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This paper is dedicated to Professor Maciej Wiewiórowski

Functional role of rRNAs in plant translation system tested with antisense strategy*

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There are regions in rRNA which are evolutionary conserved and exposed on ribosomal surface. We selected in plant material (*Lupinus luteus)* two of them: the α-sarcin domain of 26S rRNA (L-rRNA) and C loop of 5S rRNA, to be further investigated using antisense oligomers as research tools. We found inhibition of the model polypeptide biosynthesis (up to 80%) due to specific hybridization of oligomers addressed to α-sarcin domain and loop C. Based on our results we present the evidence for the key role played by these regions of rRNAs during protein biosynthesis in plant system. According to our hypothesis, conformational changes of these two regions are synchronised and cooperative during transition of pre- to post-translocation state of the ribosome. The correlation of structure and activity of rRNA domains in translation is shown.

An eucaryotic ribosome is a ribonucleoprotein complex consisting of four ribonucleic acids and about 70 proteins. 18S rRNA is a component of the small 40S ribosomal subunit. 26S–28S, 5.8S and 5S rRNAs are components of the large 60S subunit. Thirty years

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Abbreviations: L-rRNA, large ribosomal RNA, 26S and 23S for eucaryotic and procaryotic systems, respectively; (5S*L5), ribonucleoprotein complex of 5S rRNA with ribosomal L5 protein; (60S-(5S*L5)), large ribosomal subunit without the complex (5S*L5); AA-tRNA, aminoacyl-tRNA; EF1 and EF2, eucaryotic elongation factors 1 and 2, respectively; AcPhe-tRNA, acetylophenylo-tRNA^{Phe}; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TBE, Tris 45 mM, boric acid 45 mM and EDTA 1.25 mM, pH 8.3; a-DNA, antisense DNA oligomer; A-site, binding site for aminoacyl-tRNA (decoding site); P-site, site for peptidyl-tRNA; E-site, exit site for deacylated tRNA; b, base.

ago it was generally accepted that the ribosomal proteins were responsible for ribosome's activity and that rRNAs were structural elements of a ribosome. Today, when we know about catalytic activity of RNA, it is a common opinion that ribosomal nucleic acids plays a functional role in protein biosynthesis. There were some predictions that rRNA play a role of peptidyl transferase activity (Noller *et al.*, 1992). The interactions and conformational relationships between rRNAs and ribosomal proteins are responsible for the ribosome activity.

The first model of large rRNA for *E. coli* was provided by Noller (Noller *et al.*, 1981). At present, seven sequences of plant L-rRNA and about 30 of plant 5S rRNA are known (Guttel *et al.*, 1993; Guttel & Fox, 1988; Joachimiak *et al.*, 1990).

There are several regions of rRNAs which are evolutionary highly conserved (Guttel & Fox, 1988; Hartmann *et al.*, 1988). These regions are very often single-stranded and exposed on the ribosomal surface (Höpfl *et al.*, 1989). The analysis of these sequences from all the kingdoms in data bank and their comparison with the activity sites of rRNAs (Raue *et al.*, 1990; Noller *et al.*, 1990) provided a selection of functional regions which are active probably during the elongation cycle of protein biosynthesis.

One of the regions evolutionary conserved in all kingdoms and engaged in the binding of elongation factors is the α-sarcin domain of L-rRNA (Hausner *et al.*, 1987). α-Sarcin (fungal endonuclease) cuts this fragment under defined conditions in a highly specific manner (Schindler & Davies, 1977). The cleavage in the *E. coli* ribosome occurs between nucleotides 2661 and 2662 of 23S rRNA; that corresponds in rice 26S rRNA to nucleotides numbered 3018 and 3019, G3025 and A3026 of *S. cerevisiae* 26S rRNA and between G4257 and A4258 of rabbit reticulocyte 28S rRNA (Schindler & Davies, 1977; Endo *et al.*, 1990). This single cut inhibits several of the ribosome functions connected with elongation of polypeptide chain in procaryotic system (Nierhaus *et al.*, 1992). Moreover, injection of this toxin into *Xenopus* oocyte abolish protein biosynthesis in animal translation system (Saxena & Ackerman, 1990).

It was shown that short DNA oligomers are useful for testing of the structure-function relationships of rRNAs with high resolution (Nierhaus *et al.*, 1992; Hill & Tassanakajohn, 1987; Lodmell & Tappirch, 1993). There have been reports describing effective hybridization of complementary antisense oligomers to the α-sarcin domains in procaryotic and eucaryotic systems (Meyer *et al.*, 1996; Nierhaus *et al.*, 1992; Endo *et al.*, 1990; Saxena & Ackerman, 1990; Bohun & Twardowski, 1994; Hill & Tassanakajohn, 1987; Brigotti *et al.*, 1993). In our laboratory we observed complete inhibition of the ribosome translational activity in plant system through hybridization of oligomers complementary to the 3' end of this rRNA fragment (Bohun & Twardowski, 1994; Nawrot, 1994). In the *E. coli* system these oligomers do not inhibit protein biosynthesis. Probably it is the effect of conformational changes of the domain structure (Meyer *et al.*, 1996).

Ribosomal 5S ribonucleic acid (5S rRNA) is the smallest RNA component of the ribosome. Stem II and loop C (domain β) of procaryotic and eucaryotic 5S rRNAs seem to play a role in RNA-RNA and RNA-protein interactions within the ribosome (Hartmann *et al.*, 1988). However, the available data on the function(s) of 5S rRNAs from higher plants are very limited and ambiguous. In our laboratory a series of short antisense DNA oligomer (a-DNA) probes (8-10-mers) complementary to various regions of the plant 5S rRNA have been synthesised. The results of their hybridization to free 5S rRNA and to 5S rRNA within the structure of plant 80S ribosome indicated the participation of nucleotides of loop C in the elongation step of peptide biosynthesis.

Our investigations concerning the regulation of translation in the plant system using a-DNAs as a research tool indicate that α-sarcin

domain of 26S rRNA and C-loop of 5S rRNA play a crucial role in protein biosynthesis. Preliminary data of this project have been presented as a poster communication (Nawrot, 1994).

MATERIALS AND METHODS

All chemicals were from Sigma, Merck; [^{3}H]- and [^{14}C]phenylalanine as well as [γ-^{32}P]ATP were from Amersham; T4 polynucleotide kinase was from BioLabs. α-Sarcin and RNase H were generous gifts from Dr. K.H. Nierhaus and Dr. R. Brimacombe, respectively.

Lupin seeds (*Lupinus luteus* L. v. Topaz) were obtained from Poznańska Stacja Hodowli Roślin Wiatrowo near Poznań (Poland).

The presented values show the average of three experiments.

The ribosomes and elongation factors EF1, EF2 were isolated from lupin seeds (at least 3 independent isolations and different batches of plant material were used), according to procedure given in (Twardowski & Legocki, 1973). We prepared the [^{3}H]Phe-tRNA and [^{14}C]Phe-tRNA according to (Bohun & Twardowski, 1994) with specific radioactivity 320 and 110 c.p.m./pmol, respectively. For calculation, one A_{260} unit of 80S ribosomes was estimated to be equivalent to 20 pmoles.

Testing the activity of ribosomes. The activity of ribosomes was measured by testing the specific [^{3}H]AcPhe-tRNA (with radioactivity 49 c.p.m./pmol) binding to the P-site of the plant ribosomes. According to principle presented by Rheinberger and Nierhaus maximally one AcPhe-tRNA molecule per one molecule of ribosome can be bound (Rheinberger *et al.*, 1983). The AcPhe-tRNA binding experiments under P-site condition revealed that over 90% of the ribosomes were active (0.97 pmole of AcPhe-tRNA molecules were bound per 1 pmole 80S ribosomes.) The assay (according to Rheinberger *et al.*, 1988) was performed under the saturation level of AcPhe-tRNA (molar ratio AcPhe-tRNA : 80S = 1.5 :1) on poly(U) programmed ribosomes and in the absence of any factors (Bohun & Twardowski, 1994). The location of bound AcPhe-tRNA on the P-site was shown through the puromycin reaction. After 3 h of incubation at 0°C with 0.7 mM puromycin the reaction was stopped by adding sodium acetate, pH 5.5, saturated with $MgSO_4$. The Ac[^{14}C]Phe-puromycin complex was determined after extraction with ethyl acetate and liquid scintillation counting.

Preparation of a-DNA probes. The oligodeoxynucleotides were synthesised using the phosphoramidite method on the automated DNA synthesiser (Applied Biosystem, Model 380/381). Fully deblocked oligomers were separated from low molecular mass blocking groups by Qiagen®, following the manufacturer's instruction and purified using ethanol precipitation. The oligomers were labelled at the 5′ end with [γ-^{32}P]ATP (Amersham International, U.K.) and polynucleotide kinase (Biolabs, New England) following the manufacturer's instruction to specific radioactivity 200 c.p.m./pmol. The labelled DNA probe was then isolated and purified by anion exchange chromatography on a Qiagen-20 column (Quiagen®) and eluted using 1 M NaCl and 2 M urea.

Hybridization. Hybridization of a-DNA to ribosome was performed with 30-fold molar excess of oligo according to the following procedure:

- denaturation of oligomer by incubation for 3 min at 70°C,
- incubation with ribosomes – 10 min 37°C and next 2 h 0°C in the hybridization buffer (10 mM Tris/HCl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$), as described in detail in (Hill & Tassanakajohn, 1987).

α-Sarcin cleavage and detection of the α-sarcin fragment on PAGE. For detection of the 3′ fragment of 26S rRNA after the α-sarcin cleavage 6.2% polyacrylamide gel electrophoresis in the presence of SDS and TBE buffer was applied (using of the SDS/urea gels gave the same results). We used purified

5S rRNA and $tRNA^{Phe}$ as molecular mass markers. Eight pmoles of 80S ribosomes were treated with 1 μg of α-sarcin. The reaction was conducted for 10 min at 37°C. The final concentrations of ions were: 50 mM Tris/HCl, pH 7.5, 70 mM KCl, 3 mM dithiothreitol (DTT), 5 mM $MgCl_2$. The mixture was loaded on PAGE without the rRNA extraction procedure. After electrophoresis had been completed, gel was washed with ethidium bromide in water (0.5 μg/ml) and examined in ultraviolet light. For 5S rRNA analysis the 15% acrylamide gel with 7 M urea was used. Staining was performed with toluidine blue.

RNase H cleavage. Eight pmoles of ribosomes after oligonucleotide hybridization were treated with 5 μl of RNase H in buffer: 50 mM Tris/HCl, pH 7.0, 70 mM KCl, 5 mM $MgCl_2$ and DTT. Reactions were performed for 30 min in 37°C and stopped by adding 2 μl 10% SDS and 5 μl of loading buffer (xylene cyanole, bromphenol blue, sucrose). The mixture was loaded on PAGE without the rRNA extraction procedure. The cleavage product was observed on 6.2% PAGE/SDS (using of the SDS/urea gels gave the same results).

Conditions for nonenzymatic conversion of free ribosomes to:

◆Pre-translocation state:

Ribosomes (12 pmoles) in buffer A (70 mM KCl, 3 mM DTT, 50 mM Tris/HCl, pH 7.5, and 17 mM $MgCl_2$) were mixed with 10 μg poly(U) and 20 pmoles deacylated $tRNA^{Phe}$ in 13 μl and incubated for 10 min at 37°C for P-site occupation. For A-site occupation, 20 pmoles of Phe-tRNA were added and the reaction mixture was incubated for 10 min at the same temperature.

◆Post-translocation state:

Ribosomes (12 pmoles) in buffer B (70 mM KCl, 3 mM DTT, 50 mM Tris/HCl, pH 7.5, and 15 mM $MgCl_2$) were mixed with 10 μg poly(U) and 16 pmoles Phe-tRNA and incubated for 10 min at 37°C for P-site occupation. For E-site occupation, 40 pmoles of deacylated $tRNA^{Phe}$ were added and incubation was continued under the same conditions.

Conditions for enzymatic conversions of ribosomes to:

◆Pre-translocation state:

Ribosomes (12 pmoles) were enzymatically converted to pre-translocational state in two stages incubation. First a ternary complex (GTP*EF-1*Phe-tRNA) was obtained by preincubation of 30 μg of EF-1, 0.5 mM GTP, 0.5 μl (500 pmoles) of Phe-tRNA for 5 min at 37°C. Then the complex was mixed with 12 pmoles of ribosomes in post-translocational state obtained in nonenzymatic conversion and incubated for 3 min at 37°C.

◆Post-translocation state:

Ribosomes (12 pmoles) were enzymatically converted to post-translocational state as follows: first a binary complex (GTP*EF-2) was obtained by preincubation of 20 μg of EF-2 with 0.5 mM GTP for 3 min at 37°C and then it was mixed with 12 pmoles of ribosomes in pre-translocational state obtained in nonenzymatic conversion and incubated for 3 min at 37°C.

Isolation of 60S subunits, complex (5S*L5) and (60S-(5S*L5)):

60S ribosomal subunits were obtained by treatment of the ribosomes with 1 mM EDTA and centrifugation in Beckman SW40 rotor, in 10-25% sucrose gradient for 18 h. Fractions (0.5 ml) were collected and absorbancy at 260 nm was measured.

Isolation of ribosomal complex (5S*L5) was achieved by treatment of 60S with EDTA and gradient centrifugation according to the following procedure:

60S ribosomal subunits were resuspended in 250 μl of buffer (20 mM Tris/HCl, pH 7.6, 50 mM KCl, 0.9 mM EDTA). The ratio of EDTA to ribosomes was 0.09 μmoles of EDTA per one A_{260} units of ribosomes (the conditions of isolation were optimised). The mixture was incubated for 5 min at 37°C, and next directly loaded on the gradient (250 μl of sample on 12.5 ml of 10-25% sucrose gradient in 20 mM Hepes/KOH, pH 7.9, 50 mM KCl, 0.5 mM $MgCl_2$, 6 mM DTT and 0.75 mM EDTA). The sample was centrifuged at 20000

r.p.m. in Beckman swing-out rotor type SW40 for 16 h. The fractions were collected from the bottom of the tube (25 fractions, 500 μl each). The complex (60S-(5S*L5)) was found in fractions number 9–11, and ribonucleoprotein complex (5S*L5) in 16–18 fractions. These selected fractions were collected, stored at −70°C and used for further experiments.

RESULTS

Antisense strategy, the application of short oligodeoxynucleotides complementary to specific sequences, was used as a research tool for finding correlations between structure and function of the conservative fragments of rRNAs. First, we preselected oligonucleotides for hybridization based on known sequences

Table 1. List of a-DNA probes complementary to fragments of α-sarcin domain (A) and 5S rRNA (B) numbered in Roman and Arabic, respectively.

In the table, the number and sequence of each used oligomer is shown.

A. α-Sarcin domain *(Oriza sativa)*		B. Loop C of 5S rRNA *(Lupinus luteus)*	
Number of oligomer	Sequence 5'-AAUUCAACCUAGUACGAGAGGAACCGUUGAUUCA-3'	Number of oligomer	Sequence 5'-ACCGGAUCCCAUCAGAACUCCGGU-3'
I	3'-TCTCCTTGGCAACTAAGT-5'	3.0	TAGTTCTTGAG
II	3'-TCTCCTTGGCAACTAAG-5'	3.1	AGTTCTTGAG
III	3'-TCCTTGGCAACTAAGT-5'	3.2	TAGTTCTTGA
IV	3'-TCCTTGGCAACTAAG-5'	3.3	AGTTCTTGA
V	3'-CCTTGGCAACT-5'	10.	TCAGAACT
VI	3'-CTCTCCTTGGCAACT-5'		
VII	3'-CTCCTTGGCAACT-5'		
VIII	3'-TCCTTGGCAACT-5'		
IX	3'-AGTTGGATCATGCTCTC-5'		
X	3'-AGTTGGATCATGCTCT-5'		
XI	3'-AGTTGGATCATGCTC-5'		
XII	3'-AGTTGGATCATGCT-5'		
XIII	3'-TGCTCTCCT-5'		
XIV	3'-GGATCATGC-5'		
XV	3'-GATCATGCT-5'		
XVI	3'-ATCATGCTC-5'		
XVII	3'-TCATGCTCT-5'		
XVIII	3'-CATGCTCTC-5'		
XIX	3'-ATGCTCTCC-5'		
XX	3'-AAGTTGGATCATGCT-5'		
XXI	3'-ATGCTCTCCT-5'		
XXII	3'-ATCATGCTCTCCTT-5'		
C	CACATGTTA		

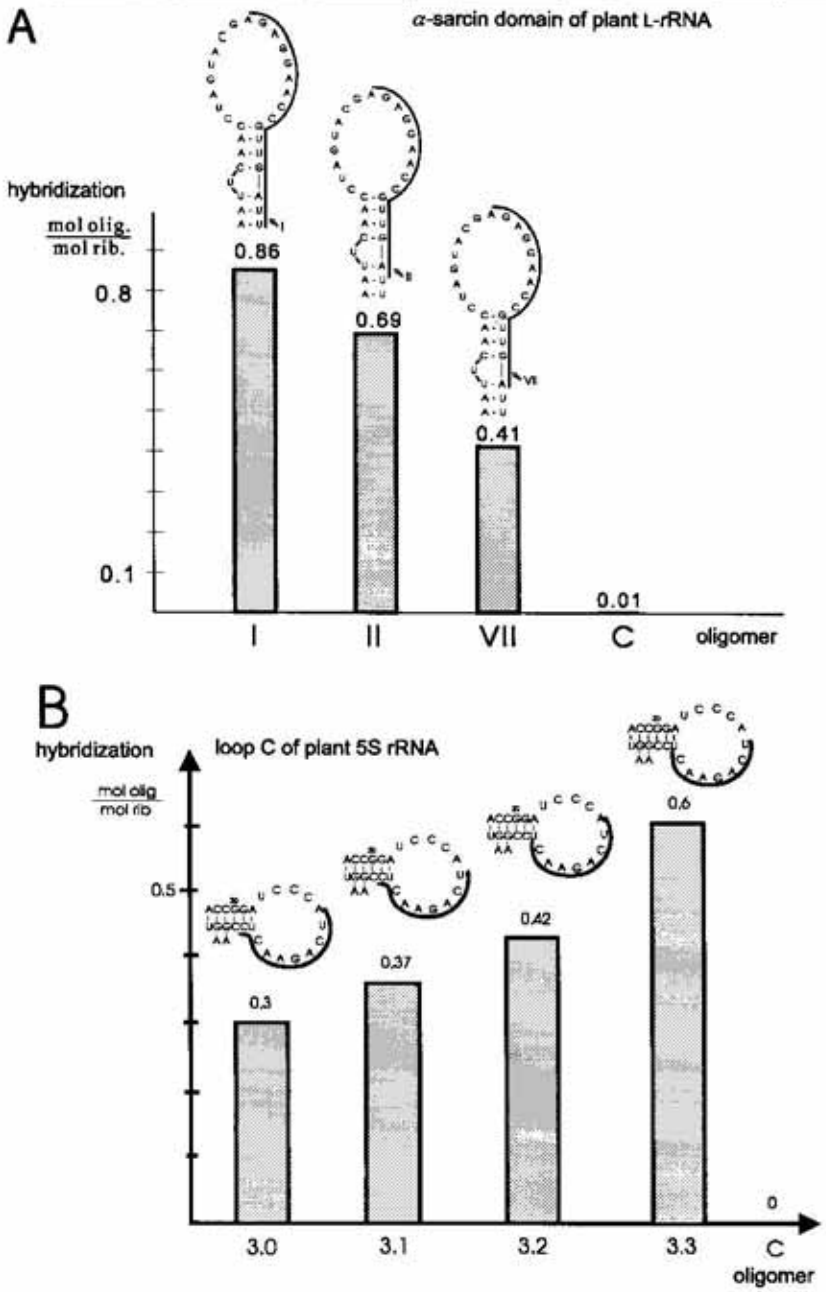

Figure 1. Hybridization of selected a-DNA probes to plant ribosomal:

A) α-Sarcin domain. The tested fragment of α-sarcin domain is shown in the upper part of the panel, the hybridization activity of a-DNA probes No. I, II, VII, and C (as control an oligomer, not complementary to any sequence in rRNA) is shown on the graph. In the abscissa the molar ratio of hybridized oligomer per ribosome is given.

B) C-loop of 5S rRNA. The tested fragment of C-loop of 5S rRNA is shown in the upper part of the panel, the hybridization activity of a-DNA probes No. 3.0, 3.1, 3.2, 3.3 , and C (as control, an oligomer not-complementary to any sequence in rRNA) is shown on the graph. In the abscissa the molar ratio of hybridized oligomer per ribosome is given.

of the α-sarcin domain of 26S-rRNA and lupin 5S rRNA (Table 1, panel A and B, respectively). As a control test, we used the oligomer without the complementary region to plant rRNA (based on database analysis). Next, we selected probes with the highest hybridization activity which appeared to be complementary to the 3′ region of the α-sarcin domain and to

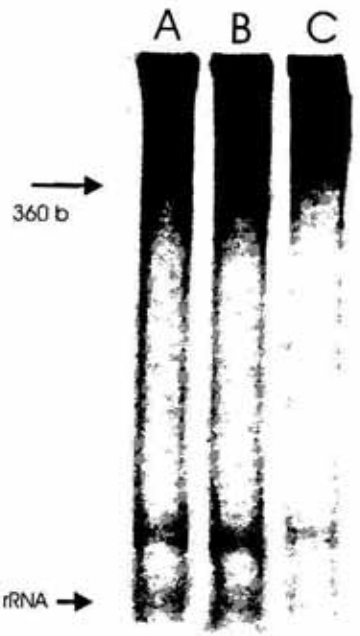

Figure 2. Polyacrylamide gel electrophoresis analysis of the protection effect of EF1 attached to ribosome against RNase α-sarcin cleavage of α-sarcin domain of L-rRNA.

The bold arrow indicates the product of α-sarcin cleavage (360 b). The short arrow shows position of marker.

A, control: 8 pmoles of ribosomes; B, 8 pmoles of ribosomes treated with 1 μg RNase α-sarcin; C, 8 pmoles of ribosomes with complexed EF1 (15 μg) treated with 1 μg α-sarcin.

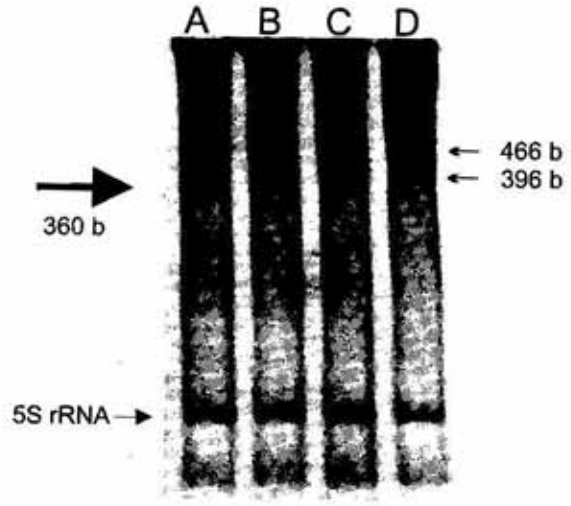

Figure 3. SDS/PAGE analysis of the protection effect of selected oligomers against RNase α-sarcin cleavage.

Two oligomers, specific (No. I) and unspecific one (No. XII), were prehybridized to ribosome and next treated with RNase α-sarcin. Arrow in bold indicates the product of α-sarcin cleavage (360 b). The short arrow shows position of marker. A. Ribosomes (8 pmoles) and α-sarcin (1 μg); B. Ribosomes (8 pmoles), oligomer No. I (240 pmoles) and α-sarcin (1 μg); C. Ribosomes (8 pmoles), oligomer No. XII (240 pmoles) and α-sarcin (1 μg); D. Control of ribosomes (8 pmoles).

the fragment between bases 38–45 of C-loop 5S rRNA, as indicated in Fig. 1 A and 1 B, respectively.

The α-sarcin domain (stem-loop structure) of L-rRNA is crucial for protein biosynthesis. Cleavage of this loop with endonuclease α-sarcin inhibits basic functions of the ribosome (Hausner *et al.*, 1987). For example binding of elongation factors is blocked which results in the inhibition of peptide synthesis. On the other hand, binding of EF1 to the ribosome protects L-rRNA against α-sarcin cut (Fig. 2). Hybridization of specific oligomers to α-sarcin domain inhibits translation and protects this domain against ribonucleolytic activity of RNase α-sarcin. The protection experiment is shown in Fig. 3. In the presence of oligomer No. I prehybridized to ribosome, complete protection of α-sarcin domain against hydrolytic activity of α-sarcin was observed. We tested hybridization of this oligomer to L-rRNA by RNase-H digestion. We observed the cleavage product on SDS/PAGE located below the fragment produced by the α-sarcin treatment (Fig. 4).

We found correlation between the level of oligomer's hybridization and inhibition of the synthesis of poly(Phe). The most effective inhibition was observed for oligo No. I (from Table 1). There is close correlation between the length and position of the oligomer and its inhibitory effect, as we reported earlier (Bohun & Twardowski, 1994) and this is presented in Table 2: oligo No. I inhibits Phe-tRNA^{Phe} binding to ribosome up to 60% and the poly(Phe) synthesis up to 70%. Other oligomers which were tested (see Table 1)

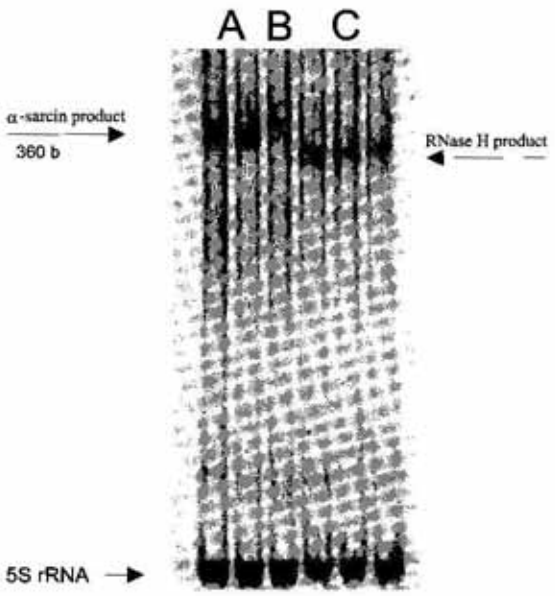

Figure 4. SDS/PAGE analysis after RNase H digestion. A and B, two lines with ribosomes after α-sarcin cleavage; C, control ribosomes; D, E, F, three lines ribosomes after RNase H cleavage.

Arrows indicate the α-sarcin and RNase H cleavage products. The short arrow shows position of a marker.

have given the much lower effect. The control oligomer (C) have no effect in translation system.

We synthesised the antisense DNA probes with sequences complementary to the selected regions of 5S rRNA (Table 1, panel B). In Table 2 the data on their inhibitory effects at particular translation steps are presented (the effect on binding of Phe-tRNA^{Phe} and poly(Phe) synthesis on poly(U)-programmed ribosomes). Probe No. 3.3 (from Table 1, panel B) gave the highest inhibitory effect. In our experiments, we demonstrated that oligomer No. 3.3 complementary to loop C of 5S rRNA hybridized to the ribosome with molar ratio 0.6 mol of oligomer per 1 mol of ribosome, while oligomer No. 3.0 hybridized with molar ratio 0.3 mol per 1 mol of ribosome. We observed about 50% inhibition of binding of Phe-tRNA to 80S ribosome after treatment with oligomer No. 3.3. Similarly, we observed an inhibitory effect on poly(Phe) synthesis of the probes hybridized to poly(U) programmed ribosomes. Using double-labelled compounds, [^{3}H]Phe-tRNA and ^{32}P probes, we showed that the inhibitory effects of oligomers No. 3.3 and No. 3.0 on poly(Phe) synthesis: 88% and 60%, are proportional to the degree of hybridization: 60% and 30%, respectively (see Table 2). The correlation between the level of hybridization and that of inhibition points out to the significance of these interactions.

Similar to the well established procaryotic model (Nierhaus *et al.*, 1992; Lodmell & Tapprich, 1993), plant ribosomes also undergo several conformational changes during the translational process (Holmberg *et al*, 1992). The formation of ribosomal pre- and post-

Table 2. Determination of inhibitory effect of complementary oligonucleotides on biological activity of lupin ribosomes (functional tests).

A. α-Sarcin domain; B. Loop C of 5S rRNA.

Oligomers/percent of hybridization	Phe-tRNA binding (percent of remaining activity)	Poly(Phe) (percent of remaining activity)
A. For α-sarcin domain		
I/86	40	30
II/69	46	40
VII/41	60	75
C/1	100	100
B. For loop C of 5S rRNA		
C. 3.3/60	50	10
3.0/30	70	40
7/10	100	60
C/0	100	100

Table 3. Level of hybridization of oligomer 3.3 complementary to C loop of 5S rRNA to 80S, 60S subunit, (60S-(5S*L5)) and to (5S*L5) complexes.

Experiments were also performed with other oligomers, and similar but lower hybridization effects were observed.

Component	Percent of hybridization
80S	60
60S	100
(60S-(5S*L5))	19
(5S*L5)	76

translocation states may be achieved in an enzymatic and nonenzymatic ways. The basic difference between these two ways is higher concentration of magnesium ions (15 mM $MgCl_2$) in the case of nonenzymatic conversion and the presence of elongation factors EF-1 and EF-2 (at 7 mM $MgCl_2$) in the case of the enzymatic one. We performed the hybridization assay with ribosomes converted to pretranslocational and post-translocational states with probe No. 3.3 (8-mer complementary to loop C with the highest hybridization activity). In the case of nonenzymatic transition we observed over two-fold higher hybridization of a-DNA to loop "C" 5S rRNA of pretranslocation in comparison with post-translocation ribosomes (see Fig. 5).

The enzymatic conversion was done with purified preparations of EF-1 and EF-2. Hybrid formations for the two states of ribosome were carried out and results are presented in Fig. 6. The enzymatic conversion results in over four-fold more effective hybridization of the specific probe to pre-translocational in comparison with post-translocation state of ribosome. Our results point out that the interaction between tRNA and 5S rRNA occurs in the post-translocation state of ribosome.

It is known that 5S rRNA is present as a complex with L5 protein inside the ribosome. This complex most probably participates in binding of EF1 and EF2 factors to ribosome (Nygard & Nilsson, 1987). We isolated (5S*L5) complex and large ribosomal subunit without this complex, from ribosomes as described in Material and Methods and shown at the Fig. 7. All the aforementioned complexes: 80S, 60S, (60S-(5S *L5)), and (5S*L5) were tested for oligomer No. 3.3 hybridization (Table 3). It is evident that the inhibitor hybridizes exclusively to the large ribosomal subunit if 5S rRNA is present. This is a very

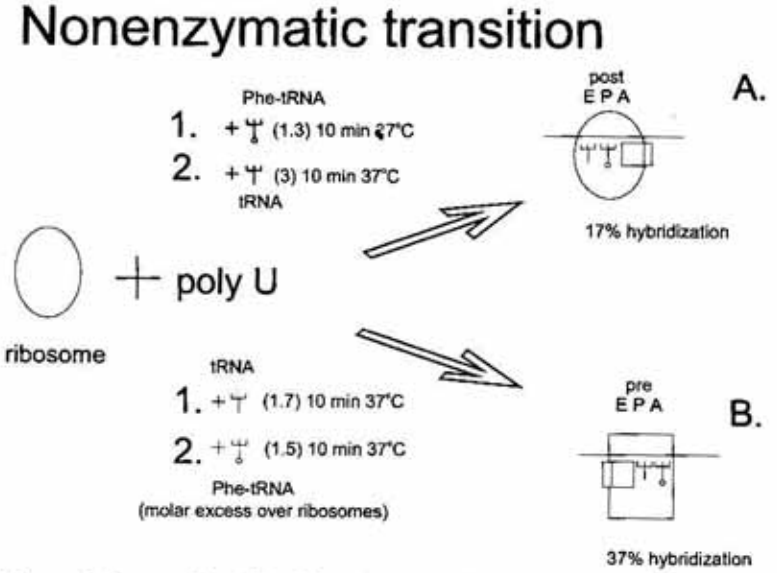

Figure 5. Hybridization of oligonucleotide No. 3.3 complementary to loop C of 5S rRNA in different states of ribosomes (nonenzymatic transition).

In panel A and B the percentage of hybridization of oligomer No. 3.3 to post- (17%) and pretranslocational state (37%), respectively is given.

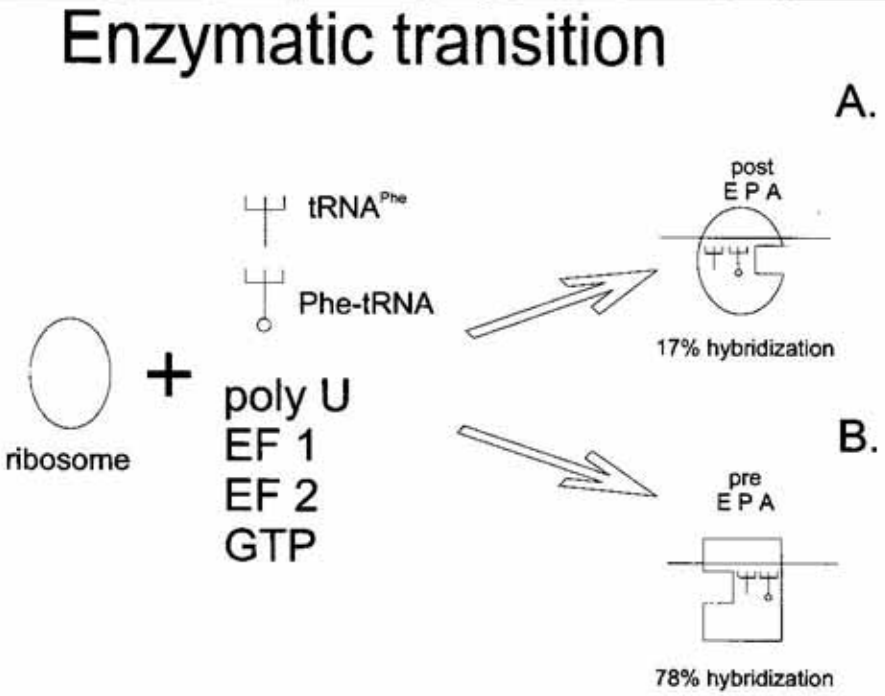

Figure 6. Hybridization of oligonucleotide No. 3.3 complementary to loop C of 5S rRNA in different states of ribosomes (enzymatic transition).

In panel A and B the percentage of hybridization of oligomer No. 3.3 to post- (17%) and pretranslocational state (78%), respectively is given.

important evidence for the specificity of the described interaction and it indicates a crucial role of loop C of 5S rRNA.

DISCUSSION

Using literature data (Guttel, 1992) and computer data base (HIBIO, 1991) we found several evolutionary conserved sequences in rRNAs. Two of them were selected for further experiments: α-sarcin domain in 26S rRNA and loop C in 5S rRNA. We accepted the structures and sequences of the selected fragments from Guttel's model of rice 26S RNA (Guttel, 1992) and Joachimiak's model for lupin 5S rRNA (Joachimiak *et al.*, 1990). The following seven sequences of plant L-rRNA

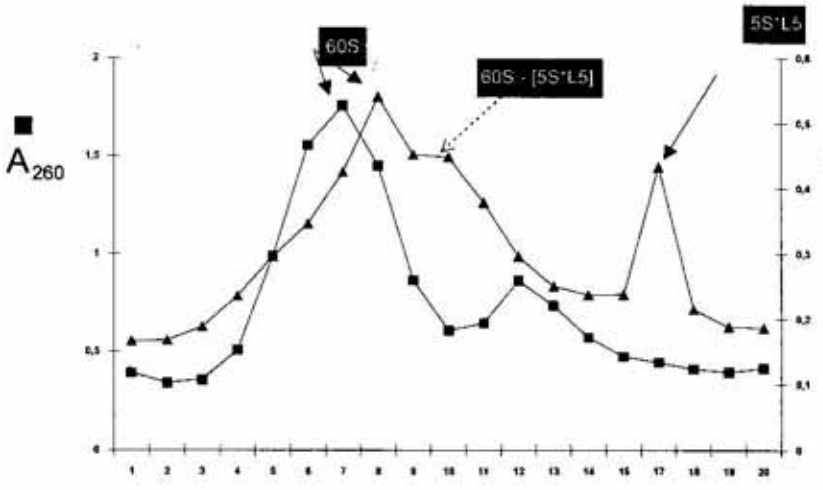

Figure 7. Isolation of ribonucleoprotein complex (5S rRNA*L5) on 10–25% sucrose gradient with EDTA.

Line indicated by squares, dissociation of ribosomes to the subunit in 1 mM EDTA; line indicated by triangles, isolation of the complex (5S rRNA*L5) from 60S subunits; double arrow indicates 60S subunits; interrupt arrow indicates 60S subunits-(5S rRNA*L5) complex; long arrow indicates (5S rRNA*L5) complex; X axis, number of fractions; Y axis, A_{260} value.

are known and all of them are characterized by ultraconservative structure of α-sarcin domain: *Oriza sativa, Fragaria ananasa, Brassica napus, Citrus limon, Lycopersicon esculentum, Sinapis alba* and *Arabidopsis thaliana* (Guttel *et al.*, 1993; Bohun & Twardowski, 1994). Preliminary data of sequencing shown the highly similar (if not identical) sequence for this domain in lupin (Nawrot & Madej, unpublished).

Interactions of 5S rRNA with other components of the ribosomal system during the translation process are most probably crucial for the efficiency and accuracy of protein biosynthesis (Nomura & Erdmann, 1970; Barciszewska *et al.*, 1990; Erdmann, 1976). Ribosomes lacking 5S rRNA show a substantially reduced biological activity which results in the inhibition of the enzymatic binding of aminoacyl-tRNA to ribosomal A-site (Nomura & Erdmann, 1970). Eucaryotic 5S rRNA displays a secondary structure with five helical regions, suggesting a coaxial tertiary stacking of either helices A and D or helices B and D. In most published eucaryotic 5S rRNA sequences the loop defined by helix C consists of 12 bases and has a semi-conserved PyNGNNPy-sequence. A conserved AGAAC-sequence in this loop was also reported in higher plants (Barciszewska *et al.*, 1990). The 5S rRNA in the plant ribosome appears to have the shape of constrained into a bent "Y" in which helix III is directed towards the 30S subunit (Evstafieva *et al.*, 1985), helix I is angled towards the upper site of the central protuberance (Shatsky *et al.*, 1980), and helix IV is directed downwards toward the peptidyltransferase center (Shatsky *et al.*, 1980; Moazed *et al.*, 1988). It means that loop C of 5S rRNA is exposed on the surface of the ribosomal subunit. It was found, in the procaryotic system, by means of site-directed crosslinking studies with tRNA, that nucleotide 47 in the elbow region of $tRNA^{Phe}$ at the P-site is located in close vicinity to nucleotide 2309 of 23S rRNA. This site is also at the loop-end of helix 84 and provides a link between the elbow region of the P-site tRNA and the top of the central protuberance of the 50S subunit.

The inhibition of the enzymatic Phe-tRNA binding and, consequently, polypeptide synthesis, in our plant system, can be explain with antisense strategy experiments, as is presented in this paper. We think, that the interaction between the conservative sequence TΨCG of tRNA and sequence GAAC of 5S rRNA is blocked by hybridized oligonucleotide. The possibility of such interactions was supposed by Erdmann (Erdmann, 1976).

The procaryotic α-sarcin domain is involved in several key steps of translation including EF-Tu dependent binding of aminoacyl-tRNA to ribosomes and EF-G/EF2 catalysed GTP hydrolysis and translocation (Hausner *et al.*, 1987; Nierhaus *et al.*, 1992; Moazed *et al.*, 1988; Holmberg & Nygard, 1994). In the case of procaryotic α-sarcin domain, both factors EF-Tu and EF-G protect nucleotides of this domain (G2655, A2660, G2661) from chemical modification; besides EF-Tu protects A2665 (Miller & Bodley, 1991). Treatment of *E. coli* ribosomes with α-sarcin results in a loss of binding of the EF-Tu-GTP-aminoacyl-tRNA ternary complex and the EF-G-GTP complex to the ribosome (Hausner *et al.*, 1987).

Our experiments show that plant (lupin) EF1 protects α-sarcin domain against cleveage by the site-specific ribonuclease α-sarcin. The hybridization of specific a-DNAs complementary to the 3′ half of this domain (oligomers No. I, II) leads to effective inhibition of poly(Phe) chain synthesis. A possible explanation for this effect in plant system is the dissociation of 80S ribosomes to the 60S and 40S subunits after hybridization of a complementary probe (Bohun & Twardowski, 1994). In our experiments with lupin system the level of inhibition of poly(Phe) synthesis and dissociation to the subunits is proportional to the molar ratio of hybridization. The inhibitory effect of this particular oligomer was also independently confirmed by (Brigotti *et al.*, 1993) using another plant system

(wheat). It is evident, that α-sarcin domain of L-rRNA is an important component of elongation factor binding site(s) of the ribosome.

Similarly to EF1 ribosome-bound elongation factor 2 completely protected A4256 located in the α-sarcin loop from ricin induced depurination (Osborn & Hartley, 1990). It suggests that EF2 formed a functional complex with the ribosomes. On the other hand, the depurination catalysed by ricin resulted in a total loss of the EF2 binding capacity of the 80S ribosomes (Holmberg & Nygard, 1994; Miller & Bodley, 1991)) and in inhibition of protein synthesis as is the case when the ribosomes are treated with α-sarcin (Endo *et al.*, 1990; Saxena & Ackermann, 1990).

During the transformation from pre- to post-translocational states significant conformational changes of rRNAs take place. Based on the presented data we suppose that hybridization of the antisense probe and elongation factors binding occur in the same region of the ribosome. The hypothesis concerning conformational changes of rRNA during the translation process was suggested in several papers (Nierhaus *et al.*, 1992; Nygard & Nilsson, 1987; Erdmann, 1976; Meyer *et al.*, 1996). However, as far as we know, there are limited experimental data in support of this speculation. The conformational changes of α-sarcin domain (Nierhaus *et al.*, 1992; Meyer *et al.*, 1996) and C loop of 5S rRNA (Nygard & Nilsson, 1987) are, in our opinion, correlated. Following the models of AA-tRNAs location on ribosome according to Dontsova and Lim (Dontsova *et al.*, 1994; Lim *et al.*, 1992), loop C and α-sarcin domain are located close to the TΨC loop of tRNA and anticodon, respectively. The molecule of AA-tRNA could play the function of a "bridge" between L-rRNA and 5S rRNA. The distance between the α-sarcin domain and loop C of 5S rRNA is the same as the distance between these two fragments (TΨC and anticodon) of tRNA. rRNAs blocked in α-sarcin domain (L-RNA) and loop C (5S rRNA) by antisense oligomers can not interact with aminoacyl-tRNA.

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