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

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It was recently demonstrated that peptide bond formation can occur using an *Escherichia coli* naked 23*S* ribosomal RNA without any of the ribosomal proteins. Here, the six domains of the 23*S* 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 23*S* 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* 23*S* 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* 23*S* 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–transfer RNA (tRNA) analog *N*-acetylphenylalanyl-tRNA (AcPhe-tRNA) and phenylalanyl-tRNA (Phe-tRNA) allowed us to observe the intrinsic peptidyltransferase activity of *E. coli* 23*S* 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 23*S* rRNA transcribed in vitro without any ribosomal proteins (*11*). Active conformation of the 23*S* 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 23*S* 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 23*S* 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 unspecific interactions between these domains and tRNAs increased the effective local tRNA concentrations, thereby enhancing the rates of unspecific reactions. This speculation is supported by the reported possibility that tRNAs contact several domains, including V (*12*), as well as our findings that neither mutations in domain V nor antibiotics (see below) completely eliminated peptide 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

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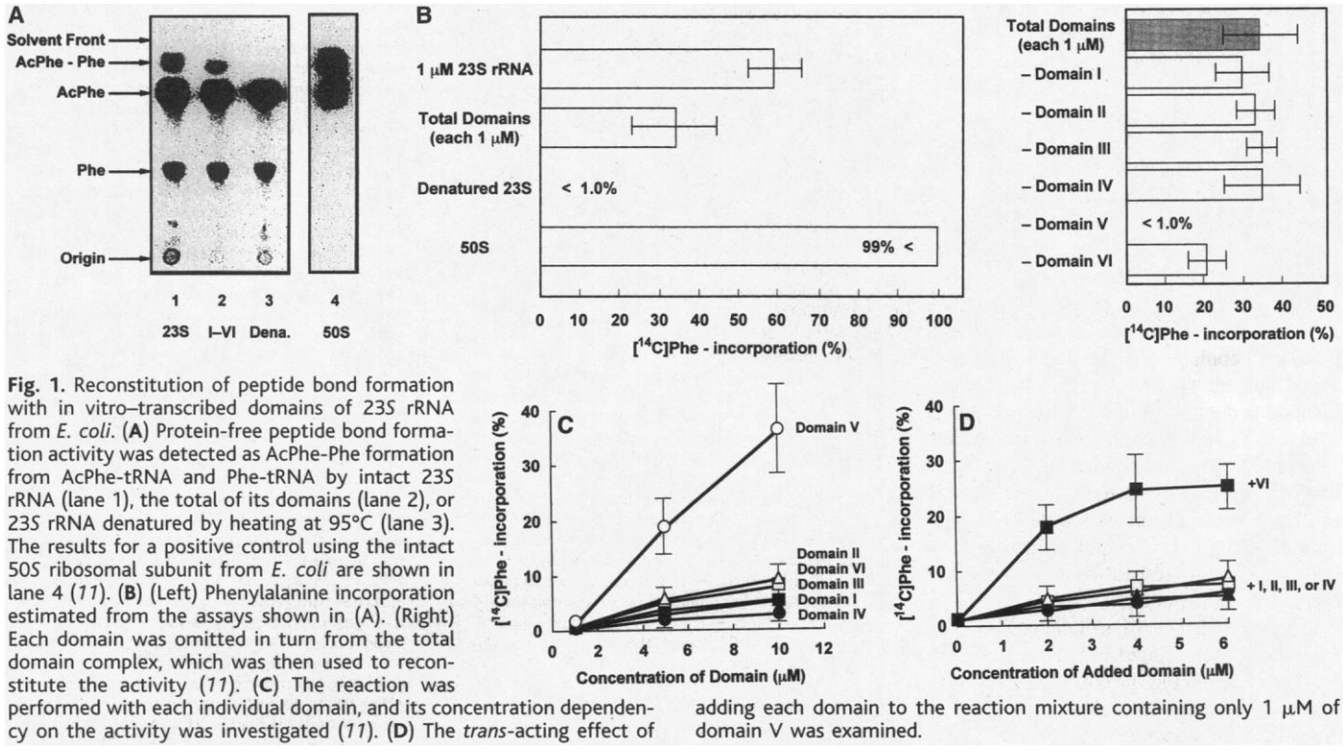

Fig. 1. Reconstitution of peptide bond formation with in vitro–transcribed domains of 23*S* rRNA from *E. coli*. (**A**) Protein-free peptide bond formation activity was detected as AcPhe-Phe formation from AcPhe-tRNA and Phe-tRNA by intact 23*S* rRNA (lane 1), the total of its domains (lane 2), or 23*S* rRNA denatured by heating at 95°C (lane 3). The results for a positive control using the intact 50*S* ribosomal subunit from *E. coli* are shown in lane 4 (*11*). (**B**) (Left) Phenylalanine incorporation estimated from the assays shown in (A). (Right) Each domain was omitted in turn from the total domain complex, which was then used to reconstitute the activity (*11*). (**C**) The reaction was performed with each individual domain, and its concentration dependency on the activity was investigated (*11*). (**D**) The *trans*-acting effect of adding each domain to the reaction mixture containing only 1 μM of domain V was examined.

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 23*S* 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 domain V mutants as well as of other domains (*16*). All five mutants in the second region of domain V failed to eliminate peptide bond formation activity; only the double mutations C2507U/G2581A and C2507Δ/G2581A (where Δ indicates deletion of the base) slightly inhibited it by ~10 to 20%, in contrast to the 60% inhibition obtained by the mutation of G2252. These findings mean that mutation at a one or two nucleotides in the conserved region near the peptidyltransferase ring affected the peptide 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.

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 aminoglycoside family, members of which are well known historically for their misreading-inducing effects in bacteria. In addition, all aminoglycosides impair translocation (*18*). However, there has been no report that neomycin inhibits peptide transfer. As shown in Fig. 3, the presence of 3 mM sparsomycin inhibited AcPhe-Phe formation by ~70%; at more than 3 mM, sparsomycin reduced the activity to the same level. The remaining activity resistant to high concentrations of sparsomycin might again be due to the effect of unspecific interactions between domain V and tRNAs. In contrast, neomycin did not inhibit the reaction at all, whatever the concentration, and even showed a tendency to slightly enhance AcPhe-Phe formation at relatively high concentrations of more than 3 mM. This agrees well with the mechanism of neomycin, which activates unspecified aminoacyl-tRNA binding to the ribosome and causes codon misreading (*19*).

We demonstrated that even without 5*S* rRNA (*20*), a catalytic RNA-activating peptide bond formation could be assembled from two of the proposed domains of *E. coli* 23*S* rRNA (*21*), with domain V possessing the

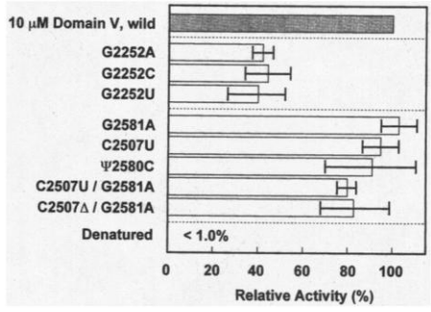

Fig. 2. Sensitivity of peptide bond formation activity toward mutations in domain V. Relative activities of AcPhe-Phe formation carried out with the wild type or two series of 10 μM domain V mutants, taking the wild-type activity as 100%. One series of mutations was G2252 to A, C, and U; the other was 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 detectable enhancement of AcPhe-Phe formation when domain V was associated not with domain II, but with domain VI, suggesting that the 3′-terminal region of 23*S* rRNA plays a central role in our system. Although domain VI contains a single universally conserved element, the so-called "α-sarcin 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 I self-splicing introns, in which it has been proposed that the catalytic core lies in two structural domains, P4-P6 and P3-P9, which were synthesized separately and shown to be able to self-assemble into a catalytically active complex (*24*). In addition, the separately synthesized P5abc domain was reported to activate the ΔP5abc deletion mutant of the intron in a *trans*-acting manner (*25*). This modular construction of ribozymes is analogous to the structure of some proteins in which multiple structural regions, or domains, interact to create a catalytic center. Certain protein enzymes, such as *E. coli* β-galactosidase (*26*) and *E. coli* isoleucyl-tRNA synthetase (*27*) are composed of multiple structural domains that can be separated and reconstituted in *trans*. Considering these results, RNA catalysts appear in some cases to be divided into functionally independent modules, or pieces, which can associate through tertiary interactions after individual folding.

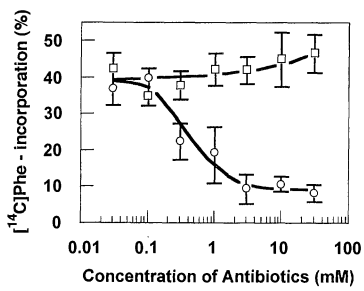

Fig. 3. Effects of antibiotics on the peptide bond formation reaction. Inhibition profiles of peptide bond formation with 10 μM domain V by sparsomycin (○) and neomycin (□) are shown (*30*).

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9. Using polymerase chain reaction (PCR) amplification, all the ribosomal genes were obtained from pEC23SN constructed in our previous study (*8*). *Escherichia coli* 23*S* 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 NH_4Cl. Before the assay for peptide bond formation, the transcripts in the self-folding buffer were heated at 65°C for 10 min and then cooled to 37°C over a period of 90 min for the complete self-folding of 23*S* rRNA or of its individual domains, or at 37°C for 20 min to reconstitute the active complex from several domains.
10. Deacylated-tRNA specific to phenylalanine from *E. coli* MRE600 was isolated from the total tRNA by using a solid-phase–attached DNA probe, 5′-TGGT-GCCCGGACTCGGAATCG-Biotin-3′. The tRNA was phenylalanylated with the S100 fraction from *E. coli* followed by acetylation with acetic anhydride. The Ac[^{14}C]Phe-tRNA and [^{14}C]Phe-tRNA obtained (specific activity: 9.2×10^{-4} pmol/cpm) were purified by high-performance liquid chromatography (HPLC) using a C_4 column, and stored at –20°C in a tRNA stock buffer [20 mM potassium acetate (pH 5.5) and 5 mM $MgCl_2$].
11. Assay for peptide bond formation: AcPhe-Phe synthesis was carried out at 37°C for 60 min in 25 μl of a reaction mixture containing 1.0 to 10.0 μM 23*S* rRNA or its one or more domains, 1.0 μM Ac[^{14}C]Phe-tRNA, 50.0 nM [^{14}C]Phe-tRNA, 50 mM Hepes-KOH (pH 8.2), 30 mM $MgCl_2$, and 160 mM NH_4Cl. Both the AcPhe- and Phe-tRNAs were labeled with [^{14}C]phenylalanine so they could be detected during purification by HPLC. Unlike in previous work, SDS was not added because AcPhe-Phe formation from the highly purified AcPhe- and Phe-tRNA was sufficiently efficient. Taking the reaction with 1.0 μM domain V as an example, the mixture was made up of 1.25 μl of 1.0 M Hepes-KOH (pH 7.8), 1.06 μl of 500 mM $MgCl_2$, 2.5 μl of 10.0 μM domain V in the self-folding buffer, 2.5 μl of 10.0 μM AcPhe-tRNA in the tRNA stock buffer, 1.25 μl of 1.0 μM Phe-tRNA in the tRNA stock buffer, 7.5 μl of self-folding buffer, 0.25 μl of tRNA stock buffer, 1.0 μl of 1 N KOH, and 7.69 μl of ddH_2O. The following factors of pmol/A_{260} were used to estimate the concentrations of the RNAs: 36 for 23*S* rRNA; 228 for domain I; 189 for II; 335 for III; 347 for IV; 222 for V; 757 for VI; 1500 for tRNA. After incubation for peptide bond formation, the reaction was stopped and the aminoacyl bond was hydrolyzed by heating for 60 min at 95°C. The products contained in 25 μl of the reaction mixture were analyzed by thin-layer chromatography (TLC) (silica gel plate, developed with *n*-butanol/acetic acid/H_2O = 4/0.9/1). The activity of peptide bond formation was assessed by the incorporation of [^{14}C]phenylalanine from [^{14}C]Phe-tRNA into Ac[^{14}C]Phe-Phe, which was calculated as the conversion of Phe-tRNA to AcPhe-Phe using the following formula: (half the amount of the radioactivity of the spot corresponding to AcPhe-Phe)/(the amount of radioactivity from Phe + half the amount of the radioactivity from AcPhe-Phe). In every assay, the reproducibility and background level were confirmed by simultaneous reactions with 1 μM self-folded and denatured wild-type 23*S* rRNAs, respectively. All the quantitative results are mean values from experiments repeated at least three times. In the reaction shown in Fig. 1B, right, the domains were preincubated at 37°C in the self-folding buffer. By contrast, before the reaction shown in Fig. 1C, each individual domain was heated at 65°C for 10 min and then slowly cooled. Since the concentration of each domain in Fig. 1B was 1 μM, the total for all the domains except V was 5 μM fragments, which gave less than 1% of the phenylalanine incorporation. In Fig. 1C, however, the average activity with 5 μM of each domain was shown to be around 5%. The most likely explanation for this inconsistency is the difference in the preheating of the domains. Identification of the products: The reaction products—AcPhe-Phe, AcPhe, and Phe—were identified by TLC with two different solvent systems (*n*-butanol/acetic acid/H_2O and $CHCl_3$/methanol) as well as by HPLC with an octadecylsilane column using authentic materials synthesized chemically. In addition, the spot corresponding to AcPhe-Phe was extracted from the TLC plate [J. G. Seidman, B. G. Barrell, W. H. McClain, *J. Mol. Biol.* **99**, 733 (1975)] and acid hydrolysis of AcPhe-Phe was carried out in 6N HCl for various time periods (1 to 12 hours) at 105°C [W. H. McClain, C. Guthrie, B. G. Barrell, *J. Mol. Biol.* **81**, 157 (1973)]. After the reaction of 1 hour, not only Phe but AcPhe could be detected on the TLC plate, the latter of 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 into Phe and acetic acid, partly via AcPhe. We 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, whereas Phe and a very small amount of Phe-Phe were detected in the case of using only Phe-tRNA, suggesting that the peptide transfer can occur between two Phe-tRNAs, but not between two AcPhe-tRNAs. For 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 that the product is really AcPhe-Phe.
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13. Additional experimental results indicated that the reaction was completely suppressed by denaturing at 95°C any of the 23*S* rRNA domains as well as by digestion of the domains with either pyrimidine-specific ribonuclease A (RNase A) or guanine-specific RNase T1 (*28*). Furthermore, RNAs unrelated to rRNA, such as polyuridylate and polyadenylate, were found to never enhance AcPhe-Phe synthesis at high concentrations on either a mole or weight basis, whereas 16*S* rRNA did so at the same level as that of the domains other than V (*28*). These findings give weight to the proposition that tRNAs are unspecifically gathered by 23*S* rRNA domains other than domain V.
14. Effective activation in the presence of domain VI was further supported by the following facts. First, the amount of AcPhe-Phe formed by domain V together with domain I, II, III, or IV was similar to that formed by each of these domains without domain V (compare Fig. 1, C and D), whereas the amount formed by domain V with VI reached about the same level as that formed by the total domain complex when the concentration of domain VI was 4 μM or more. Second, at relatively low concentrations of additional domain VI, the incorporation of phenylalanine increased linearly with increments in the concentration of domain VI, but leveled off at around 5 μM, indicating that domain V was the central module for peptide bond formation and VI was the activator. Finally, the activity with 1 μM of domain V and 2 μM of domain VI was twice that with 3 μM of domain V only (Fig. 1, C and D).
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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 23*S* 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 domain V wild type or its mutants with either RNase A or RNase T1 (*28*).
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19. The lowest concentration of sparsomycin needed to almost suppress peptide bond formation with the naked rRNA, 3 mM, was much higher than the concentrations required in the in vitro system containing intact ribosomes, which are on the order of less than 100 μM. A likely explanation is that the binding of antibiotics affecting ribosomes probably requires some of the ribosomal proteins. This likelihood is supported by the result obtained previously (*8*). In the conventional puromycin reaction with intact ribosomes which follows the formation of a single peptide bond uncoupled from the numerous other processes of translation [R. R. Traut and R. E. Monro, *J. Mol. Biol.* **10**, 63 (1964)], 0.5 mM was a sufficient 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 23*S* rRNA.
20. Although *E. coli* 5*S* rRNA is essential for reconstituting the active 50*S* subunit, 5*S* rRNA had no effect on peptide bond formation with the naked 23*S* rRNA or its domains (*28*). The most likely explanation is that 5*S* 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 5*S* and 18*S* rRNA in eukaryotes, it has been proposed that 5*S* rRNA is involved in an interaction with 16*S* 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* 23*S* rRNA are essential for peptide bond formation between *N*-acetylmethionine-(3′)ACCAAC(5′) and puromycin [R. Green and H. F. Noller, *RNA* **2**, 1011 (1996)]; by contrast, unmodified 23*S* 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.
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29. Using an in vitro mutagenesis kit (Bio-Rad), which follows the conventional Kunkel's method, plasmids carrying 23*S* rRNA genes mutated in the first region were prepared from pEC23SN. On the other hand, using PCR, 23*S* 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 sparsomycine or neomycin (*28*).
31. We thank K. H. Nierhaus and C. M. T. Spahn for providing plasmids containing mutant 23*S* 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.

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