geometrical and stereochemical refinement using FRODO subroutine REFI. Motifs were combined into larger domains. Loops C and D were modeled to resemble closely at their 3' and 5' ends of tRNA\textsuperscript{Phe} anticodon loop, including U turns at positions 38 and 88. Single loopout nucleotides connected to double helical regions were modeled following rules of stereochemistry. At the end of this initial stage the model was still imperfect and displayed locally poor sequences which still give alternative models, suggested that only minor alternations in the sequence might be

Fig. 5. Analysis of lupin SS rRNA fragment - domain γ. A, 15% polyacrylamide gel electrophoresis in 7 M urea after purification on Sephadex G-75 column (lane 1) and pure SS rRNA as a standard (lane 2); B, enzymatic sequencing [21]; C, the nucleotide sequence with marked digestions sites for structure specific nuclease (see Material and Methods)
required for changing the calculated secondary structure. In line with this reasoning we asked what single base substitutions can be sufficient to affect the calculated secondary structure model. Several sites have been selected on the basis of sequence differences of plant 5S rRNA (Fig. 2). These are: 1, 3, 23, 24, 49, 73, 93, 107, 116, 117, 118, 119 and 120. Nucleotides 1, 3, 116, 117, 118, 119 and 120 are part of a common helix I (Fig. 6), so they cannot account for alternative folding. However, nucleotides in positions 23, 24, 49, 73, 93 and 107 seem to play an important role. The base in position 49 has been proposed earlier [12] as a conformation discriminatory site. In fact, single substitution C to G in position 49 can cause lupin 5S rRNA to fold into a new conformation [28]. We combined experimental and theoretical data and constructed a wire model of the tertiary structure of 5S rRNA from plants (Fig. 7). It consists of three domains $\alpha$, $\beta$ and $\gamma$. Domain $\alpha$ is simply helix I, domain $\beta$ contains stems II and III and loops B and C, and domain $\gamma$ consists of stems IV and V and loops D and E (Fig. 6).

Domain $\alpha$ should form a rather typical RNA-A as it was proposed for E. coli and yeast 5S rRNAs [8, 27]. Conformation of RNA in the other two domains is strongly affected by the presence of three bulges at nucleotides 49 - 50, 63 and 105. The possible sequence dependent constraints may influence the conformation of loops C and D as visualized with digestion by
Fig. 8. Computer model of tertiary structure of plant 5S rRNA

Fig. 9. Stereopicture of the refined computer model of higher plant 5S rRNA
stereochemistry, mainly within regions connecting separate motifs. The model was assembled to test feasibility of the proposed tertiary contacts between loops C and D. However, no attempt has been made to impose any direct contacts between residues from loops C and D. Rather the major goal at this stage of modeling was to provide the best stereochemistry and geometry of the individual secondary structure motifs.

The initial model was subjected to further refinement using energy minimalization with the molecular dynamic programme package XPLOR [19]. The Powell method combined with the SHAKE method was applied. In this approach coordinates of all atoms including hydrogen atoms were used as variables. During the refinement significant improvements of stereochemistry and geometry was noticed (Fig. 8). The contribution to overall stabilization energy $E_{\text{Total}}$ came mainly from hydrogen bonds, van der Waals interactions and electrostatic interactions. The stereo picture of the model is shown on Figs. 8 and 9.

DISCUSSION

Up to now there has been no information on the tertiary structure of eukaryotic 5S rRNA in solution. The only hypothesis concerning a prokaryotic model of *E. coli* 5S rRNA was published many years ago [12, 31]. Therefore, we attempted to probe the structure of plant 5S rRNA in solution using various methods [32]. Using differential scanning calorimetry we provided previously evidence for some tertiary interactions in lupin 5S rRNA, in contrast to wheat germ 5S rRNA [32]. In this paper we present a new model for plant 5S rRNAs, based on experimental data and theoretical calculations, using known algorithms [17, 26]. For this purpose, we evaluated the nucleotide sequence of all known to date higher plant 5S rRNAs (Table 1). As can be seen from the model (Fig. 2) for 13 positions out of 120, heterogeneity is observed. The model is composed of 5 helical regions (including 3 bulges) and 5 loops (Fig. 6). Four positions: 23, 24, 49 and 73 out of 13 are present in bulges and loops. The most heterologous are 3' and 5' ends of the molecule. An analysis of these positions suggested sites which could be important for the stability of the RNA structure, i.e. nucleotide 49 and base pair 3'-116.

Application of the computer programme revealed some new elements in the secondary structure of 5S rRNA [10, 12, 33, 34]. The domains of the molecule subjected to rearrangement consist of stems IV and V and loops A, D, E. The most important difference in our model, is a larger D loop and a new bulge C105. There is also evidence supporting the proposed tertiary interaction between loops C and D. On the basis of the digestion pattern and accessibility of phosphodiester bonds in 5S rRNA and assuming the proposed model of secondary structure, we were able to fold 5S rRNA into a three-dimensional structure and build a wire model (Fig. 7). This was supported by computer modeling of 5S rRNA of lupin (Figs. 8 and 9).
In this model of 5S rRNA it is composed of three domains and is reminiscent of the three-dimensional model of tRNA. The major grooves of the helices are fully accessible to the solvent and open for interaction with transcriptional factor IIIA. Our model explains the observation of Huber & Wool [35] that, in Xenopus laevis oocytes, TF IIIA protects in 5S rRNA only the fragment G64-G106 (domains γ and α), and not C14-C63 (domain β). Since it has been observed that 5S rDNA exists in the B-form under TF IIIA binding conditions [36], 5S rRNA should adopt a proper conformation in order to interact with specific protein, if the interaction is realized by the same structural elements in protein, RNA and DNA. Alternatively, the interacting structural elements should be flexible enough to adjust specifically to each other (good candidates are junctions between stems and loops and zinc fingers). There is also the possibility that TF IIIA changes its structure to interact with DNA/RNA molecules. Differences in stability between wheat germ and lupin 5S rRNAs detected by differential scanning calorimetry [32] may be explained by a tertiary interaction affected by substitution of C to A in position 49. This is also supported by the cleavage of domain γ. The overall shape of plant 5S rRNA tertiary structure is supported by the small angle diffraction data for rat liver 5S rRNA which are very similar to those for tRNA^Phe [34]. The proposed model accommodates well within the measured radius of gyration and maximum dimensions proposed for rat liver and E. coli 5S rRNA [34, 37]. The major feature of the new model is the almost parallel alignment of domains β and γ. Both domains form a continuous helical segment with domain α. Loops C and D are within the interacting distance. Further modeling is necessary to determine whether the proposed tertiary interactions can be arranged as suggested.

In our model of lupin 5S rRNA, we have 11 A-U base pairs, 20 G-C base pairs and 2 G-U base pairs [25]. These numbers are not identical with those proposed by Li & Marshall [27] on the basis of FT-IR measurements and await further verification by other methods. Our model is further supported by new experiments with alpha sarcin, ribonuclease H and chemical modifications [38].

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