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Conserved 5S rRNA complement to tRNA is not required for protein synthesis

(ribosome/ribosome reconstitution)

BERNADETTE PACE*, ELIZABETH A. MATTHEWS†, KIMBERLY D. JOHNSON*, CHARLES R. CANTOR†, AND NORMAN R. PACE*‡

*Department of Molecular and Cellular Biology, National Jewish Hospital and Research Center, and Department of Biochemistry, Biophysics and Genetics, University of Colorado Medical Center, 3800 East Colfax Avenue, Denver, Colorado 80206; and †Department of Chemistry, Columbia University, New York, New York 10027

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ABSTRACT **The notion that tRNA and 5S rRNA interact through evolutionarily conserved complementary sequences has been tested by nucleolytic modification of the 5S rRNA, using the modified rRNA to reconstitute the large ribosomal subunit, and assaying for poly(uridylic acid)-directed polyphenylalanine synthesis. The 5S rRNA sequence C-G-A-A (residues 43–46) and several residues surrounding it are not essential for protein synthesis.**

Considerable is known about the sequence of events involved in protein synthesis, and a detailed three-dimensional structure for the prokaryotic ribosome is beginning to emerge. We have few details regarding the molecular mechanics of ribosome function, but it seems certain that transitory base pairings between the involved nucleic acids will prove to be of importance. Transient interactions between tRNA and mRNA (1, 2), and between mRNA and 16S rRNA (3) have been demonstrated. Another potential RNA–RNA interaction that has attracted substantial attention is between 5S rRNA and tRNA. Forget and Weissman (4) pointed out that the 5S rRNA of *Escherichia coli* contains a sequence (C-G-A-A-C; see Fig. 1) complementary to a sequence (G-T-Ψ-C-G) in the common arm of most tRNAs. Subsequent oligonucleotide binding studies with tRNA and 5S rRNA (6–8), and the observations that all prokaryotic 5S rRNAs whose sequences have been determined contained the common arm complement (5), lent credence to the notion that the molecules might interact during protein synthesis. It has been suggested that the 5S RNA–tRNA association might stabilize the tRNA–ribosome complex, or perhaps be involved in the manipulation of tRNA on the ribosome. There is, however, no direct evidence that the sequences, or indeed the RNA molecules in question, interact with each other during protein synthesis.

In this communication we report a direct test of whether the proposed binding site for tRNA in the 5S rRNA sequence is required for protein synthesis. 5S rRNAs from which various portions had been enzymatically excised were used to reconstitute 50S ribosomal subunits, which were assayed for their ability to conduct poly(U)-dependent poly(Phe) synthesis. The results show clearly that the phylogenetically conserved 5S rRNA sequence C-G-A-A-C is not essential for the mechanics of protein synthesis.

EXPERIMENTAL PROCEDURES

RNA Preparations. For the preparation of 5S rRNA, 50 g of *E. coli* MRE600 frozen cell paste was suspended in 50 ml of 50 mM Tris·HCl (pH 7.3)/5 mM $MgCl_2$/0.5 M NH_4Cl/20 μg of DNase per ml and passed through a French pressure cell at 20,000 pounds/inch^2 (138 MPa). After dilution with the same buffer and clarification at 48,000 × *g* for 30 min, ribosomes were pelleted at 100,000 × *g* for 3 hr and suspended in 10 mM Tris·HCl (pH 7.3)/1 mM EDTA/0.5% sodium dodecyl sulfate, and RNA was purified by three phenol extractions and two precipitations with ethanol. Dried RNA precipitates were dissolved in 10 mM Tris·HCl (pH 7.5)/1 mM $MgCl_2$ at 5 mg/ml, adjusted to 2 M NaCl, held at 2°C overnight, and, after sedimentation of the high molecular weight rRNA, the salt-soluble fraction (mostly 5S rRNA) was recovered by precipitation from ethanol. In the preparation of tRNA, 50 g of cells was suspended in 150 ml of STE buffer [50 mM Tris·HCl (pH 7.3)/0.15 M NaCl/1 mM EDTA] and directly extracted with phenol, and the RNA was precipitated with ethanol. The final purifications of tRNA and 5S rRNA were by chromatography on a 2.5 × 90 cm Sephadex G-150 column in STE buffer. High molecular weight rRNA was recovered from phenol-extracted total rRNA as the excluded material from Sephadex G-150 chromatography in 10 mM Tris·HCl (pH 7.3)/0.1 M NaCl/0.1 mM $MgCl_2$.

Preparation of 5S rRNA Fragments. Before partial nuclease digestions, the 5S rRNA in TM2 buffer [10 mM Tris·HCl (pH 7.3)/10 mM $MgCl_2$] was heated to 60°C for 10 min and slowly (ca. 2 hr) cooled to 45°C. RNA concentration in digestion reactions was 5 mg/ml and the RNase concentrations were RNase T1 at 1–20 units/ml, RNase T2 at 2 units/ml, and RNase U2 at 12.5 units/ml. RNases T1 and T2 digestions were carried out in 55 mM Tris·HCl (pH 7.3)/25 mM $MgCl_2$/0.2 M NaCl, and RNase U2 digestions were in 10 mM morpholinoethanesulfonate (pH 5.0)/10 mM $MgCl_2$. After 15 min at 10°C, the digests were frozen, lyophilized thoroughly, dissolved in 10 M urea, and layered onto a 12.5 × 0.3 × 20 cm long 8% acrylamide/0.4% bisacrylamide gel in 8 M urea/50 mM Tris–borate (pH 8.3)/1 mM EDTA. Electrophoresis was in the same Tris/borate/EDTA buffer at 2°C at 200 V for 6 hr. RNA bands were detected by UV shadowing (9), excised, and eluted overnight with 0.5 M NaOAc/1 mM EDTA/50 mM Tris·HCl (pH 7.3). The filtered eluate was precipitated twice with ethanol.

Determination of Primary Structure of 5S rRNA Fragments. Two-dimensional chromatograms of complete T1 digests of uniformly ^{32}P-labeled 5S rRNA fragments and molar ratios of the resulting oligonucleotides were as described (10). The primary structures of the 5S rRNA fragments were examined by comparative sequencing gels (11) from limited RNase T1 and alkali hydrolysates of 3′- or 5′-^{32}P-labeled molecules. The homogeneity of terminal nucleotides was examined by paper elec-

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‡To whom reprint requests should be addressed.

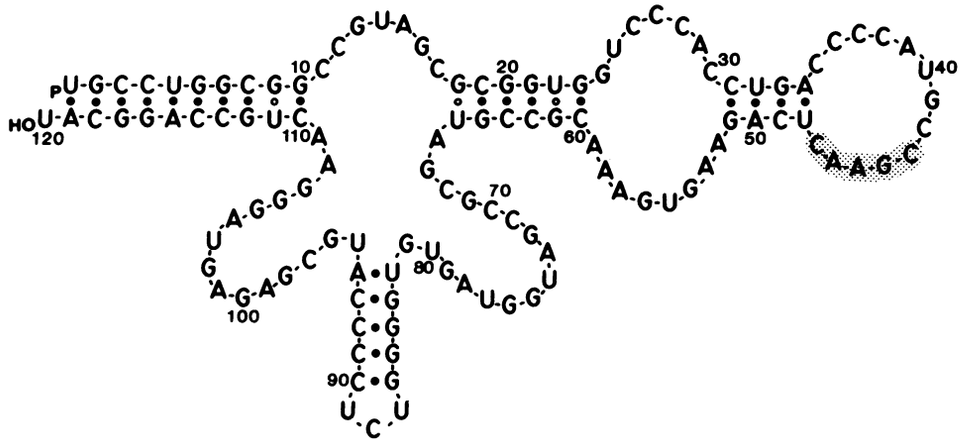

FIG. 1. Structure of *E. coli* 5S rRNA, as suggested by Fox and Woese (5). ∘ indicates non-Watson–Crick hydrogen bonding. The shaded sequence is putatively involved in tRNA binding to the ribosome.

trophoresis in pH 3.5 pyridine/acetate of complete base hydrolysates and venom phosphohydrolase digestions of the 3′- and 5′-^{32}P-labeled fragments, respectively.

Reconstitution of 50S Particles. 50S ribosomes were reconstituted from chromatographically purified high molecular weight rRNA (above), total proteins from purified 50S subunits (12), and intact or reconstructed 5S rRNA by using the two-step procedure of Amils *et al.* (12), except that the Mg^{2+} concentration in the first step was 6 mM. The 5′ and 3′ 5S rRNA fragments were annealed in 10 mM Tris·HCl (pH 7.3)/1 mM $MgCl_2$ by heating to 70°C and slow cooling. The activities of the reconstituted particles were determined by assaying aliquots of the reconstitution mixtures for poly(U)-directed poly(Phe) synthesis (13), using tRNA that had been purified from 5S rRNA by Sephadex chromatography as above.

RESULTS

Identification of Fragments and Construction of Modified 5S rRNAs. The basic strategy of these experiments was to generate fragments of 5S rRNA by limited nuclease digestions and then to reassemble, by annealing, 5S rRNAs lacking certain regions, for reconstitution into ribosomes. These were tested for the ability to synthesize protein. To generate the fragments, purified *E. coli* 5S rRNA was digested under carefully controlled conditions with RNase T1, T2, or U2. Because of the high degree of secondary structure of 5S rRNA and the specificity of the nucleases, discrete cuts are made in the RNA when digestions are carried out at low temperature and enzyme concentrations, and care is taken to terminate the digestions. The resultant fragments were isolated from denaturing polyacrylamide gels, all as detailed in *Experimental Procedures*. Fig. 2 shows a UV shadow of one such preparative gel.

We were extremely careful regarding the proofs of structure of the fragments. Their nucleotide sequences were evaluated in several ways. Some of the fragments characterized in pilot experiments with uniformly ^{32}P-labeled 5S rRNA were examined by two-dimensional analysis of complete RNase T1 digests (not shown). Many of the fragments were not isolated uniformly labeled, so their structures were examined by comparative sequencing gels on RNase T1 and alkali hydrolysates of appropriately 3′- or 5′-end-labeled molecules. The 3′ termini were labeled by the RNA ligase-catalyzed addition of cytidine [5′-^{32}P]bisphosphate and the 5′ termini by polynucleotide kinase-mediated transfer from [γ-^{32}P]ATP. Fig. 3 is a sequencing gel of some of the important 5′-labeled fragments. Length differences in the fragments are readily discerned in the figure; lines drawn on the autoradiogram align the equivalent residues in the various fragments.

Sequencing gels are convenient for the analysis of the major components of fragment isolates, but they do not provide good quantitative analysis of any terminal heterogeneity. The distributions of the 3′-terminal nucleotides were determined in a nearest-neighbor analysis by hydrolyzing fragments containing cytidine [5′-^{32}P]bisphosphate at their 3′ termini with alkali and resolving the resultant nucleoside 3′(2′)-monophosphates by paper electrophoresis at pH 3.5. 5′-^{32}P-Labeled fragments were digested completely with venom phosphohydrolase and the 5′-mononucleotides were resolved similarly. The results of all the two-dimensional analyses, sequencing gels, and terminal nucleotide analyses are summarized in Table 1. Terminal heterogeneity in many of the fragments is evident. Most notably, those derived from RNase U2 and T2 digestions often lack U-120. The absence of this residue probably has no effect on protein synthesis, however, because 5S rRNA isolated from cells often has submolar quantities (less than 0.8) of the 3′-terminal U.

5S rRNAs lacking the putative binding site for tRNA were constructed by annealing the appropriate fragments, as detailed in *Experimental Procedures*. In order to avoid conformational stress in the gapped constructs, the remaining nick at G-41/C-42 or gap beyond G-41 was not sealed. All of the examined fragments annealed well with their expected complements and

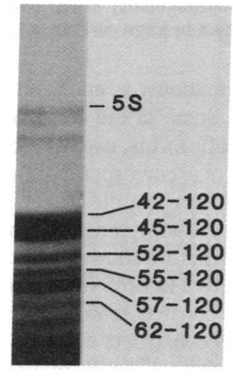

FIG. 2. Preparative resolution of RNase T1 fragments. *E. coli* 5S rRNA was digested in limited fashion with RNase T1 and resolved by denaturing gel electrophoresis. A photograph of a "UV shadow" (9) is shown. Inclusive residue numbers are given on the right.

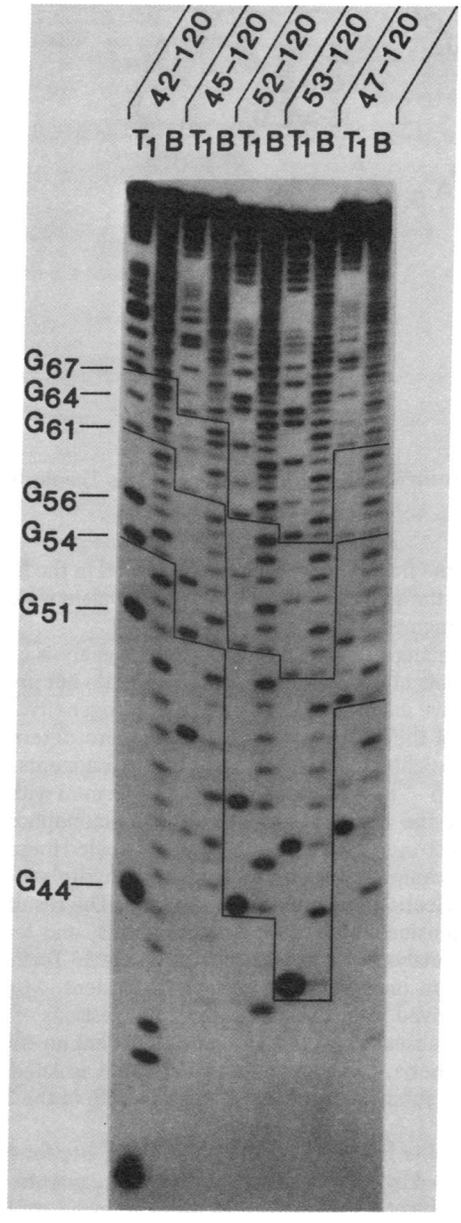

FIG. 3. Sequencing gel characterization of some 5S rRNA fragments. 5′-^{32}P-Labeled fragments, as identified by residue numbers in the figure, were partially digested with RNase T1 (lanes T_1) or alkali (lanes B) and resolved on a sequencing gel (11). The relative lengths and levels of terminal heterogeneity of the fragments are evident in the figure. Lines are drawn to align corresponding residues in the gel.

generally their conformations, as analyzed on appropriate gels (14), seemed correct. Some of the constructs displayed partly abnormal electrophoretic forms, usually more rapidly migrating than expected. These may correspond to the "denatured" form, which intact 5S rRNA can assume under some refolding conditions (15). Gel tracts showing annealed constructs with normal and partly abnormal forms are shown in Fig. 4. The "denatured" intact 5S rRNA is inactive in protein synthesis, so ribosome reconstitutions with the constructs were carried out with a 2-fold molar excess of the 5S constructs over 23S rRNA, to assure an adequate pool of the "correct" conformation during particle formation. It was determined in titration experiments that the constructs saturated the reconstitution mixtures at the same input as native 5S rRNA; minor species are not responsible for any observed activities.

Table 1. Terminal nucleotides of 5S rRNA fragments

		5′ terminus		3′ terminus	
No.	Fragment	% major	% minor	% major	% minor
1	42–120	100 C-42	—	88 U-120	12 A-119
2	45–120	94 A-45	6 C-42	86 U-120	14 A-119
3	52–120	100 A-52	—	88 U-120	12 A-119
4	55–120	100 U-55	—	85 U-120	15 A-119
5	57–120	89 A-57	7 U-55	88 U-120	12 A-119
6	62–120	74 C-62	22 A-57	85 U-120	15 A-119
7	65–120	46 U-65	34 C-62	87 U-120	13 A-119
8	53–120	100 A-53	—	49 U-120	43 A-119
9	47–120	80 C-47	20 A-46	49 U-120	40 A-119
10	1–41	100 U-1	—	100 G-41	—
11	1–52	86 U-1	6 C-3	100 A-52	—

Terminal heterogeneity in the fragments was evaluated as described in the text. Fragments 1–7 and 10 were derived from RNase T1 digests, 8 and 12 were from RNase T2 digests, and 9 was from a RNase U2 digest.

Tests for Protein Synthesis. 50S ribosomes were reconstituted (*Experimental Procedures*) from total isolated 50S proteins, the 5S constructs, and a mixture of 16S and 23S rRNA obtained by passing bulk rRNA through a Sephadex G-150 column to remove 5S rRNA. Particles reconstituted with more rigorously purified 23S rRNA had comparable activities, but the mixture of 16S and 23S rRNA is more conveniently obtained in the quantities needed and is generally more intact. The reconstitution mixtures were assayed directly for poly(U)-directed poly(Phe) synthesis, without prior isolation of the particles. Protein synthesis was carried out in the "polymix" system of Jelenc and Kurland (13), adding 30S particles (2:1 molar excess of 30S rRNA to input A_{260} unit of 23S rRNA), poly(U), and tRNA that had been freed of 5S rRNA by Sephadex G-150 chromatography. Although we found that intact 5S rRNA included in reactions does not exchange with particle-bound RNA, we wished to avoid any ambiguity in the experiments.

The protein-synthesizing activities, in single-time-point assays, of reconstituted particles containing the 5S rRNA con-

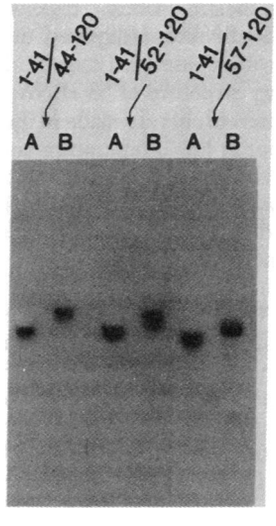

FIG. 4. Nondenaturing gel analysis of annealed fragments. The indicated 5′-^{32}P-labeled fragments were electrophoresed alone (lanes A) or after annealing with an equimolar amount of the indicated nonradioactive fragment (lanes B).

Table 2. Activities of ribosomes containing 5S rRNA constructs

No.	Reconstituted 5S rRNA (residues)*	Sequence deleted	[^{14}C]Phe incorporated,† cpm	% activity
1	Intact 5S	—	15,806	—
2	1–41/42–120	—	12,199	[100]
3	1–40/42–120	G-41	11,436	94
4	1–41/45–120	C-C-G-44	10,166	83
5	1–41/47–120	C-C-G-A-A-46	9,027	74
6	1–41/53–120	C-42–A-52	9,784	80
7	1–41/55–120	C-42–G-54	1,954	16
8	1–41/57–120	C-42–G-56	2,997	25
9	1–41/62–120	C-42–G-61	3,145	26
10	1–52/42–120	C-42–A-52 extra	10,004	82
11	1–52/53–120	—	5,475	45
12	1–52/57–120	A-53–G-56	2,856	23
13	1–52/62–120	A-53–G-61	2,579	21
14	1–41	C-42–U-120	1,440	12
15	42–120	U-1–G-41	3,217	26
16	No 5S	Entire	1,584	13
17	30S alone	—	1,093	9

Reaction mixtures (100 μl) contained, in addition to the other components (12, 13), 50S particles reconstituted from 0.5 A_{260} unit of 23S rRNA and 0.04 A_{260} unit of 5S rRNA constructs (2:1 molar ratio of 5S to 23S rRNA), 0.5 A_{260} unit of purified (12) 30S subunits, 250 μg of 5S rRNA-free tRNA, 10 μg of poly(U), and 5 × 10^4 cpm (500 pmol) of [^{14}C]phenylalanine.

* See Table 1 for any heterogeneity in terminal residues.

† Average of two independent, always closely consistent assays.

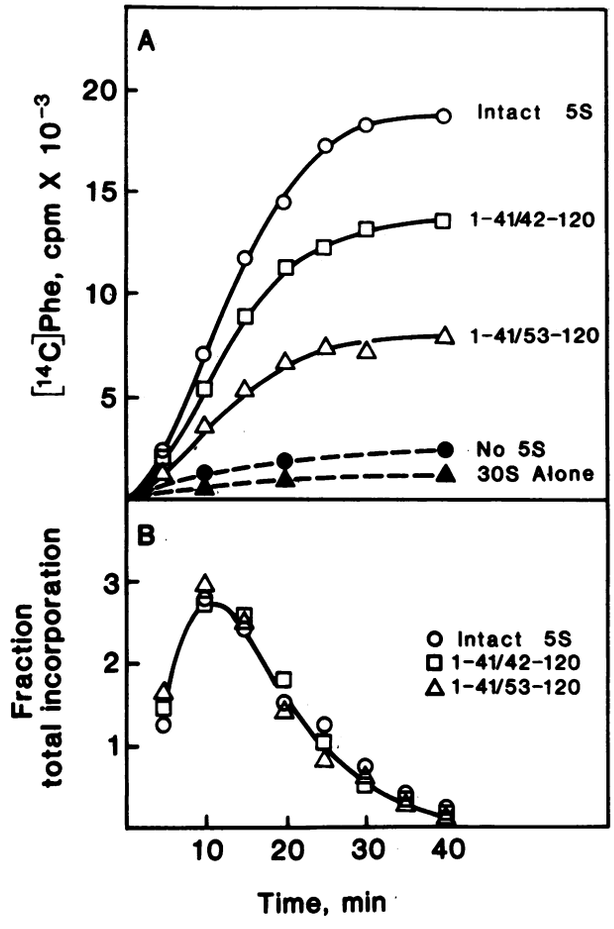

FIG. 5. Kinetics of [^{14}C]phenylalanine incorporation by some reconstituted particles. Reaction mixtures were as for Table 2, containing the indicated 5S rRNA constructs. Reactions were initiated by warming to 37°C. (*A*) Aliquots (100 μl) were withdrawn at the indicated times and examined for hot trichloroacetic acid-precipitable [^{14}C]phenylalanine. (*B*) The amounts of incorporation between the successive time points are plotted.

structs are shown in Table 2. It is evident that protein synthesis requires 5S rRNA and that any alteration of the molecule, even the simple nick at G-41/C-42 (construct 2, Table 2) reduces activity relative to particles reconstituted with the intact 5S rRNA. Successive deletions beyond G-41 do not result in much further diminution of activity until A-52, beyond which it plummets.

The kinetics of poly(Phe) synthesis with some of the important constructs are shown in Fig. 5*A*; Fig. 5*B* plots the rates of synthesis. No preferential retardation of initiation or premature termination is evident in the synthesis kinetics of the less active particles, and the reaction mixtures, in fact, were saturated with respect to 50S particles. The reduced activities of the functioning 5S construct-containing particles therefore probably reflect reduced rates of chain elongation, although there are other possibilities. The important point is that 5S rRNA deletions spanning the putative tRNA binding site, C-43–C-47, display good activity. Therefore this interaction, if it ever occurs, is not obligatory in peptide chain elongation. We have not examined the character of the product polypeptides, but the kinetics of synthesis do not suggest premature termination.

Constructs with deletions spanning C-42–A-52 (construct 6, Table 2) are active, but removal of the next few residues (constructs 7 and 8, Table 2) destroys function. We focused specifically on this locale with the construct 1–52/57–120 (construct 12, Table 2), which also proved to be poorly active, although even a nick in this region (construct 11, Table 2) is deleterious to activity. This suggests some critical role for the A-53–G-56 region, either structural or by interaction with another element of the translation apparatus. The inactivity of particles containing 5S constructs must be interpreted cautiously, however, because we cannot be certain that the "native" conformation is assumed in the annealing process. With that caveat, these experiments offer the most decisive demonstration that 5S rRNA is actively involved in protein synthesis other than by serving to bind the ribosomal proteins L18 and L25, which would be absent in particles lacking 5S rRNA (16). Their binding sites in the 1–52/57–120 construct are present (16), although we have not yet analyzed their content in the reconstituted particles. Interestingly, ribosomes containing only the 3′-terminal 80 nucleotides (construct 15, Table 2) display low but reproducible activity. This portion of the molecule also contains important binding points for L18 and L25. In contrast, the 5′-terminal 41 residues do not impart any activity on the particles beyond that observed when 5S rRNA is omitted from the reconstitution mixture.

Finally, Table 2 shows a test of the effect of removing residue G-41 from the 5S rRNA. This is an interesting nucleotide because it is one of the most accessible in the 5S rRNA to chemical modification or tritium exchange, even while in the ribosome (17, 18). The exposure of G-41 might suggest that it is some sort of interacting point. Residue G-41 was removed by periodate oxidation and β elimination from the RNase T1 fragment containing residues 1–41. As analyzed by nearest-neighbor transfer from 3′-appended cytidine [5′-^{32}P]bisphosphate, removal of G-41 was more than 88% complete. The activity of ribosomes containing this fragment, in the pair 1–40/42–120 (construct 3, Table 2), was quite good, so G-41 evidently is not crucial to poly(U)-directed poly(Phe) synthesis.

DISCUSSION

The notion, originally from sequence comparisons, that tRNA and 5S rRNA interact through complementary, phylogenetically conserved sequences has received indirect support from several lines of evidence. Enzymatic and nonenzymatic aminoacyl-tRNA binding to the ribosome could be inhibited by the tRNA fragment T-Ψ-C-G, albeit at high concentrations (6–8). This fragment was found to bind to a 5S rRNA–protein complex, and the binding was inhibited by *N*-oxidation of two adenine residues in single strands (6). Additionally, it was reported (19) that codon–tRNA interactions in solution and in the presence of the ribosome enhance the equilibrium binding of the oligonucleotide C-G-A-A to tRNA, implying that a message-dependent conformational change in tRNA exposes the normally unavailable common arm to the environment. The most persuasive argument for the 5S rRNA–tRNA interaction, however, has been the cumulative finding that these complementary sequences are phylogenetically conserved in all elongation tRNAs and all eubacterial 5S rRNAs whose sequences have been determined to date.

The T-Ψ-C-G oligonucleotide binding data cannot be interpreted straightforwardly, and there now exist structural data of a more detailed and direct nature showing that the sequence C-G-A-A-C in 5S rRNA is not available for interaction in the free molecule or in the ribosome. Chemical modification by kethoxal shows that G-44 is generally inaccessible (17, 20). The A residues responsible for any T-Ψ-C-G binding to the 5S rRNA–protein complex (5) probably are not in the loop containing the conserved sequence, and tritium exchange experiments also are consistent with the inaccessibility of the 5S rRNA conserved sequence to the solvent (18, 21). Additionally, the conserved common arm sequence of tRNA is firmly engaged in tertiary interactions in the free molecule (22). The report (17) that tRNA rearranges upon binding a cognate codon, to render the common arm available to bind the complementary C-G-A-A oligonucleotide, seems to be incorrectly interpreted. Kethoxal modification of a codon–$tRNA^{Lys}$ complex showed that the primary allosteric effect of codon binding was disruption of a base triplet involving the extra loop and the D loop (23). There was no significant disengagement of the common arm. High-resolution NMR data also argue against a significant, mRNA-induced perturbation of tRNA structure (24). There are no data regarding conformational rearrangements of tRNA upon binding to the ribosome.

An interaction between 5S rRNA and tRNA cannot be ruled out by the experiments presented here. We have demonstrated unequivocally, however, that any interaction between the phylogenetically conserved C-G-A-A-C sequence in 5S rRNA and tRNA is not required for peptide bond formation and translocation. The question remains then, why are these complementary sequences conserved? It may be a coincidence that two highly conserved sequences in the small RNAs involved in protein synthesis are complementary. These sequences may have evolved independently for unrelated, albeit specific, structural reasons. It also remains possible that ribosome functions other than simple peptide chain elongation, as assayed here, require the putative 5S rRNA–tRNA contact. An example of such function might be the ribosome-dependent synthesis of ppGpp and pppGpp, as occurs during the stringent response. Additionally, we have not tested the fidelity with which ribosomes containing the modified 5S rRNAs translate the genetic code; it is conceivable that translational accuracy is enhanced by a transient tRNA–5S rRNA interaction. Finally, we do not yet know whether the modified ribosomes can properly initiate and terminate synthesis on a natural message, in contrast to the much simpler poly(U) template used here. Certainly, however, poly(U)-directed poly(Phe) synthesis reflects the essence of protein synthesis, and these results show that the phylogenetically conserved 5S rRNA sequence C-43–C-47, which is complementary to part of the tRNA common arm, is not essential for that process.

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