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QUARTERLY

Evidence for plant ribosomal 5S RNA involvement in elongation of polypeptide chain biosynthesis*

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A series of short oligo-DNA probes (8–10-mers) complementary to various regions of the plant ribosomal 5S ribonucleic acid (5S rRNA) have been synthesized. The results of their hybridization to free 5S rRNA and to ribosomes pointed to the availability of nucleotides in loop "C" for complexation. We found a correlation between hybridization of selected oligonucleotides and their inhibitory effect on enzymatic binding of Phe-tRNA and poly(Phe) synthesis on wheat germ 80S ribosomes. Evidence was obtained for involvement of 5S rRNA in the elongation of polypeptide chain during protein biosynthesis. 5S rRNA seems to play a critical role in protein biosynthesis, probably through causing conformational changes of loop C.

Ribosomal 5S ribonucleic acid (5S rRNA) is the smallest RNA component of ribosomes. Interactions of 5S rRNA with other components of the ribosomal system during the translation process are crucial for the efficiency and accuracy of protein biosynthesis [1]. Ribosomes lacking 5S rRNA show a substantially reduced biological activity, e.g., enzymatic binding of aminoacyl-tRNA to the ribosomal A-site was particularly affected [2]. Stem II and loop C (domain β) of prokaryotic and eukaryotic 5S rRNAs seem to play an important role in RNA-RNA and RNA-protein interactions within the ribosome [3]. Structural and functional analysis of prokaryotic 5S rRNA suggested the importance of some fragments of this molecule in translation. Mutants of *E. coli* with the loop C of 5S rRNA lacking G41 or those with removed G41, CCG44 or CCGAA46, yielded reconstituted 50S subunit particles with reduced poly(Phe) synthesizing activity [4]. In other experiments it has been shown that nucleotides in loop C, i.e., positions 42–47, in free eubacterial 5S rRNA are available for oligonucleotide hybridization, but not in the 5S rRNA-protein complexes. Thus, prokaryotic 5S rRNA in the 70S ribosome and in the ribosomal subunit does not interact with the oligonucleotide probe [5]. However, the available data on the function of 5S rRNAs from higher plants are very limited and ambigous.

In a recent study, RNase H and complementary oligodeoxynucleotides were used for testing the tertiary structure of plant 5S rRNA [6]. Loops C, E and D were found to be the regions of RNA-DNA duplex formation most suscep-

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tible to RNase H hydrolysis. This model has further been extended to other plants [7] and confirmed by n.m.r. data [8].

In this communication we have extended this approach to topography and function of 5S rRNA within the ribosome. Our results support participation of loop C of 5S rRNA structure in protein biosynthesis machinery.

MATERIALS AND METHODS

5S rRNA was isolated and purified as previously described [7].

Isolation and aminoacylation of $tRNA^{Phe}$, binding, polymerization assays and isolation of ribosomes were performed according to the protocols outlined earlier [9, 10].

The activity of 80S plant ribosomes was tested by binding of Ac[^{14}C]Phe-tRNA in standard conditions [11]. We found our ribosomes over 90% active.

Protein synthesis assay. The translational activity of the ribosomes was tested by measuring the poly(U)-directed [^{3}H]phenylalanine incorporation into polypeptide chain as described previously [10]. The effect of DNA probes on the binding and polymerization steps was determined after hybridization of the oligomers. The standard volume of hybridization mixture (25 µl) was increased to 50 µl by addition of the components of the poly(Phe) synthesis, or of the Phe-tRNA binding assays. The concentration of ions remained unchanged.

Antisense DNA probes. The oligodeoxynucleotides were synthesized by the phosphoramidite method using an automated DNA synthesizer (Applied Biosystem, Model 380/381). Fully deblocked oligomers were separated from low-molecular-mass blocking groups by ethanol precipitation. The oligomers were labeled at the 5′ end with [γ-^{32}P]ATP (Amersham International, U.K.) and T4 polynucleotide kinase (Biolabs, New England) following the manufacturer's instructions. The labeled DNA probe was then isolated and purified by anion exchange chromatography on a Quiagen-20 column (Quiagen) and eluted using 1 M NaCl and 2 M urea following the manufacturer's instruction. The tested oligomers are listed in Table 1, panel C.

Hybridization assays. The assays were performed by incubating 12 pmol of 80S ribosomes with a 30-fold molar excess of (5′-^{32}P)-end-labeled probe for 10 min at 37°C in 25 µl of binding buffer (10 mM Tris/HCl, pH 7.4; 100 mM KCl, 5 mM $MgCl_2$) followed by 2 h incubation at 0°C; on average 1 pmol ^{32}P-labeled probe was equal to 200 c.p.m. The conditions of hybridization test were as in [11]. Then the reaction mixture was diluted to 1 ml with the same buffer, filtered through Millipore nitrocellulose filter and washed five times with 1 ml aliquots of the same buffer. Radioactivity retained on the filters was determined by liquid scintillation counting.

Hybridization of DNA probe to free 5S rRNA was also assayed by molecular filtration on Sephadex G-50 (column 0.9 × 42 cm). In this particular experiment the hybridization conditions were as described above except that: in the same volume 600 pmol of 5S rRNA was incubated with a 3-fold molar excess of the labeled probe. The incubation mixture was 5-fold diluted with the binding buffer directly before being loaded on the column. The elution volume of the 5S rRNA-oligomer complex coincided with column void volume (10 ml) and the elution volume of the free oligomers was about 25 ml. The column was preeluted (at 4°C with flow rate of 0.5 ml/min) with the binding buffer containing 10 nM oligomer analogous to that used for complex formation. Fractions of 0.5 ml were collected and assayed for migration of the [^{32}P]oligomer-5S rRNA hybrid and free [^{32}P]DNA probe by radioactivity measurement.

RESULTS AND DISCUSSION

We selected the antisense DNA probes with sequences complementary to those of single stranded regions, and tentatively chosen as potentially crucial for protein biosynthesis (cf. Table 1 and Fig. 1). In Table 2 the experimental data on their hybridization to ribosomes and on their inhibitory effect on particular translation steps, are presented (the effect on binding of $Phe\text{-}tRNA^{Phe}$ and poly(Phe) synthesis on poly(U)-programmed ribosomes). The probes no. 3 and 7 gave the highest inhibitory effects. They were further tested for hybridization to naked 5S rRNA. Since probes no. 4 and 9 showed no effect either in the binding or the

Table 1

Sequences of the tested 5S rRNA of wheat germ (A) and complementary antisense DNA probes (B).
Sequence of 5S rRNA is given from the 5', to 3' end, the complementary oligomers are shown from the 3' to 5' end. The tested probes are separately listed (C).

```
     5'
      1          11         21         31         41
A)   GGAUGCGAUC-AUACCAGCAC-UAAAGCACCG-GAUCCCAUCA-GAACUCCGAA-

        3'GCTAG-TATGG5'   G-ATTACGTG          TAGT-CTTGAG
B)                                                       GCTT-
                                                           TT-

      51         61         71         81         91
     -GUUAAGCGUG-CUUGGGCGAG-AGUAGUACUA-GGAUGGGUGA-CCUCCUGGGA-

                 GAACCCGCTC                      CACT-GGAG  CCCT-
     -CAATTC                C-TCATCATGA
     -CAATTC

      101        111       3'
     -AGUCCUCGUG-UUGCAUUCC

     -TCAGGA
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C) LIST OF TESTED OLIGONUCLEOTIDES

# 1	5' GGT ATG ATC G 3'
# 2	GTG CAT TAG
# 3	GAG TTC TGA T
# 4	CTT AAC TTC G
# 5	CTT AAC TT
# 6	CTC GCC CAA G
# 7	AGT ACT ACT C
# 8	GAG GTC AC
# 9	AGG ACT TCC C

polymerization tests, we assumed that they could serve as the controls.

The molecular gel filtration results presented in Fig. 2 show that probe no. 3 complementary to the nucleotides of loop C hybridizes to free 5S rRNA. To stabilize the complex, molecular filtration was performed at 4°C and the column was preequilibrated with low concentration of the oligomer. Although when using this technique the DNA probe hybridization to 5S rRNA should be evident, we consistently found only about 1% of the 5S rRNA bound to the probe in the eluate; presumably, conditions of the complex migration through the column were responsible for its dissociation.

Previously, using the RNase H assay, the accessibility of this region and interior loop E for antisense DNA probes has been demonstrated [6]. The molecular filtration results are in agreement with the RNase H digestion experiments, which allows us to expect some specificity of the RNA-DNA hybrid formation. We also observed hybridization of probe no. 7 to loop E. In comparison with probes no. 3 and 7, hybridization of probe no. 9 to loop E was significantly lower. Hybridization of probe number 4 was not detected by molecular filtration (Fig. 2). In the model of the tertiary structure of plant 5S rRNA proposed by Barciszewska *et al.* [6–8], the tertiary base pairing U-53 and A-100 stabi-

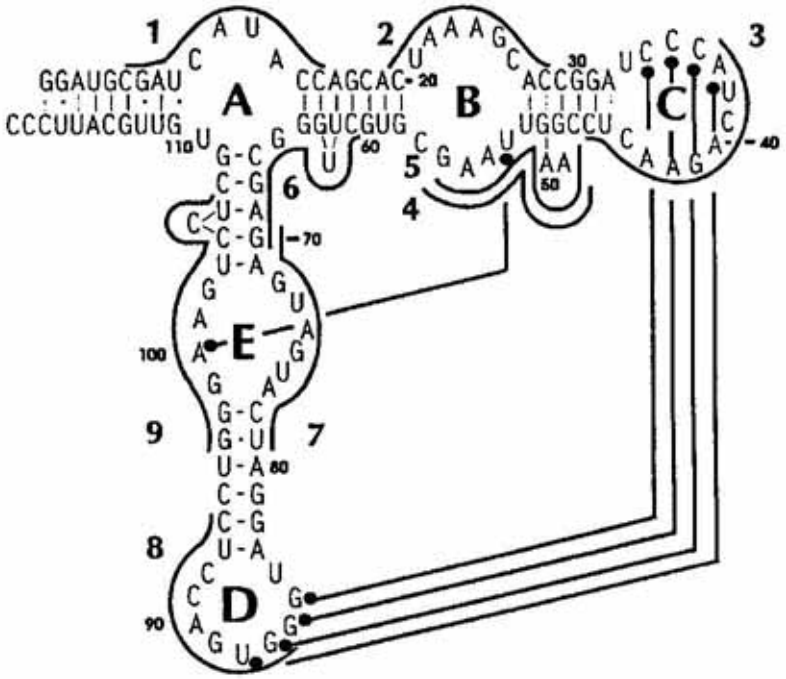

Fig. 1. The secondary structure of ribosomal 5S rRNA from wheat germ, with the tertiary interactions between loops B and E, and C and D, respectively [7].
The tested probes are indicated by lines and numbers; for the sequence of DNA probes see Table 1.

lizes the interaction between loop B and loop E [8] (as shown in Fig.1). The very low hybridization of probes no. 9 and 4 to these regions, supports this model.

The accessibility of loop C in wheat germ 5S rRNA is also a common feature of prokaryotic molecules as shown for *E. coli* and *B. stearothermophilus* [5]. In modification studies on *E. coli* 5S rRNA, G41 was shown to be the most reactive nucleotide, both in isolated 5S rRNA and in its ribosome bound form ([12] and references therein). However, Lorenz *et al.* [7] reported a decrease in RNase H susceptibility of loop C at positions 42–47 in the presence of d(GTTCGG) for *E. coli* and *B. stearothermophilus* 5S rRNA when complexed with their respective binding proteins. The 70S ribosomes and ribosomal subunits did not interact with this oligonucle-

Table 2

The hybridization data and inhibitory effect of oligonucleotide probes on binding of Phe-tRNA^{Phe} and poly(Phe) synthesis on poly(U)-programmed ribosomes.

As 100% hybridization of 12 pmoles of probe to 12 pmoles of ribosomes was taken, for details see Materials and Methods and our ealier articles cited therein.

Probe no.	DNA probe attachment position on 5S rRNA	Hybridization to target	Inhibition of poly(U)-programmed ribosome	
			Phe-tRNA binding	poly(Phe) synthesis
		%	%	%
1	6–15	0	0	0
2	20–29	0	0	0
3	37–46	30	30	60
4	45–55	2	3	3
5	49–55	3	0	10
6	61–70	0	2	0
7	70–79	10	0	40
8	87–94	0	0	0
9	97–106	2	0	0

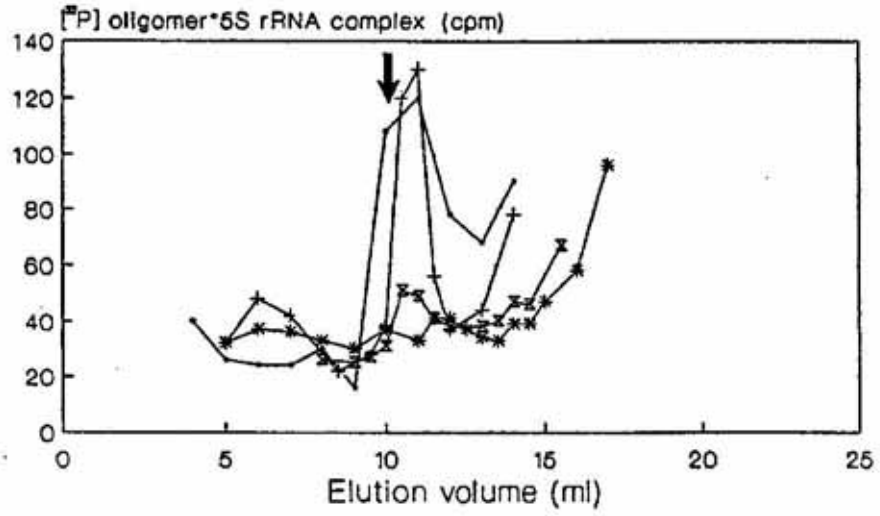

Fig. 2. Hybridization of oligonucleotide probes to naked 5S rRNA.
For details of molecular filtration of hybrids see Materials and Methods. The collected fractions were monitored for radioactivity. Symbols: ●, +, ✳, × represent the 5S rRNA-oligomer hybrids no.: 3, 7, 4 and 9, respectively. The arrow indicates the void volume of the column.

otide [5]. In our experiments (see Fig. 3) we demonstrated, that oligomer #3 complementary to loop C of wheat germ 5S rRNA undergoes hybridization to the ribosome. The detected molar ratio was 0.3 mol of oligomer per 1 mol of ribosome for DNA probe #3. This apparent contradiction between our results and the cited paper is understandable in the light of the fact that, unlike Lorenz *et al.*, we performed the experiments with poly(U)-programmed wheat germ ribosomes and not with nonprogrammed prokaryotic ribosomes. The interaction between the 5S rRNA loop C and DNA probe #3 within wheat germ ribosomes was achieved as the result of poly(U) induced conformation. The other oligomers, except probe #7 did not hybridize to the ribosome (Fig. 3).

Based on literature data, concerning the prokaryotic system, we could expect that also in the plant system 5S rRNA is of functional significance and participates in binding of aminoacyl-tRNA to the ribosomal A-site [1, 2]. This process depends on elongation factor 1 and that is why we tested the inhibitory effects of the listed probes on the enzymatic binding of Phe-tRNA. The activity of ribosomes used in those experiments exceeded 90%, as proved by binding Ac-Phe-tRNA [11]. Figure 3 shows that the inhibition occurred only in the case of pre-hybridized oligomer no. 3. We observed about 30% inhibition of binding of Phe-tRNA to 80S ribosomes in comparison with the control. Other oligonucleotides had no effect on this substep of the elongation cycle (Fig. 3 exemplifies the results for oligomers no. 3, 4, 7 and 9).

Similarly, we observed an inhibitory effect on poly(Phe) synthesis of the probes hybridized to poly(U) programmed ribosomes. The levels of inhibition by oligomers no. 3 and 7 were about 60% and 40%, respectively (Fig. 3). Using double-labeled compounds, [^{3}H]Phe-tRNA and ^{32}P-labeled probes, we showed that the

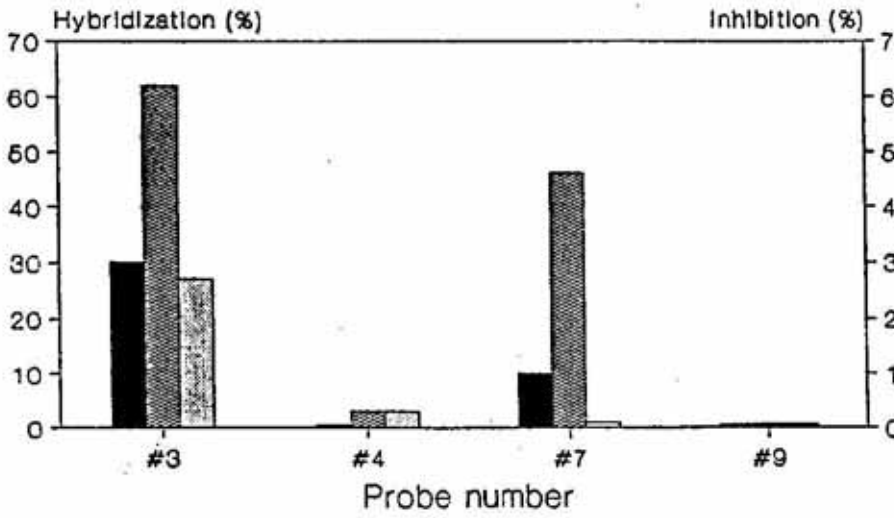

Fig. 3. The inhibitory effect of selected ^{32}P-labeled oligonucleotides hybridized to poly(U)-programmed 80S ribosome (cf. Table 1).
The following ribosome activities have been tested: binding of Phe-tRNA^{Phe} and poly(Phe) synthesis (see Materials and Methods for details). ▨ inhibition of poly(Phe) synthesis ▩; inhibition of Phe-tRNA binding; ■ hybridization of probes to 5S rRNA.

inhibitory effects of oligomers no. 3 and 7 on poly(Phe) synthesis are proportional to the degree of hybridization: 30% and 10%, respectively. The correlation between the level of hybridization and that of inhibition points to the significance of these interactions. Other oligonucleotides had no effect on the elongation cycle. In Fig. 3, oligomers no. 4 and 9 used as controls gave negative results. These experiments indicate that the conserved loop C is the only available site for hybridization of an antisense DNA probe.

Although DNA probe #7 also inhibited poly(Phe) synthesis and hybridized with the ribosome, we cannot exclude some interaction of probe #7 with 18S rRNA. According to computer searching through plant gene data base, RNA sequences in small subunits of different plant ribosomes have a high degree of similarity to 5S rRNA loop E. Upon hybridization of probe no. 3 to loop C, binding of aminoacyl-tRNA to A-site and polypeptide synthesis are inhibited. The ribosomal attachment site for elongation factor 2 (EF-2) contains the 5S rRNA-L5 particle, and this factor is located in close vicinity (less than 4 Å) to the 5S rRNA within the EF-2*ribosome complex [13]. The presented experimental and literature data allow us to hypothesize that the 5S rRNA molecule may be involved in the switching (conversion) between different active states of ribosomes.

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