Reconstitution of Peptide Bond Formation with *Escherichia coli* 23S Ribosomal RNA Domains

Itaru Nitta, Yoshie Kamada, Hiroe Noda, Takuya Ueda, Kimitsuna Watanabe*

It was recently demonstrated that peptide bond formation can occur using an *Escherichia coli* naked 23S ribosomal RNA without any of the ribosomal proteins. Here, the six domains of the 23S ribosomal RNA were individually synthesized and shown to be capable, when complexed together, of stimulating the reaction. Omission and addition experiments indicated that the activity could be reconstituted solely by domain V at a concentration 10 times higher than that of the intact 23S ribosomal RNA, whereas domain VI could enhance the activity in trans. These findings suggest that fragments of an RNA molecule have the ability to associate into a functional whole.

The complete sequence of *E. coli* 23S ribosomal RNA (rRNA) was first determined in 1980; in the following year, a model of its likely secondary structure was proposed (1). Subsequent extensive studies on rRNA sequences of the ribosomal large subunit spanning a wide range of organisms revealed a common secondary structure comprising six domains (2). The distinctive features of the rRNA secondary structure led to the emergence of two interesting speculations concerning the origin of the ribosome in relation to “the RNA world,” in which RNA is hypothesized to have acquired its genetic information and the ability to replicate itself (3). One is that the prototype of ribosomes was composed solely of functional RNAs, several parts of which remain conserved within the present-day rRNA—and there is abundant biochemical and genetic evidence suggesting the direct involvement of conserved nucleotides in critical ribosomal functions (4). Following a series of substantive studies to define the minimal components necessary for the ribosome to carry out its functions (5–7), we were finally able to conclude that peptide bonds can indeed be formed solely by rRNA without the need for any of the ribosomal proteins (8). The second speculation advances a possible solution to the problem that rRNA molecules are too long to have been formed by chance in a few simple evolutionary events. Because conserved regions of large subunit rRNAs are flanked by variable regions (2), it can be inferred that RNA fragments, associating through base-pairing and tertiary interaction, are likely to have preceded the contemporary rRNA. To substantiate this idea experimentally, the proposed six domains of *E. coli* 23S rRNA were synthesized individually and then assayed for the reconstitution of peptide bond formation, one of the main ribosomal activities, by associating some of the domains.

N-acetylphenylalanylphenylalanine (AcPhe-Phe) formation from the peptidyl–tRNA (tRNA) analog N-acetylphenylalanyl-tRNA (AcPhe-tRNA) and phenylalanyl-tRNA (Phe-tRNA) allowed us to observe the intrinsic peptidyltransferase activity of *E. coli* 23S rRNA transcribed by T7 RNA polymerase in the complete absence of ribosomal proteins (8). To obtain a sufficient signal-to-noise ratio to detect AcPhe-Phe formation, the system required “self-folded” rRNA (9) and highly purified AcPhe- and Phe-tRNA (10). As shown (Fig. 1, A and B), peptide bond formation activity was successfully reconstituted using 23S rRNA transcribed in vitro without any ribosomal proteins (11). Active conformation of the 23S rRNA transcript was a prerequisite for the activity. Denaturing by heating at 95°C for 5 min followed by quenching on ice completely eliminated the activity. When all the 23S rRNA domains that were transcribed separately in vitro were incubated together in a self-folding buffer at 37°C for 20 min, peptide bond formation activity was still evident, even though it was reduced by half. To identify the one or more domains directly involved in peptide bond formation, bonds were formed using total domain complexes from which single domains were omitted in turn. Each complex was incubated at 37°C for 20 min in the self-folding buffer. Omission of domains I to IV did not significantly affect the activity of the complex, whereas a lack of domain V or VI, respectively, eliminated the activity or reduced it by half (Fig. 1B, right). This suggests that the catalytic center for peptide bond formation might be located in domain V or VI, or in both.

To clarify this, each domain was individually self-folded in the same manner as used for intact 23S rRNA. Only domain V had readily detectable—though significantly reduced (by ~10-fold)—peptide bond formation activity, especially at higher concentrations (Fig. 1C). At 10 μM, domain V enhanced AcPhe-Phe formation to the same level as that obtained by 1 μM of the active complex in which all the domains were assembled (Fig. 1, B and C). Figure 1C reveals that at a high concentration (10 μM), the other domains also showed slight but discernible levels of phenylalanine incorporation, ranging from 10 to 25% of the activity obtained with domain V. The most likely explanation for the low levels of activity with domains other than V is that unspecified interactions between these domains and tRNAs increased the effective local tRNA concentrations, thereby enhancing the rates of specific reactions. This speculation is supported by the reported possibility that tRNAs contact several domains, including V (12), as well as by our findings that neither mutations in domain V nor antibiotics (see below) completely eliminated peptidyl bond formation by domain V alone, the level of remaining activity being similar to the activities of the other domains (13).

The fact that peptide bond formation activity was eliminated altogether without domain V, whereas an absence of domain VI caused only a modest decrease in the activity (Fig. 1B, right), indicates that the catalytic center for peptide bond formation was in domain V, while domain VI enhanced the reaction. In experiments that were essentially the reverse of the omission experiments, we examined the *trans*-acting effect of each domain with domain V. The results were basically as expected (Fig. 1D). In this reaction, the final concentration of domain V was kept constant at 1 μM, and the concentrations of the other domains were varied from 0 to 6 μM. Each additional domain enhanced phenylalanine incorporation, but the activity with 2 μM of domain VI was four times that with 2 μM of domains I to IV (Fig. 1D). This suggests that domain V did not cooperate with the other domains except for VI and that domains I to IV had little involvement in peptide bond formation (14).

To verify that the AcPhe-Phe synthesis activity was attributable to an intrinsical catalytic reaction in the rRNA transcript, the effects on the activity of site-directed mutageneses at the two universally conserved regions close to the so-called “peptidyltransferase ring” of domain V (4) were investigated (Fig. 2). One of these regions is the eighth helical segment in domain V, formed by the
nucleotides from G2246 to C2258; the other is the 16th segment from U2506 to G2583. Both regions have been suggested to be involved in peptidyltransferase activity (15). In the first region, replacement of the wild-type G2252 of domain V by the other three bases similarly suppressed AcPhe-Phe formation to around 40% of the activity of the wild type. In a result apparently inconsistent with our findings, when a tRNA fragment was used, the U2252 mutation of 23S rRNA eliminated the ribosomal activity of peptide bond formation almost completely in the presence of ribosomal proteins (15). This discrepancy can be explained by the effect of the unspecific gathering of tRNAs as described earlier. Because, within the limits of experimental error, the amounts of AcPhe-Phe synthesized by these mutations were similar to those obtained with individual domains other than domain V (Fig. 1C), the remaining low activity levels might have been due to the relatively high concentrations of do-
mother than domain V (Fig. 1C), the remaining low activity levels might have been due to the relatively high concentrations of do-
mother than domain V (Fig. 1C), the remaining low activity levels might have been due to the relatively high concentrations of do-
mother than domain V (Fig. 1C), the remaining low activity levels might have been due to the relatively high concentrations of do-
mother than domain V (Fig. 1C), the remaining low activity levels might have been due to the relatively high concentrations of do-
mother than domain V (Fig. 1C), the remaining low activity levels might have been due to the relatively high concentrations of do-
mother than domain V (Fig. 1C), the remaining low activity levels might have been due to the relatively high concentrations of do-
mother than domain V (Fig. 1C), the remaining low activity levels might have been due to the relatively high concentrations of do-
mother than domain V (Fig. 1C), the remaining low activity levels might have been due to the relatively high concentrations of do-

To further confirm that the activity of domain V was actually due to peptidyltransferase, we examined the effects on AcPhe-Phe synthesis of sparsomycin and neomycin. Sparsomycin is a universal and powerful inhibitor of peptide bond formation. All organisms so far studied are sensitive toward this antibiotic, suggesting that the drug recognizes a universally conserved, functionally important site for peptide transfer (17). By contrast, neomycin belongs to the aminoglyco-
cide bond formation activity and that the variation in the amount of AcPhe-Phe depended on which nucleotide was replaced. This indicates that the activity of domain V was not due to the effect of unspecific interactions between domain V and tRNAs; domain V actually participated in peptide bond formation in an enzymatic manner, and the tRNAs did not recognize the whole of domain V but just several local regions, probably around the peptidyltransferase center.

In contrast, neomycin did not inhibit the reaction at all, whatever the concent-
rination was investigated (17). (C) The reaction was performed with each individual domain, and its concentration depend-

Fig. 2. Sensitivity of peptide bond formation activity toward mutations in domain V. Relative activities of AcPhe-Phe formation carried out with the wild type or two series of 10 μM domain V mutants, taking the wild-type activity as 100%. One series of mutations was G2252 to A, C, and U; the other was site-
directed mutagenesis at G2581 and its neighboring nucleotides in the secondary structure, such as C2507 (29).
essential catalytic core and domain VI as the trans-acting activator. Extensive mapping studies on RNA-RNA interaction within ribosomes led to the proposal of functional interaction between the conserved region spanning nucleotides 878 to 811 in domain II and the peptidyltransferase ring in domain V (22). In contrast to these findings, our study shows determinable activation of AcPhe-Phe formation when domain V was associated not with domain II, but with domain VI, suggesting that the 3′-terminal region of 23S rRNA plays a central role in our system. Although domain VI contains a single universally conserved element, the so-called ‘α-sarcom loop’, this loop is the target site of ribotoxins and is involved in translocation, not in peptide transfer (23). To our knowledge, no evidence directly implicating domain VI in peptide transfer has so far been reported. Further argument requires structural information on domain VI, with respect to tRNA and the interaction site of domain V.

Another instance of a domain-like relationship has been found in the *Tetrahymena* group, *S*.*galactosidase* (26) and *E*. coli *sarcin loop* (66). This finding is interesting because the 3′-terminal region of 23S rRNA is involved in translocation, not in peptide transfer has so far been reported. Further argument requires structural information on domain VI, with respect to tRNA and the interaction site of domain V.

**References and Notes**


9. Using polymerase chain reaction (PCR) amplification, all the ribosomal genes were obtained from *E* coli* MRE600* constructed in our previous study (8). *Escherichia coli* 23S rRNA and its proposed domains prepared by T7 RNA polymerase were dialyzed against a “self-folding” buffer: 50 mM Hepes-KOH (pH 7.5), 20 mM magnesium acetate, and 400 mM NH₄Cl. Before the assay for peptide bond formation, the transcripts in the self-folding buffer were heated at 65°C for 10 min and then denatured in 90% H₂O for the complete self-folding of 23S rRNA or of its individual domains, or at 37°C for 20 min to reconstitute the active-enzyme complex for several domains.

10. Decapsidated-tRNA was prepared by gel filtration from *E. coli* MR600 was isolated from the total tRNA by using a solid-phase–attached DNA probe, 5′-GGTT-GCCCGGACTCGGAATCG-Biotin-3′. The tRNA was purified by a 1100 fraction from *E. coli* followed by acetylation with acetic anhydride. The Ac³⁻Phe-tRNA and Ac³⁻Phe-tRNA obtained (specific activity: 9.2 × 10⁶ pmol/cpm) were purified by high-performance liquid chromatography (HPLC) using a C₄ column, and stored at −20°C in a tRNA stock buffer (20 mM potassium acetate, pH 5.5 and 5 mM MgCl₂).

11. Assay for peptide bond formation: AcPhe-Phe synthetase was carried out at 37°C for 60 min in 25 µl of a reaction mixture containing 1.0 to 10.0 µM 23S rRNA or its one or more domains, 1.0 µM Ac³⁻Phe-tRNA, 50.0 µM Ac³⁻Phe-tRNA, 50.0 µM Hepes-KOH (pH 8.2), 30 mM MgCl₂, and 160 mM NH₄Cl. Both the Ac³⁻Phe- and Phe-tRNAs were labeled with [³⁻¹⁴C]Phe. After the reaction of 1 hour, not only Phe but also 16S RNA did not reach the same level as that of the domain other than V (28). These findings give to the product is really AcPhe-Phe.


13. Additional experimental results indicated that the reaction was completely suppressed by denaturing at 95°C any of the 23S rRNA was degraded into Phe and a very small amount of Phe-tRNA, which disappeared quickly in the longer reaction periods. After 12 hours of reaction, AcPhe-Phe was completely converted to Phe. This means that AcPhe-Phe is degraded in Phe and activated by Phe-tRNA synthase. In addition, the spot corresponding to AcPhe-Phe was then examined what happened if only one of the two input tRNAs was used. When only AcPhe-tRNA was incubated with domain V, AcPhe was solely recovered by the above procedure. Further confirmation of the products, we carried out the following experiments. When peptide bond formation was performed with domain V using radiolabeled AcPhe-tRNA and unlabeled Phe-tRNA, we found only spots corresponding to AcPhe-Phe and AcPhe. In contrast, two spots for AcPhe-Phe and Phe were observed in the cases of unlabeled AcPhe-tRNA and labeled Phe-tRNA, which are the same results as already reported (8). This also serves to confirm the result is really AcPhe-Phe.
16. In our experiment, we used native tRNA instead of a tRNA fragment. According to footprinting data, intact tRNA has multiple contact sites with rRNA [M. Dabrowski, C. M. T. Spahn, K. H. Nierhaus, EMBO J. 14, 4872 (1995)], so local mutation of the 23S rRNA did not seem to be critical with regard to tRNA-rRNA interaction, and high domain V mutant concentrations resulted in an increment of local concentrations of tRNAs due to unspecific tRNA-rRNA interactions. In support of this notion, the AcPhe-Phe synthesis reaction was completely eliminated by digesting the intact tRNA has multiple contact sites with rRNA [M. Dabrowski, C. M. T. Spahn, K. H. Nierhaus, EMBO J. 14, 4872 (1995)], so local mutation of the 23S rRNA did not seem to be critical with regard to tRNA-rRNA interaction, and high domain V mutant concentrations resulted in an increment of local concentrations of tRNAs due to unspecific tRNA-rRNA interactions. In support of this notion, the AcPhe-Phe synthesis reaction was completely eliminated by digesting the intact rRNA, 3 mM, was much higher than the concentration of puromycin for use as an aminoacyl-tRNA analog in peptide transfer; however, even higher concentrations of the antibiotic had no effect in the previous system with naked 23S rRNA.

20. Although E. coli 55 rRNA is essential for reconstituting the active SOS subunit, 55 rRNA had no effect on peptide bond formation with the naked 23S rRNA or its domains (28). The most likely explanation is that 55 participates not in peptide transfer but in association of the subunit or translocation, or both, probably cooperating with ribosomal proteins. By analogy with known interactions of 5S and 18S rRNA in eukaryotes, it has been proposed that 5S rRNA is involved in an interaction with 16S rRNA in the ribosome function [A. A. Azad, Nucleic Acids Res. 7, 1913 (1979)].

21. It has been recently reported that modifications of E. coli 23S rRNA are essential for peptide bond formation between N-acetylmehtionyl-3'ACCAAC(5') and puromycin [R. Green and H. F. Noller, RNA 2, 1011 (1996)], by contrast, unmodified 23S rRNA can work in the system with AcPhe-tRNA and Phe-tRNA, but our unpublished data revealed that the system was abolished by replacement of Phe-tRNA with puromycin. These findings imply that the role of modified nucleotides is not peptide transfer itself but interaction with the antibiotic. W. Stiege, C. Glotz, R. Brimacombe, Nucleic Acids Res. 11, 1687 (1983); D. Moazed and H. F. Noller, Biochimie 69, 879 (1987).


28. I. Nitta and K. Watanabe, data not shown.

29. Using an in vitro mutagenesis kit (Bio-Rad), which follows the conventional Kunkel’s method, plasmids carrying 23S rRNA genes mutated in the first region were prepared from pEC235N. On the other hand, using PCR, 23S rRNA genes in the second region possessing mutations were amplified from plasmids kindly given by C. M. T. Spahn, and were embedded into pUC119. All the mutant genes were confirmed by dideoxy sequencing. Domain V genes mutated in both regions were obtained from these plasmids by PCR amplification, with the forward primer carrying the class III T7 promoter fused with the 5’ end of domain V, and were in vitro transcribed with T7 RNA polymerase.

30. Sparsomycin was a gift from K. Igarashi. Neomycin sulfate, a mixture of 85% neomycin B and 15% neomycin C, was obtained from Sigma. Stock solutions of 100 mM antibiotics were prepared so as to have a pH of 7.5 by adjustment with KOH just before use. Before the reaction, the domain V transcript was treated with the antibiotic at 0°C for 15 min. The slight activities observed with 10 μM of domains other than V were unaffected by either sparsomycin or neomycin (28).

31. We thank K. H. Nierhaus and C. M. T. Spahn for providing plasmids containing mutant 23S rRNA genes and critical reading of the manuscript, K. Igarashi for sparsomycin, and H. F. Noller and W. H. McClain for advice on recovering the products from TLC plates and their analysis. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture (Japan) and by the Japan Society for the Promotion of Science under the “Research for the Future” program.

17 April 1998; accepted 2 July 1998