BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **248,** 51–56 (1998)
ARTICLE NO. RC988778

Inhibition of Ribosomal Subunit Association and Protein Synthesis by Oligonucleotides Corresponding to Defined Regions of 18S rRNA and 5S rRNA

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Received May 6, 1998

Strong complementarity between a conserved sequence near the 3′ end of 18S (16S) rRNA of the small ribosomal subunit and a conserved sequence in the 5S rRNA of the large ribosomal subunit supported the suggestion that base-paired interaction between the two RNA molecules could be responsible for the reversible association of ribosomal subunits during protein synthesis. If this were true then oligonucleotides corresponding to defined regions of the 18S and 5S rRNAs should have profound effects on the association of ribosomal subunits and protein synthesis. In this report we show that oligonucleotides, corresponding to a defined region of eukaryotic 18S rRNA, when bound to wheat embryo 60S ribosomal subunits, inhibited association with 40S ribosomal subunits and also inhibited *in vitro* protein synthesis. Similarly oligonucleotides corresponding to a defined region of 5S rRNA when bound to 40S ribosomal subunits also inhibited the formation of 80S ribosomes and *in vitro* protein synthesis. The minimum sequences responsible for the inhibition of ribosomal subunit association and *in vitro* protein synthesis corresponded to the 5′ strand of the m_2^6A m_2^6A hairpin structure near the 3′ end of 18S rRNA and nucleotides 91-100 of 5S rRNA which are complementary to each other. Sequences at identical positions of *Escherichia coli* 16S and 5S rRNAs are also complementary to each other.

Ribosomal RNAs, which have commonly been regarded as structural scaffolds within the ribosomal subunits, could play very important functional roles in view of the fact that base-paired interactions between limited stretches of ribosomal RNAs provides a convenient mechanism for reversible intermolecular and intramolecular interactions. On the basis of efficient and selective hybridization, *in vitro*, between eukaryotic 5S rRNA and 18S rRNA (1-4), it was proposed that base-paired interaction between 5S rRNA in the large (60S) ribosomal subunit and 18S rRNA in the small (40S) ribosomal subunit could contribute to the reversible association of ribosomal subunits. A number of studies support this hypothesis. A region of wheat embryo 5S rRNA that survives RNase digestion of *in vitro* 5S-18S rRNA complex (5) is complementary to a totally conserved region of eukaryotic 18S rRNA that constitutes the 5′ strand of the m_2^6A m_2^6A hairpin structure near the 3′ end of 18S rRNA (6,7,36-40). This sequence of 18S rRNA, in turn, is complementary to a highly conserved region of eukaryotic 5S rRNAs (6, 7). Sequences present at an identical position in a variety of prokaryotic 5S rRNAs were also found to be complementary to the 5′ strand of the m_2^6A m_2^6A hairpin structure near the 3′ end of prokaryotic 16S rRNA (6) suggesting that similar mechanisms could operate in both eukaryotes and prokaryotes. These putative interactions should be very stable as the calculated ΔG (8) range from -15 to -24 Kcal (6,7). The 3′ end of 18S (16S) rRNA is essential for function of the small (40S/30*S*) ribosomal subunits (9-14), and is present on the surface of the small subunit (15-20). 5S rRNA is also essential for ribosome function and protein synthesis (21-25). If the hypothesis that base-paired interaction between highly conserved regions of 5S and 18S rRNA (6,7) contributes to the reversible association of ribosomal subunits (1) is correct, then oligonucleotides corresponding to a region of 5S rRNA complementary to the 5′ strand of the m_2^6A m_2^6A hairpin structure of 18S rRNA and this region of 18S rRNA itself should inhibit the association of 50S and 60S subunits to form 80S ribosomes, and consequently protein synthesis. In this study we demonstrate that this is precisely what happens when synthetic oligonucleotides corresponding to defined regions of 18S and 5S rRNA are added to dissociated wheat embryo ribosomal subunits.

MATERIALS AND METHODS

Preparation of ribosomal subunits. Wheat embryo ribosomes and ribosomal subunits were prepared essentially as described before

0006-291X/98 $25.00

TABLE 1

Oligonucleotides Used to Study the Inhibition of Association of 40S and 60S Ribosome Subunits

Oligonucleotide number	Sequence	Complementarity
	Eukaryotic 18S rRNA (Orientated 3′ → 5′)	
1	^{41}GTAACAAGGTTTCCGTAGGT^{22}	Residues 91-110 of plant 5S rRNA
2	^{32}TTTCCGTAGGT^{22}	
3	^{41}GTAACAAGG^{33}	
	Plant 5S rRNA (Orientated 5′ → 3′)	
4	^{91}CCTCCTGGGAAGTCCTCGTC^{110}	Residues 22-41 (from 3′ end) of eukaryotic 18S rRNA
5	^{91}CCTCCTGGGA^{100}	(5′ strand of hairpin)
6	^{101}AGTCCTCGTC^{110}	
	Control oligonucleotides	
7	d(CCCAAAGTACCCAGAG)	None
8	d(GGACGCCGGTCCGGTTGT)	None

(26). Briefly, a ribosomal pellet obtained from commercially available wheat germ was suspended in dissociation buffer consisting of 2 mM Tris-HCl (pH 7.5), 500 mM KCl, 4 mM magnesium acetate and 5 mM β-mercaptoethanol and left on ice for 1 h and then layered onto 10-30% linear sucrose gradients made up in dissociation buffer. Ribosomal subunits were separated by centrifugation (14,000 r.p.m., 15 h, 0°C) in a Beckman SW 28 Ti rotor.

Association of ribosomal subunits. The 50S and 60S ribosomal subunits were resuspended in association buffer consisting of 10 mM Tris-HCl (pH 7.5), 60 mM KCl, 10 mM $MgCl_2$ and 5 mM β-mercaptoethanol (27), and 1 A_{260} unit of each subunit was mixed and incubated at 37°C for 6 h. The samples were then chilled on ice for 5 min before loading onto 10-30% linear sucrose gradients containing the association buffer. The gradients were then centrifuged at 14,000 r.p.m. for 5 h at 0°C in a SW 28 Ti rotor. Gradients were fractionated and the absorbance of each fraction monitored at 260nm to determine the efficiency of 80S ribosome formation.

To determine the effects of oligonucleotides on subunit association, 1 μg of the complementary oligonucleotides were incubated with one A_{260} unit of the appropriate ribosomal subunit prior to mixing and subsequent incubation with the other subunit. Subunit association was monitored as described above.

Design and analysis of oligonucleotides. The oligonucleotides used in this study were designed on the basis of the 18S rRNA structure and the 18S-5S rRNA complex shown in Fig. 1. These oligonucleotides are shown in Table 1.

To determine their effects on ribosomal subunit association and protein synthesis, preliminary studies were carried out to see if the above oligonucleotides would bind to rRNAs and ribosomal subunits. As would be predicted from the oligonucleotide design shown in Fig 1 and Table 1, and from previous studies (6, 7), oligonucleotides 1-3 efficiently and specifically hybridized with 5S rRNA and bound to the large 60S ribosomal subunits, while oligonucleotides 4-6 efficiently and selectively hybridized with 18S rRNA and bound to the small 40S ribosomal subunit (P. Failla, unpublished data). Oligonucleotides 7 and 8 did not hybridize to either rRNA or ribosomal subunits. Oligonucleotide saturation assays showed that saturation was reached at oligonucleotide to ribosomal subunit ratios of between 10 to 15, confirming specificity of bindings (P. Failla, unpublished data). Specificity of binding between defined regions of 5S and 18S rRNA is also suggested from the isolation of a 5S rRNA fragment (residues 90-114), that survives RNase T1 digestion of an *in vitro* complex between 5S and 18S rRNA (5), and corresponds essentially to oligonucleotide 4 used in the present study.

Oligonucleotide synthesis and purification. Oligonucleotides were chemically synthesised using phosphoramidite chemistry on an ABI 381 DNA Synthesizer. They were purified by electrophoresis on 20% polyacrylamide gels, visualising the bands by UV shadowing, cutting out the appropriate bands and eluting them in water overnight at 4°C. The eluted oligonucleotides were then concentrated on a Speedivac concentrator (Dynavac), resuspended in association buffer (1 mg/ml) and stored at −20°C.

Translation assays. Oligonucleotides (2 μg) were preincubated at 37° with 2 A_{260} units of the appropriate ribosomal subunit in 200 μl of association buffer, and 15 μl of this mixture was then added to an *in vitro* translation mix (Amersham), consisting of 10 mM potassium acetate, pH 7.2, 10 μg TMV mRNA, 60 pM amino acids (minus L-leucine) and 1.3 μCi L-[4,5^{3}H] leucine and 5 μl of amino acid depleted and nuclease treated wheat germ extract. The *in vitro* translation mixtures were made up to 40 μl with water, and then incubated at 25°C for 1 h. A 1 ml aliquot of a 1 M sodium hydroxide/5% hydrogen peroxide mixture was then added to the reaction mixtures and the tubes were incubated at 37°C for 10 min and then chilled on ice. To each tube was added 2ml of ice cold 25% trichloroacetic acid (TCA) containing 2% casein hydrolysate. The controls were mixed and left on ice for 1 h. The TCA precipitates were recovered by filtering the mixtures through 0.45 μm HA filter discs (Millipore). The discs were washed twice with 3 ml ice cold 8% TCA and allowed to air dry. The acid precipitable incorporation of tritiated leucine was measured in a Hewlett-Packard Scintillation Counter.

RESULTS

Effects of oligonucleotides on association of ribosomal subunits. The effects of oligonucleotides, corresponding to defined regions of 18S or 5S rRNA (see Fig. 1), on ribosome subunit association are shown in Fig. 2. In the absence of any oligonucleotides the large (60S) and small (40S) ribosomal subunits, when mixed together in equal amounts in association buffer, formed 80S ribosomes (Fig. 2a). Using commercial wheat germ

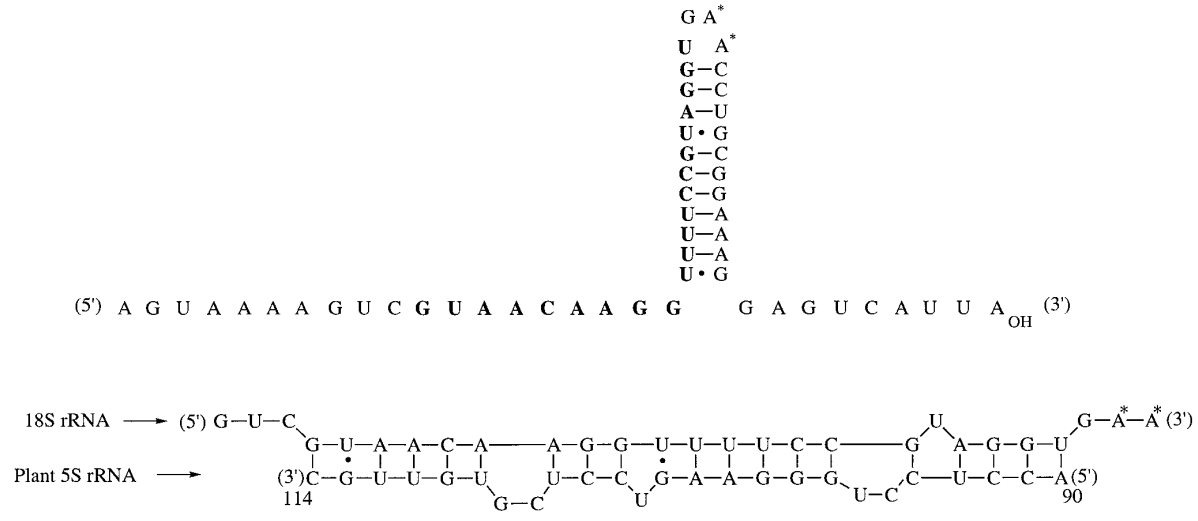

FIG. 1. (Top) Eukaryotic 18S rRNA. This region of 18S rRnA has been shown to be totally conserved in wheat embryo, hen reticulocyte, mouse sarcoma, rat liver, rabbit reticulocyte, barley embryo, yeast, and *Xenopus laevis* (5-7, 36-40). ΔG of m_2^6A m_2^6A hairpin = −7 Kcal. Bold/lined letters signify the sequence complementary to 5S rRNA. (Bottom) Complex between 18S and 5S rRNA. ΔG = −22.2 Kcal.

and the described association buffer, we could never get all the 40S and 60S subunits to combine and a nearly constant residual amount of uncomplexed 40S and 60S subunits could be seen in every experiment where the subunits were combined in the absence of oligonucleotides. Preincubation of the subunits with

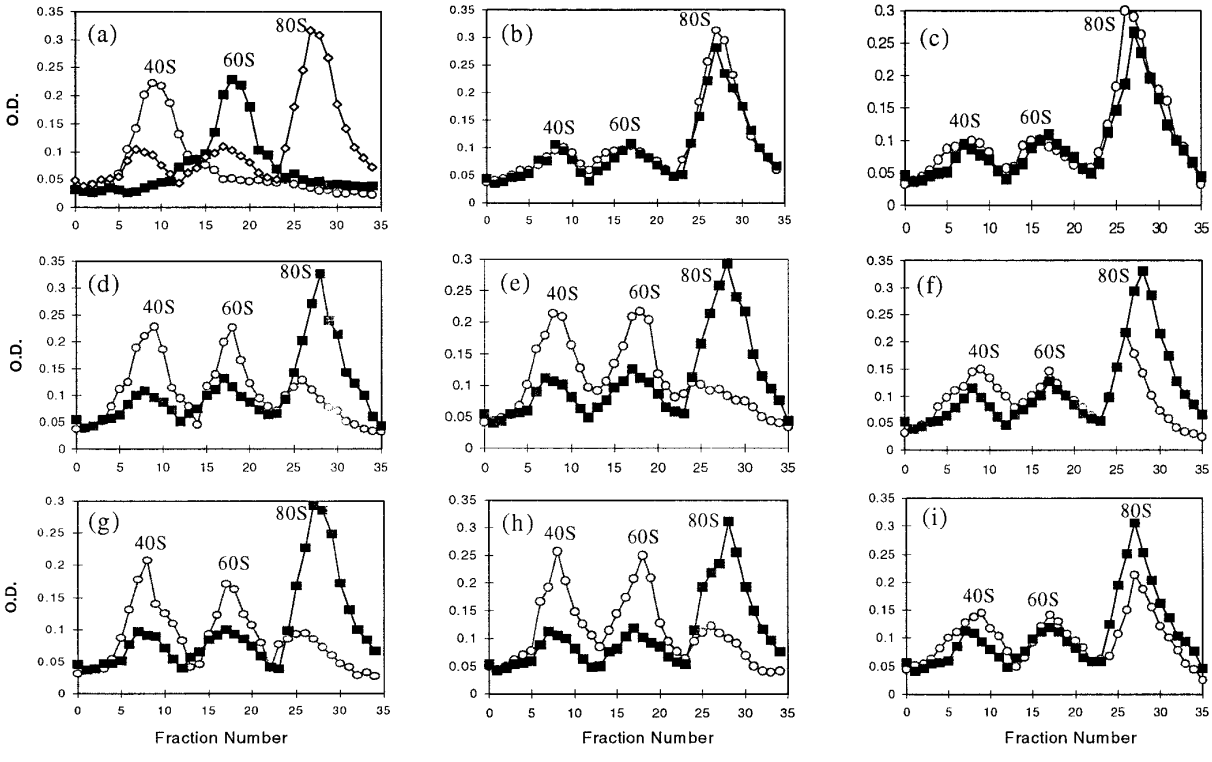

FIG. 2. Effects of oligonucleotides on association of ribosomal subunits analysed by sucrose density gradient centrifugation. Figure 2a shows the relative positions of the ribosomal subunits and the ribosome when 40S ribosomal subunits (○) or 60S subunits (■) were centrifuged individually or after mixing in equal amounts to form 80S ribosomes (◆). Figures 2b–2i show the results obtained in the presence (○) or absence (■) of control oligonucleotides 7 (2b) and 8 (2c); 18S rRNA oligonucleotides 1 (2d), 2 (2e) and 3 (2f); and 5S rRNA oligonucleotides 4 (2g), 5 (2h), and 6 (2i).

the control oligonucleotides 7 (Fig. 2b) or 8 (Fig. 2c) had no effect on the formation of 80S ribosomes, when compared with subunits which had not been preincubated with any oligonucleotides.

Preincubation of the 60S subunits with oligonucleotides 1 and 2 had significant inhibitory effects on their ability to combine with the partner subunit to form 80S ribosomes. Oligonucleotide 1, that includes the 5′ strand of the m_2^6A m_2^6A hairpin structure near the 3′ end of 18S rRNA and a contiguous sequence 5′ to it (see Fig. 1), significantly inhibited formation of 80S ribosomes when compared to 80S ribosome formation in the absence of oligonucleotide 1 (Fig. 2d). Oligonucleotide 2, which corresponds to the 5′ strand of the hairpin alone, had a similar effect to oligonucleotide 1, but had an even greater inhibitory effect on the formation of 80S ribosomes (Fig. 2e). Oligonucleotide 3, which corresponds to the region contiguous to the hairpin, is significantly less effective in preventing the formation of 80S ribosomes (Fig. 2f). These results show that the binding of sequence homologous to the 5′ strand of the m_2^6A m_2^6A hairpin structure near the 3′ end of 18S rRNA to the 60S ribosomal subunit is sufficient to inhibit the association of ribosomal subunits to form 80S ribosomes.

Preincubation of oligonucleotides corresponding to a defined region of 5S rRNA to 40S ribosomal subunit also adversely affected the ability of 40S subunits to combine with 60S subunits. Oligonucleotide 4, which corresponds to residues 91-110 of 5S rRNA, when preincubated with 40S ribosomal subunits, significantly inhibited the formation of 80S ribosomes (Fig. 2g). Oligonucleotide 5, which corresponds to residues 91-100 of 5S rRNA, appeared to be even more effective than oligonucleotide 4 in preventing ribosomal subunit association, as measured by the presence of uncomplexed ribosomal subunits (Fig. 2h). Oligonucleotide 6, which corresponds to residues 101-110 of 5S rRNA, had very little inhibitory effect on the formation of 80S ribosomes (Fig. 2i). These results show that oligonucleotide 5, which is complementary to the 5′ strand of the m_2^6A m_2^6A hairpin structure, is sufficient to inhibit the association of ribosomal subunits.

The above results show that the 5′ strand of the m_2^6A m_2^6A hairpin of 18S rRNA and a complementary sequence of 5S rRNA are involved in the association of ribosomal subunits in the *in vitro* association assay employed in this study. The interaction between these regions seems to be quite specific since the control oligonucleotides 7 and 8 had no effect in preventing subunit association.

Effect of oligonucleotides on in vitro protein synthesis. This experiment was carried out to determine if oligonucleotides, that inhibited ribosomal subunit association, also inhibited translation *in vitro*. The strategy adopted was to bind a complementary oligonucleotide to the 40S or 60S subunit, and then add this subunit to the translation mix containing wheat embryo extract. It was expected that the ribosomal subunit to which the complementary oligonucleotide had been bound would compete with the homologous subunit in the wheat embryo extract in binding to the partner subunit thereby inhibiting or reducing the formation of 80S ribosomes and polyribosomes. Unbound oligonucleotides carried over to the translation mix could also contribute to inhibition of subunit association.

TABLE 2

Effect of Binding of Complementary Oligonucleotides to Ribosomal Subunits on *in Vitro* Protein Synthesis

Oligonucleotide number (see Table 1)	Complementarity	Acid-precipitable incorporation (%)
None	None	100
1	5S rRNA	28
2	5S rRNA	39
4	18S rRNA	16
5	18S rRNA	20
7	None	92
8	None	94

The *in vitro* translation results are shown in Table 2. The acid precipitable radioactivity in the control sample, in which water was added instead of any nucleotide, was taken as 100% incorporation of [^{3}H]-leucine into newly synthesized protein translated from TMV mRNA. The binding of oligonucleotides 1 and 2 to 60S ribosomal subunits, and oligonucleotides 4 and 5 to 40S subunits, reduced the acid precipitable counts by 60 to 84%. In contrast, oligonucleotides 7 and 8, which do not bind to either subunit or to the 18S and 5S rRNAs, caused a much smaller reduction of acid precipitable counts. These results suggest that the reduction in *de novo* protein synthesis could be due to inhibition of base-pairing between defined regions of 18S and 5S rRNA on the surface of the 40S and 60S ribosomal subunits, respectively.

The most pronounced decreases in protein synthesis were seen with oligonucleotides 2 and 5, which are shortened versions of oligonucleotides 1 and 4, respectively. Oligonucleotide 1 corresponds to the 5′ strand of the m_2^6A m_2^6A hairpin near the 3′ end of 18S rRNA, while oligonucleotide 5 corresponds to residues 91-100 of 5S rRNA. These two sequences are complementary to each other. These studies provide strong support for the hypothesis (1, 6) that base-paired interaction between specific regions of 18S rRNA and 5S rRNA could be important for ribosome subunit association and protein synthesis.

DISCUSSION

Using synthetic oligodeoxyribonucleotides and wheat embryo ribosomal subunits we have shown an oligode-

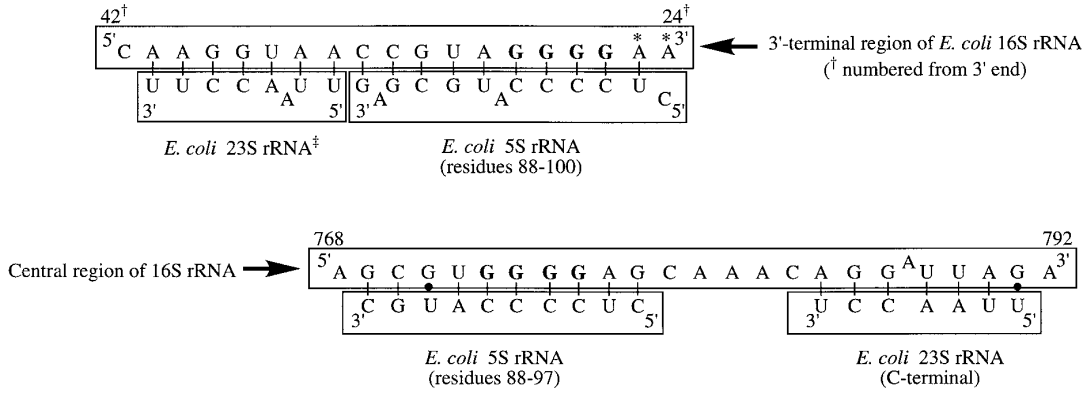

FIG. 3. Complementarity of the 3′-terminal and central regions of *E. coli* 16S rRNA with same regions of 5S rRNA and 23S rRNA. ‡The base-pairing between the 3′-terminal region of 16S and 23 rRNAs is based on Van Duin *et al.* (35) who suggested that such interaction between the RNAs of each subunit could provide a convenient way of forming a 70S couple.

oxyribonucleotide with the sequence d(TTTCCGTAGGT), which is homologous to the 5′ strand of the m_2^6A m_2^6A hairpin structure near the 3′ end of 18S rRNA, or an oligodeoxyribonucleotide with the sequence d(CCTCCTGGGA), which corresponds to residues 91 to 100 of 5S rRNA, are individually capable of inhibiting ribosomal subunit association and *in vitro* protein synthesis. The results reported here, therefore, support the original hypothesis that base-paired interaction between 5S rRNA in the 60S ribosomal subunit and 18S rRNA in the 40S ribosomal subunit could be involved in the reversible association of ribosomal subunits during protein synthesis. These studies also identify the precise regions of 18S and 5S rRNA that are involved in the interaction. While these studies have been carried out with wheat embryo ribosomes, and need to be confirmed in other systems, it is highly likely that such interactions are universal as the 18S rRNA sequence identified in this study is totally conserved in eukaryotes, while the 5S rRNA sequence involved in the interaction is totally conserved in plants and highly conserved in other eukaryotes (6, 7). Sequences at identical positions in the prokaryotic 16S and 5S rRNAs are also highly conserved (6). The *in vivo* relevance of these findings is supported by the fact that the 3′ end of both 16S and 18S rRNA are very important for ribosome function and are present at the ribosome interface (9-20), and 5S rRNA has been shown to be indispensible for ribosome function and protein synthesis (21-25).

If interaction between 5S and 18S rRNA were to occur then the dimethyl A hairpin stem needs to open up. It is worth noting that the 3′ strand of this hairpin shows homology to the 5′ non-coding region of a number of eukaryotic mRNA (7, 28-31). If this region of the mRNA bound to the 3′ strand of the dimethyl A hairpin during initiation of protein synthesis it would leave the 5′ strand single-stranded and free to interact with 5S rRNA, leading to subunit association. The region of the 5S rRNA complimentary to the 18S rRNA also needs to be single-stranded for the interaction to occur. On the basis of comparative sequence analysis it has been suggested (34) that prokaryotic 5S rRNA can exist in two thermodynamically stable forms, and 5S rRNA functions by a switch between these forms. In one of these conformations (B-form) the region of the 5S rRNA implicated in binding to 16S rRNA in this study is single-stranded. Similar mechanisms could also operate in the case of eukaryotic 5S rRNA.

The 5′ strand of the dimethyl A hairpin structure of *E. coli* 16S rRNA is complementary to 5S RNA (6), and a contiguous region to 23 rRNA (35). As pointed out before (6), these same regions of *E. coli* 5S rRNA (residues 88-97) show complementarity to residues 769-778 of *E. coli* 16S rRNA, and a contiguous region of 16S rRNA (residues 784-791) shows complementarity to 23 rRNA, as shown in Fig. 3. This region of 16S rRNA had previously been shown to be present at the ribosome interface (16, 17). Subsequent studies have shown that residues 787-795 of 16S rRNA and specific bases within this region are involved in ribosomal subunit association and initiation of protein synthesis (27, 32, 33). It is perhaps no coincidence that two regions of 16S rRNA in the 30S ribosomal subunit that are separated by over 700 bases but are present at the ribosome interface both contain sequences complementary to 5S rRNA and 23S rRNA, which are present in the 50S ribosomal subunit. This leaves open the possibility that the 50S subunit could bind to alternate regions of 16S rRNA on the surface of the 30S subunit and this could be important for a step in protein synthesis. This can only be ascertained by further experimentation and it would be interesting to see if a sequence complementary to 5S rRNA is present in another region of 18S rRNA and if a sequence complementary to 5S rRNA is present in another region of 18S rRNA and if such a mechanism also operates in eukaryotes.

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