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Functional interaction between bases C1049 in domain II and G2751 in domain VI of 23S rRNA in *Escherichia coli* ribosomes

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ABSTRACT

The factor-binding center within the *Escherichia coli* ribosome is comprised of two discrete domains of 23S rRNA: the GTPase-associated region (GAR) in domain II and the sarcin–ricin loop in domain VI. These two regions appear to collaborate in the factor-dependent events that occur during protein synthesis. Current X-ray crystallography of the ribosome shows an interaction between C1049 in the GAR and G2751 in domain VI. We have confirmed this interaction by site-directed mutagenesis and chemical probing. Disruption of this base pair affected not only the chemical modification of some bases in domains II and VI and in helix H89 of domain V, but also ribosome function dependent on both EF-G and EF-Tu. Mutant ribosomes carrying the C1049 to G substitution, which show enhancement of chemical modification at G2751, were used to probe the interactions between the regions around 1049 and 2751. Binding of EF-G-GDP-fusidic acid, but not EF-G-GMP-PNP, to the ribosome protected G2751 from modification. The G2751 protection was also observed after tRNA binding to the ribosomal P and E sites. The results suggest that the interactions between the bases around 1049 and 2751 alter during different stages of the translation process.

INTRODUCTION

The 23S and 28S rRNAs of prokaryotic and eukaryotic large ribosomal subunits form the largest class of non-coding functional RNAs. They are composed of six domains (I–VI) (1) and contain multifunctional regions that cooperatively participate in translation (2). The sites that interact with elongation factors are found in two distinct regions of *Escherichia coli* 23S rRNA: one site around residue 1070 in domain II, which includes helices H42, H43 and H44, is termed the GTPase-associated region (GAR), and the other, around residue 2660 in domain VI, is termed the sarcin–ricin loop (SRL) (Figure 1) (3–5). The GAR has been identified as a binding site of the antibiotic thiostrepton, which inhibits the GTPase-associated events dependent on translation elongation factors (6–11), presumably by locking a post-GTPase conformation of the ribosome (12). The GAR forms a platform upon which the L10.L7/L12 pentameric complex and L11 cooperatively bind (13–16) to constitute a highly mobile protuberance (17–26), termed the stalk, in addition to the stalk base region in the large subunit. On the other hand, the SRL has been identified as the target site of ribotoxins α-sarcin and ricin, which also inhibit factor-dependent steps of translation elongation (27–29).

Recent analyses by X-ray crystallography (30–33) and cryo-electron microscopy (EM) (34,35) have revealed that, in ribosomes, the GAR and SRL are located in positions neighboring each other at the stalk base, although part of the stalk and stalk base around the GAR is not resolved at high resolution. In the cryo-EM analyses, the SRL is observed at a fixed position in various complexes of the ribosome with EF-G and EF-Tu in the presence of GMP-PNP or GDP (34,35), and the SRL interacts with the regions near the GTP/GDP-binding sites of both factors (35,36). In contrast, the GAR is apparently mobile, its position depending on the specific nucleotide states of EF-G and EF-Tu (34,35,37,38). There seems to be a dynamic cooperativity between the SRL and GAR in the process of translation elongation. It is, however, unlikely that the SRL and the GAR directly interact with each other, because no physical contact has been observed between them in the crystal structures of the large ribosomal subunits.

The crystal data revealed alternative interactions between base C1049 within the GAR and base G2751 of H97 within domain VI (Figure 5A); the data from *E. coli* (32) and *Thermus thermophilus* (33) suggest a Watson–Crick C1049–G2751 pair, whereas the data from *Haloarcula marismortui* (30) suggest that only one of the three usual hydrogen bonds that exist in a Watson–Crick

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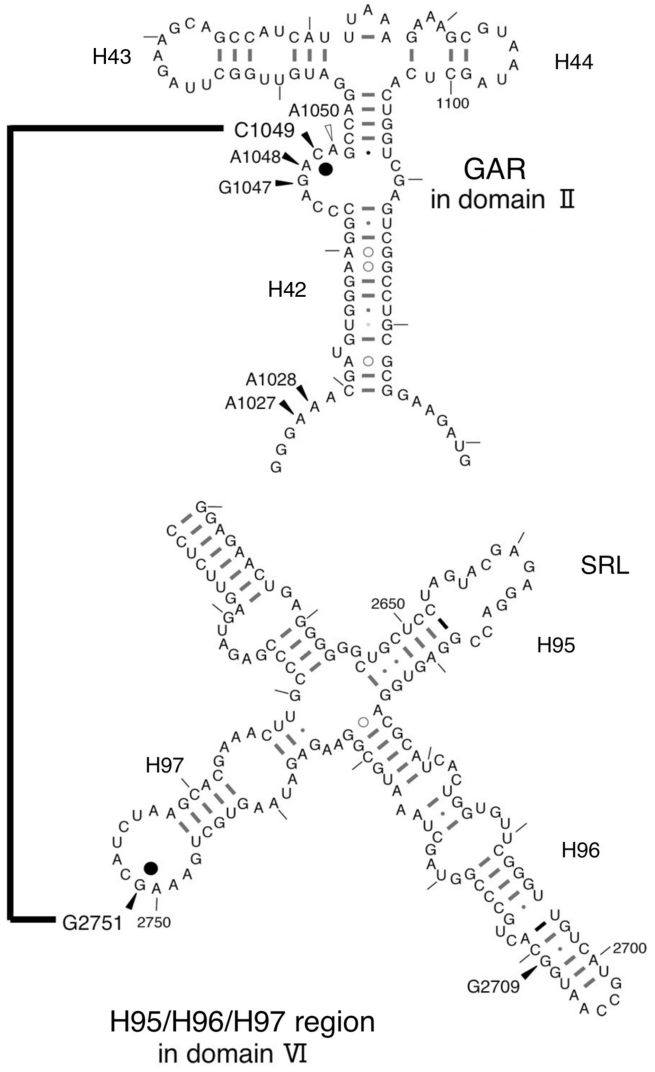

Figure 1. Interaction between bases C1049 and G2751 of 23S rRNA in the large ribosomal subunit. The secondary structures of segments of the 23S rRNA including the GTPase-associated region (GAR) (residues 1024–1131) and the H95/H96/H97 region in domain VI covering the sarcin/ricin loop (SRL) (residues 2630–2788). The positions of the interactions investigated in the present study are indicated by filled circles. Small arrowheads indicate the bases whose chemical modification is enhanced (filled symbol) or protected (open symbol) by the disruption of the C1049–G2751 pair, as shown in Figure 2.

C–G pair, the one between the N4 position of C and the O6 position of G, occurs with C1049 and G2751. In the case of *Deinococcus radiodurans*, however, non-Watson–Crick interactions between A1050–G2751, A1050–C2752 and G1051–C2752 were resolved instead of the C1049–G2751 pair (31). These conflicting structural data concerning the C1049–G2751 pair suggest that this interaction is not firmly fixed; rather it is dynamic and depends on the state of the ribosome. However, other possible explanations for the differences in the crystal structures, i.e. variations in the rRNA sequences or ribosome structure between species, differences in the preparation of ribosomes and crystals, or varying approaches to the interpretation of the data, cannot be excluded. In the present study we investigated the C1049–G2751 interaction in the *E. coli* ribosome by site-directed mutagenesis and biochemical approaches. We confirmed this interaction and clarified its role in ribosomal accessibility to EF-G and EF-Tu. Furthermore, we observed that binding of EF-G or tRNA changes the nature of the interaction between the regions around 1049 and 2751. The results are discussed in terms of the significance of these interactions, in addition to the higher-order structure of the rRNA, as part of a dynamic process of translation.

MATERIALS AND METHODS

Plasmids and bacterial strains

Plasmid pLK45 containing the *E. coli rrnB* operon, which is expressed under the control of the λ PL promoter (39), was provided by H. F. Noller (University of California-Santa Cruz). The PCR-based site-directed mutagenesis of C1049 and G2751 in *E. coli* 23S rRNA was carried out on pLK45 (39) as described by Kunkel *et al.* (40). The *E. coli* Δ7 prrn strain SQ380 (Δ*rrnE* Δ*rrnB* Δ*rrnA* Δ*rrnH* Δ*rrnG::lacZ* Δ*rrnC::cat* Δ*rrnD::cat* Δ*recA*/ptRNA67-SpcR) supplemented with an rRNA-coding plasmid, pCsacB-KmR, was provided by Catherine L. Squires (Tufts University School of Medicine). Expression of *sacB* from pCsacB-KmR in *E. coli* was lethal in the presence of sucrose (41).

Plasmid replacement

Plasmid pLK45 encoding the wild-type rRNA or rRNA mutated at C1049 or G2751 was transformed into strain SQ380. The plasmid pCsacB-KmR in strain SQ380 was then replaced by pLK45 in the presence of sucrose. Exclusive expression of the mutated rRNA in the resulting strains was confirmed by primer extension analysis of total rRNA (42).

Ribosomes, elongation factors and aminoacyl-RNAs

Salt-washed ribosomes from the transformed *E. coli* SQ380 strains were prepared according to our previous study (43). The isolated ribosomes showed no contaminating EF-G or EF-Tu activity. *E. coli* EF-Tu and EF-G were over-expressed in *E. coli* cells using the expression vector pET3a (Novagen) and purified basically according to the method described by Arai *et al.* (44). *E. coli* total tRNA (Roche) was charged with [^{14}C] phenylalanine (GE Healthcare) using partially purified aminoacyl tRNA synthetase from the supernatant S-100 fraction of *E. coli* Q13 (45). The specific radioactivity of the [^{14}C]Phe-tRNA was 400 cpm/pmol.

GTPase activity and polyphenylalanine synthesis

EF-G-dependent GTPase activity was measured as described previously (43), except that the reaction mixture contained 5 pmol of 70S ribosomes and 7 mM $MgCl_2$. The EF-Tu/EF-G-dependent polyphenylalanine synthesis was performed with 10 pmol of 70S ribosomes, as described (46).

EF-Tu-dependent aminoacyl-tRNA binding

EF-Tu-dependent aminoacyl-tRNA binding was assayed, as described (47). In brief, 20 µl of a solution containing 10 pmol of 70S ribosomes, 10 µg of poly(U), 80 µg of deacylated-tRNA (total), 10 mM $MgCl_2$, 100 mM NH_4Cl, 5 mM dithiothreitol and 20 mM Tris–HCl pH 7.6 was preincubated at 37°C for 5 min. The mixture was further incubated at 37°C for 10 min after the addition of 0.6 µg of EF-Tu, 12 pmol of [^{14}C]Phe-tRNA and 0.5 mM GTP. The reaction mixture was filtered through a nitrocellulose membrane (type HA, 0.45 µm pore size; Millipore). The membrane was washed with 3 ml of ice-cold buffer containing 10 mM $MgCl_2$, 100 mM NH_4Cl and 20 mM Tris–HCl, pH 7.6. Radioactivity retained on the filter was counted using a liquid scintillation counter (Aloka).

Chemical probing

An aliquot (50 µl) containing 10 pmol of wild-type or mutant 70S ribosomes in the presence of 7 mM $MgCl_2$, 50 mM NH_4Cl, 5 mM 2-mercaptoethanol and 50 mM potassium cacodylate, pH 7.2 (for DMS, dimethyl sulfate) or 50 mM boric acid pH 8.0 [for CMCT, 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate] was mixed with 1 µl of DMS (1:4 dilution in ethanol) or 25 µl of CMCT (42 mg/ml) and incubated at 37°C for 10 min (Figure 2). The ribosome•EF-G•GDP•fusidic acid complex was formed in the CMCT reaction buffer described earlier to which 10 pmol of 70S ribosomes, 30 pmol of EF-G, 0.5 mM GTP and 1 mM fusidic acid were added. The ribosome•EF-G•GMP-PNP complex was formed by combining 10 pmol of 70S ribosomes, 30 pmol of EF-G and 0.5 mM GMP-PNP in CMCT reaction buffer. The ribosome•poly(U)•deacylated-$tRNA^{Phe}$ complex was formed by combining 10 pmol of 70S ribosomes, 5 µg of poly(U) and 40 µg of total deacylated-tRNA in CMCT reaction buffer. Individual mixtures were incubated for 10 min at 37°C, followed by incubation for 5 min on ice. The complexes formed were treated with CMCT, as described earlier. The modified rRNA was extracted from each sample with phenol and analyzed by primer extension, followed by gel electrophoresis, as described by Moazed and Noller (48). The primers used were 5′-GGCCGACTCGACCAGTGA GC-3′ for the H42 region, 5′-AATGGCGAACAGCCA TACCC-3′ for H89 region and 5′-GTCTTCAACGTTC CTTCAGG-3′ for the H97 region, complementary to residues 1099–1118, 2543–2562 and 2805–2824 of 23S rRNA, respectively.

RESULTS

Preparation of ribosomes carrying mutations at 1049 and/or 2751 of 23S rRNA

The site-directed mutations at C1049 in the GAR and G2751 in domain VI of 23S rRNA were introduced into the *E. coli rrnB* operon contained in pLK45 (39). We prepared the following single-base substitution mutants: C1049G, C1049A, C1049U, G2751A, G2751U and G2751C, as well as single-base deletion mutants, ΔC1049 and ΔG2751. Individual plasmids carrying these mutations were transformed into the *E. coli* Δ7 prrn strain. All the resulting strains except the one carrying the ΔC1049 mutation were viable and grew without retardation. We isolated ribosomes from individual strains and confirmed that the ribosomes exclusively contained mutated 23S rRNAs, not wild-type rRNA. Using the same method with the Δ7 prrn strain, we also isolated strains carrying the following double mutations: C1049G•G2751C, C1049G•G2751A, C1049G•G2751U, C1049A•G2751C, C1049A•G2751A, C1049A•G2751U, C1049U•G2751C, C1049U•G2751A and C1049U• G2751U. The ribosomes from these mutant strains were also used in some of the experiments described later. A double deletion mutation, ΔC1049•ΔG2751, was lethal.

Chemical probing of the interaction between positions 1049 and 2751 of 23S rRNA

The state of the two bases, C1049 and G2751, within wild-type and mutant ribosomes was probed using the chemical reagents DMS and CMCT. Modification of these bases was analyzed by primer extension, followed by gel electrophoresis (Figure 2). In the case of the wild-type ribosomes, the modification of C1049 with DMS (Figure 2A, lane 1) and of G2751 with CMCT (Figure 2D, lane 1) was observed only weakly. After the mutation of G2751, the DMS modification of C1049 was markedly enhanced in all the mutants, i.e. G2751A (Figure 2A, lane 2), G2751U (lane 3) and G2751C (lane 4). The modification of other bases, in addition to C1049, was affected by these same mutations. In the vicinity of C1049, the modification of G1047 and A1048 was slightly enhanced and A1050 was protected (Figure 2A). The base substitution of G2751 also enhanced the DMS modification of A1027 and A1028 in the upstream single-strand region of H42 (Figure 2B, lanes 2–4), and the CMCT modification of U2477 and U2493 within H89 (Figure 2E, lane 3). When mutations were introduced at position 1049 of 23S rRNA, the CMCT modification at G2751 was enhanced in all the mutants, i.e. C1049G (Figure 2D, lane 2), C1049A (lane 3) and C1049U (lane 4). Furthermore, these mutations in C1049 enhanced the CMCT modification of G2709 at the tip of H96 (Figure 2C, lanes 2–4); G2709 and G2751 are located at opposite ends of the long H96/H97 structure in the large ribosomal subunit (Figure 5A). The mutation of either C1049G or G2751C also enhanced the CMCT modification of U2477 and U2493 in H89 of domain V (Figure 2E, lane 2 or 3). Unexpectedly, the compensatory double mutation of C1049G and G2751C resulted in further enhancement of the modification of U2477 and U2493 (Figure 2E, lane 4). On the other hand, the markedly enhanced reactivity of U2477/U2493 observed in the G2751A mutant (lane 6) decreased to some extent in the double mutant C1049U•G2751A (lane 7), suggesting that an interaction between positions 1049 and 2751 is partially recovered by this double mutation. The modification data for the ribosome mutants representing all possible combinations of mutations at positions 1049 and

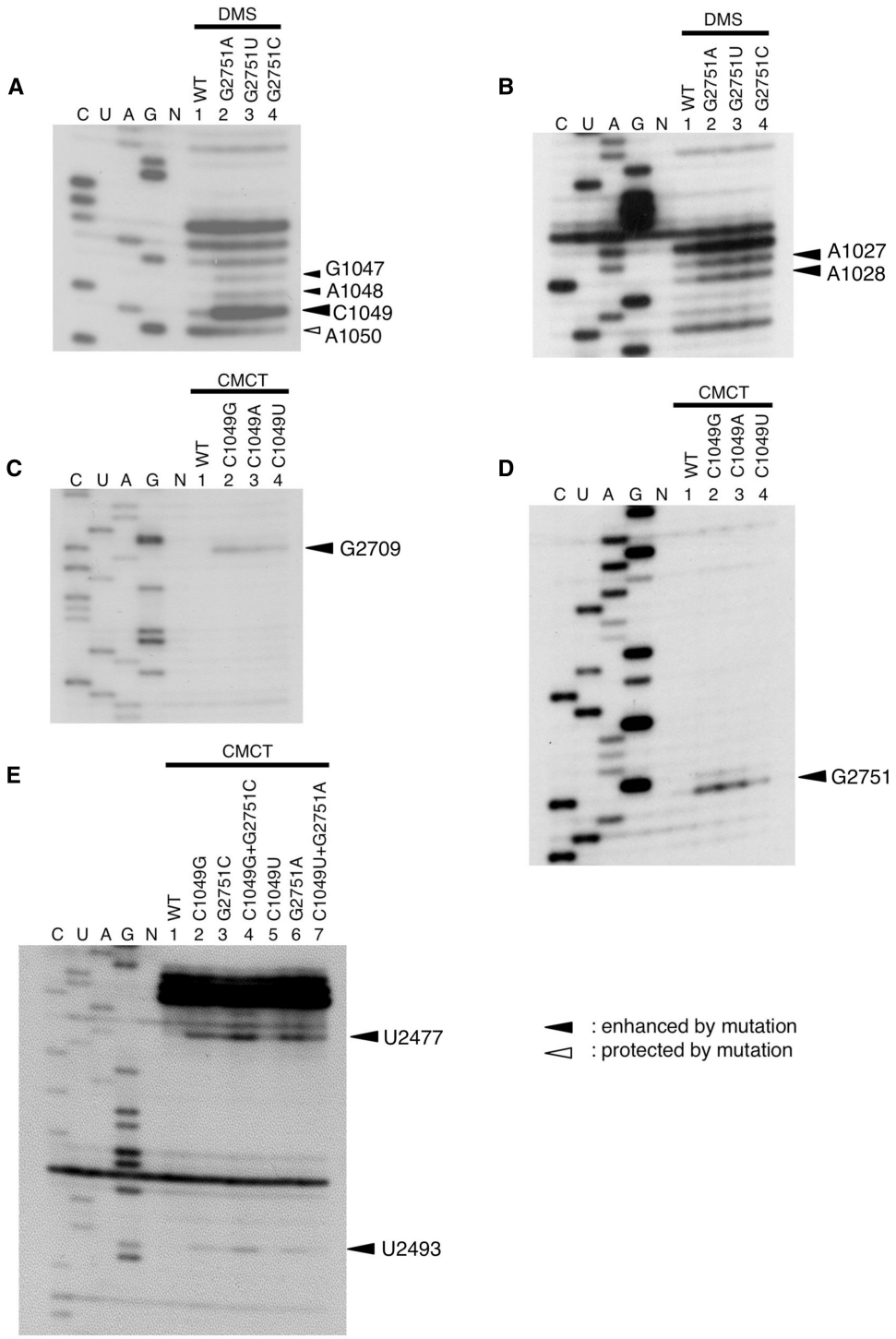

Figure 2. Effects of substitution of base C1049 or G2751 of 23S rRNA in the large ribosomal subunit on chemical modifications. (**A**, **B**) Wild-type 70S ribosomes (lane 1), G2751A mutant ribosomes (lane 2), G2751U mutant ribosomes (lane 3) and G2751C mutant ribosomes (lane 4) were treated with DMS. The rRNA extracted from each ribosome sample was analyzed by primer extension using a primer complementary to residues 1099–1118 of 23S rRNA. (**C**, **D**) Wild-type 70S ribosomes (lane 1), C1049G (lane 2), C1049A (lane 3) or C1049U (lane 4) mutant ribosomes were treated with CMCT. The rRNA extracted from each ribosome sample was analyzed by primer extension using a primer complementary to residues 2805–2824 of 23S rRNA. (**E**) Wild-type 70S ribosomes (lane 1), C1049G mutant (lane 2), G2751C mutant (lane 3), C1049G•G2751C double mutant (lane 4), C1049U mutant (lane 5), G2751A mutant (lane 6) or C1049U•G2751A double mutant (lane 7) ribosomes were treated with CMCT. The rRNA extracted was analyzed by primer extension using a primer complementary to residues 2543–2562 of 23S rRNA. The products of the primer extension were analyzed on a sequencing gel. Lanes G, A, U and C correspond to sequencing lanes. Lane N shows a sample from unmodified ribosomes. Bases whose reactivity was altered as a result of one of the mutations are marked.

2751 of 23S rRNA are shown in Supplemental Table S1. The results indicate that among the 16 possible combinations of base pairs at 1049 and 2751, only the wild-type C1049•G2751 pair shows very low or no reactivity of the bases, not only at positions 1049 and 2751, but also at the other affected positions: 1027, 1028, 1047, 1048, 2477 and 2493. It can be inferred that an interaction occurs between C1049 and G2751 within the ribosome in aqueous solution, although presumably a regular Watson–Crick base pair is not formed. This base pairing seems to affect not only some local structures in domains II and VI, but also the structure of H89 in domain V.

Functional effects of base substitutions at positions 1049 and 2751 of 23S rRNA

To examine the effects of the base substitutions at C1049 and G2751 on ribosome function, we assayed EF-G/EF-Tu-dependent poly(Phe) synthesis and EF-G-dependent GTPase activity (Figure 3A and B). In the presence of a single mutation at either position 1049 or 2751, the poly(Phe) synthetic activity was reduced to 45–70% (Figure 3A), and the GTPase activity was reduced to 60–75% of that of the control wild-type ribosomes (Figure 3B). Even after the introduction of double mutations the mutant ribosomes, with the exception of the C1049G•G2751C mutant, retained 65–100% of poly(Phe) synthetic activity (Figure 3A) and 50–90% of the GTPase activity (Figure 3B). Only the C1049G•G2751C mutant showed markedly reduced activities. The effect of the mutations on EF-Tu-dependent [^{14}C]Phe-tRNA binding to poly(U)-programmed ribosomes was also tested by filter-binding assay (Figure 3C). The ribosomes carrying either single or double mutations, with the exception of the C1049G•G2751C double mutant, retained 50–85% of the control wild-type ribosome Phe-tRNA-binding activity. Only the C1049G•G2751C mutant showed a markedly reduced binding ability. All the functional data show that mutation at either position 1049 or 2751 of 23S rRNA affects both activities of EF-G and EF-Tu on the ribosome, although the effects are modest.

Effect of EF-G/tRNA binding on the interactions between the regions around 1049 and 2751 in 23S rRNA

Ribosomes with the C1049G mutation, which showed enhanced reactivity of G2751 with CMCT, maintained an EF-G/EF-Tu-dependent activity more than 70% of that of the wild-type ribosomes. We therefore followed the CMCT reactivity of G2751 using C1049G mutant ribosomes to probe the dynamic characteristics of the interactions between the two regions around 1049 and 2751. First, we examined the effect of EF-G binding to the ribosome on modification by CMCT, which was detected by primer extension (Figure 4A). The chemical modification at G2751 detected in the C1049G ribosomal mutant (Figure 4A, lane l) was protected by the addition of EF-G, GTP, and fusidic acid, which freezes the ribosome-EF-G-GDP complex (lane 2). In contrast, the modification was not protected in the ribosome-EF-G-GMP-PNP complex, a state before GTP hydrolysis (lane 3), despite efficient

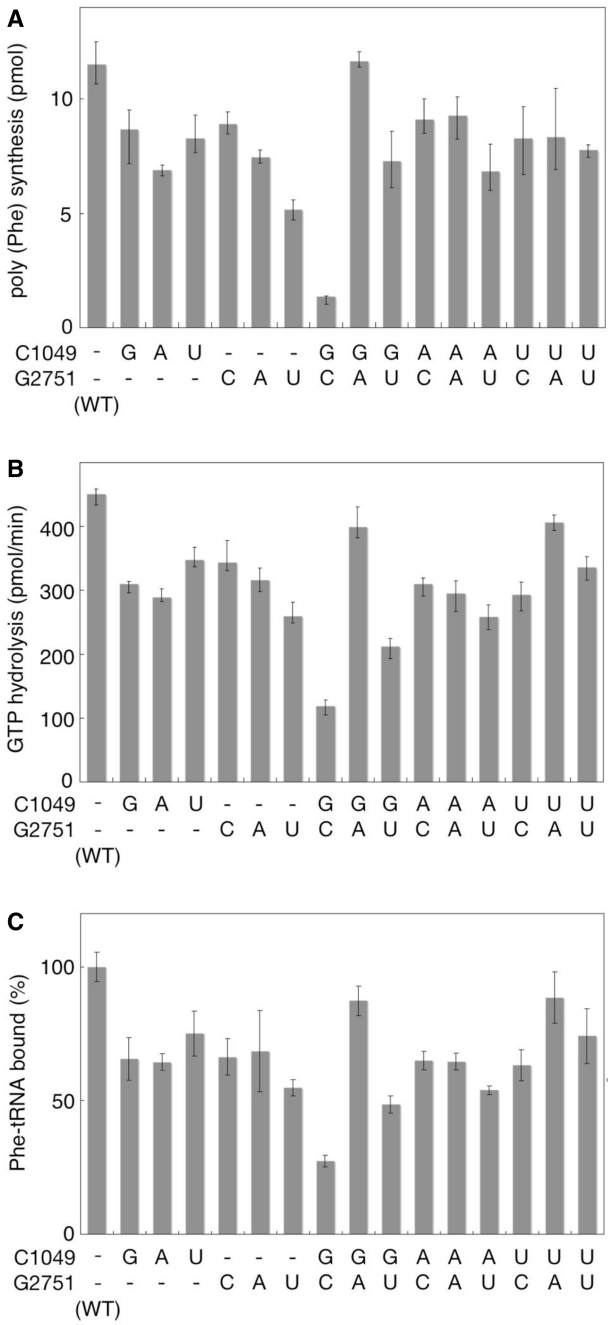

Figure 3. Effects of the C1049 and G2751 mutations on the elongation-factor dependent ribosomal activity. (**A**) Polyphenylalanine synthesis was assayed for each ribosome sample in the presence of 10 pmol of 70S ribosomes. (**B**) EF-G-dependent GTPase activity was assayed for each ribosome sample in the presence of 5 pmol of 70S ribosomes. (**C**) EF-Tu-dependent aminoacyl-tRNA binding was assayed for each ribosome sample in the presence of 10 pmol of 70S ribosomes.

complex formation confirmed by sucrose sedimentation analysis (data not shown).

We also analyzed the effect of tRNA binding to the ribosome on the CMCT modification at G2751 (Figure 4B). It is known that deacylated tRNAs bind non-enzymatically to the P and E sites or the hybrid P/E site (49). We used C1049G mutant ribosomes

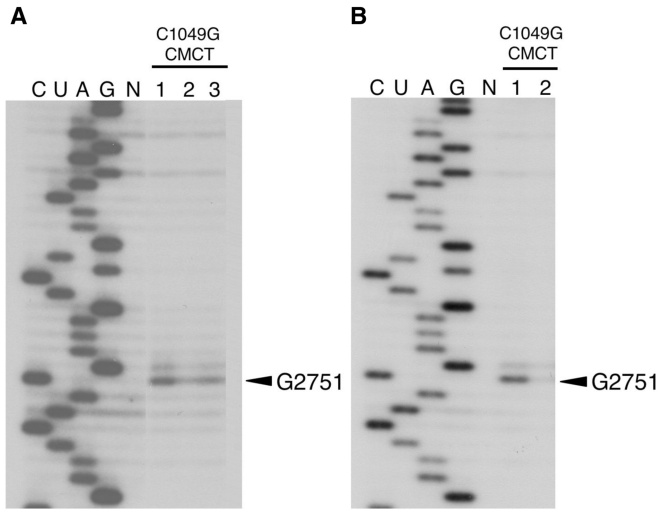

Figure 4. Dynamic features of the interactions between regions around positions 1049 and 2751. (**A**) C1049G mutant 70S ribosomes were preincubated without EF-G (lane 1), or with EF-G, GTP and fusidic acid (lane 2) or EF-G and GMP-PNP (lane 3). The complexes formed were then treated with CMCT. The rRNA extracted from each sample was assayed by primer extension as described in the legend for Figure 2. (**B**) C1049G mutant 70S ribosomes were preincubated alone (lane 1) or in the presence of poly(U) and deacylated tRNA (lane 2). The samples were treated with CMCT and analyzed as described in (A). Lanes G, A, U and C indicate dideoxy sequencing lanes. Lane N shows a sample from unmodified ribosomes.

preincubated with poly(U) and deacylated tRNA and analyzed their reactivity with CMCT by primer extension. The tRNA binding completely protected G2751 from CMCT modification (Figure 4B, lane 2).

The results using the C1049G mutant ribosomes suggest that the nature of the interaction between the two regions around bases 1049 and 2751 of 23S rRNA is changed by the binding of EF-G-GDP-fusidic acid and tRNA, at least in the mutant ribosome. We failed to detect a similar ligand-dependent protection at G2751 in the wild-type ribosome, because the reactivity of base G2751 with chemical reagents was very low in this case (see lane 1 of Figure 2D, and also Supplemental Figure S1).

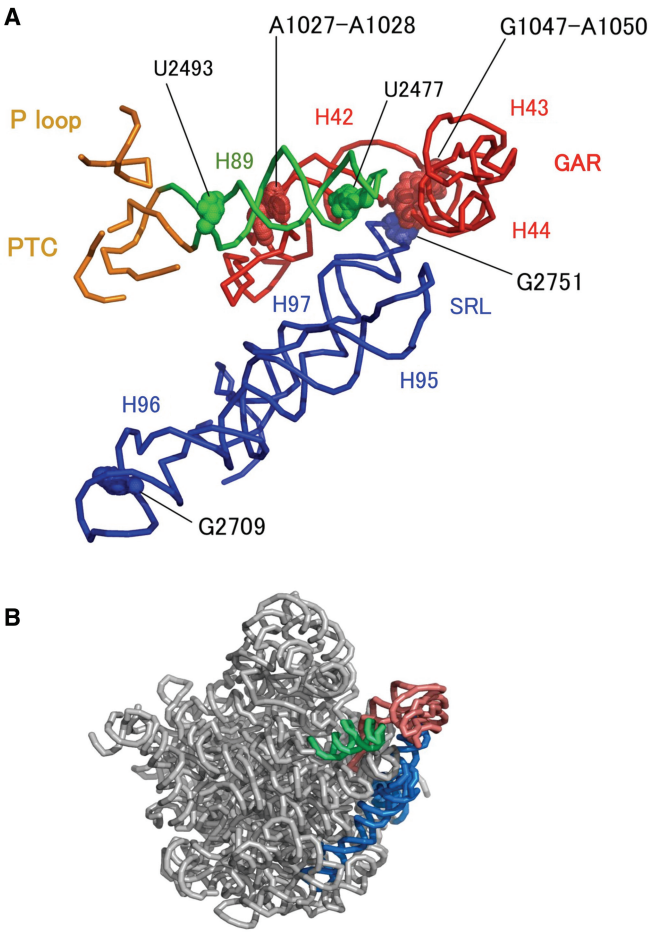

Figure 5. Spatial relationship of bases whose chemical modification was affected by disruption of the C1049–G2751 pair. (**A**) The tertiary structures and mutual locations of the GAR (red), the H95/H96/H97 domain (blue), H89 (green) and parts of the peptidyltransferase center (PTC, yellow) of 23S rRNA within the *E. coli* large ribosomal subunit are represented. This structure is from the PDB entry 2AWB, which seems to correspond to the open conformation. Bases affected by the mutation of C1049 or G2751 are shown within the tertiary structure of the *E. coli* large ribosomal subunit as van der Waals sphere representations. (**B**) The locations of the GAR, the H95/H96/H97 domain, and H89 within the large ribosomal RNA are shown using the same colors as in (A). The representations in A and B were created using PyMOL.

DISCUSSION

The factor binding center of the ribosome is constructed of parts of domains II and VI of 23S rRNA. In particular, the GAR in domain II and the SRL in domain VI have been identified as sites to which EF-G and EF-Tu directly bind (4). However, the molecular details of cooperativity between the two domains in factor-dependent events have not been characterized. The present study demonstrates that C1049 in the GAR in domain II base pairs with G2751 in H97 of domain VI within the *E. coli* large ribosomal subunit in aqueous solution. This is consistent with the crystal structure data for the large ribosomal subunits from *E. coli* (PDB; 2AWB, 2AW4) and *T. thermophilus* (PDB; 1VSA), which show a Watson–Crick C1049–G2751 pair. The results indicate that the C1049–G2751 pair participates in the interaction between the GAR and domain VI of 23S rRNA, although other non-Watson–Crick interactions are also observed around the C1049–G2751 pair in the five crystal structures of the large subunit reported so far: C1110–G2751, A1048–G2751, A1050–G2751, A1050–C2752 and G1051–C2752 (PDB; 1FFK, 1NKW, 2AWB, 2AW4 and 1VSA). The C1049–G2751 pair presumably plays a central role and the other base interactions may help to associate the mobile GAR more strongly with the tip of H97 in domain VI (Figure 5A).

Mutations at either C1049 or G2751 have at least partial effects on both activities of EF-G and EF-Tu (Figure 3), suggesting the C1049–G2751 pair is involved in construction of the functional higher-order structure of the ribosomal factor binding center. The only partially inhibitory effect of most mutations at C1049 and/or

G2751 is probably due to the maintenance of a functional interaction between the two regions in spite of the mutations. Unexpectedly, the largest inhibitory effect is observed in ribosomes carrying the double mutation C1049G•G2751C. Because G1049 in these ribosomes shows high reactivity with CMCT (Table S1), base pairing does not seem to be recovered by this double mutation. It is noteworthy that the reduced activity of the C1049G and G2751A single mutant ribosomes is entirely restored by the C1049G•G2751A double mutation (Figure 3). Furthermore, this double mutation results in a decreased level of chemical modification, not only to G1049 but also to the bases at positions 1027, 1028, 1047 and 1048, compared to the other mutant ribosomes (Table S1). A similar restorative effect by double mutation is also observed in the C1049U•G2751A mutant (Figures 2E and 3, and Table S1). It seems likely that these double mutations partially reinstate a base–base interaction, which is favorable for ribosome function. We infer that the C1049–G2751 interaction in the wild-type ribosomes may be an irregular Watson–Crick pair, as shown in crystal data for the *H. marismortui* ribosome (30), in which only one of the three possible hydrogen bonds, the one between the N4 position of C1049 and the O6 position of G2751, is suggested to exist. This single hydrogen bond seems to be unstable and may be compensated for by the C1049G•G2751A or C1049U•G2751A double mutations. The double mutation C1049G•G2751C may not be able to recover this single hydrogen bond, or rather may disrupt the interaction between the two regions to a greater extent than the others. The present results imply that the interactions between the two regions around C1049 and G2751 connect the two domains of the factor-binding center and contribute to the cooperative function of the two RNA domains in enabling the actions of the translation factors.

Due to the disruption of the base pairing between C1049 and G2751, chemical modification of these two bases is apparently enhanced (Figure 2A and D). Furthermore, an additional eight bases are also weakly affected by the disruption of this base pair. Enhancement of G1047 and A1048 reactivity and protection of A1050 by mutations at G2751 may be explained by destabilization of the local structure of H42. It is interesting that the modification of A1027 and A1028, which are located in the upstream single-strand region of H42 is enhanced. Because A1027 and A1028 contact the middle of H89 in the crystal structure of *E. coli* ribosomes (PDB; 2AW4), the stabilization caused by the C1049–G2751 pair seems to support the interaction of A1027 and A1028 with H89 in domain V. The modification of U2477 and U2493 in H89 is also enhanced by the disruption of the C1049–G2751 pair. Because U2477 interacts with ribosomal protein L36 and U2493 base pairs with A2459 in the H89 stem in the crystal structure of the 50S subunit, the C1049–G2751 interaction is likely to influence the structure of H89. Furthermore, it is unexpected that disruption of the C1049–G2751 pair causes enhancement of CMCT modification of G2709, which is located at the opposite end of the long H96/H97 stem to G2751. Because G2709 base pairs with U2698 within the loop region at the tip of H96 in the crystal structure of the ribosome (PDB; 2AWB, 2AW4), the C1049–G2751 interaction at one end appears to affect the loop structure at the other end of the long H96/H97 stem. These chemical probing data indicate that the interactions between the regions around C1049 and G2751 influence the structural features not only of the GAR and domain VI, but also of H89, which links to the peptidyltransferase center (Figure 5A). Our results are, at least in part, consistent with the effects of another mutation within the GAR reported by Sergiev *et al*. (50). They introduced an extra base pair (C1030–G1124) into H42. The mutant ribosomes carrying this mutation showed enhanced chemical modification of several sites including A1027, A1028, U2477 and U2493, similar to the present study. Their mutant also showed enhanced modification of G2702–A2705 in the loop at the tip of H96. These bases are adjacent to G2709, whose enhanced modification is described here. Their insertion of one extra base pair and our disruption of the C1049–G2751 pair in the GAR seem to have in part the same effect on the ribosomal structure.

We used the C1049G mutant ribosomes to probe the interactions between regions around positions 1049 and 2751 of 23S rRNA. Protection of G2751 was detected after binding of EF-G•GDP•fusidic acid, but not EF-G•GMP-PNP, implying that the interaction between the 1049 and 2751 regions is stronger in the ribosome• EF-G•GDP•fusidic acid complex than the ribosome• EF-G•GMP-PNP complex. It has been observed by cryo-EM that the small and large ribosomal subunits undergo a ratchet-like motion relative to one another following the binding of EF-G (51,52). This causes many local conformational changes, particularly during the process of GTP hydrolysis (34). The location of the GAR moves between two positions during this process: in the position there is an open space between the GAR and H89, and in the other position the GAR lies in close proximity to H89. These configurations are referred to as 'open' and 'closed' forms, respectively (50). Moreover, two crystal structures of the *E. coli* 50S subunit are currently available (PDB; 2AWB, 2AW4, ref. 32): in one crystal structure, the open space between the GAR and H89 is present, and the GAR tightly interacts with the tip of H97 through the base pairs C1049–G2751, A1050–G2751 and A1050–C2752 (PDB; 2AWB); in the other crystal structure, the GAR is in close proximity to H89, and interacts less tightly with the tip of H97 through base pairs C1049–G2751 and A1050–G2751 (PDB; 2AW4). Considering these lines of evidence, it is likely that the change in interaction between positions 1049 and 2751 observed in this study reflects the conformational change between the open form in the ribosome•EF-G•GDP•fusidic acid complex and the closed form in the ribosome•EF-G•GMP-PNP complex.

Interestingly, the protection of G2751 was also detected after binding of deacylated tRNA to the P and E sites of the ribosome. It has been reported that the binding of deacylated tRNA in a P/E hybrid state stimulates EF-G-dependent GTPase turnover as the result of enhancement of EF-G accessibility (53,54). These previous results indicate that tRNA binding to the P/E site allosterically changes the factor-binding center. Taking the present

results into consideration, we infer that the tRNA binding tightens the interaction between the bases at positions 1049 and 2751 and presumably stabilizes the open conformation described earlier. This may explain, in part, the allosteric mechanism of enhancement of EF-G accessibility by tRNA binding. It can be postulated that H89 also participates in the allosteric signaling as discussed by Sergiev *et al.* (50), because this helix forms a bridge between the peptidyltransferase center and the factor-binding center (Figure 5A).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Noller,H.F., Kop,J., Wheaton,V., Brosius,J., Gutell,R.R., Kopylov,A.M., Dohme,F., Herr,W., Stahl,D.A. *et al.* (1981) Secondary structure model for 23S ribosomal RNA. *Nucleic Acids Res.*, **9**, 6167–6189.
2. Noller,H.F. (1991) Ribosomal RNA and translation. *Annu. Rev. Biochem.*, **60**, 191–227.
3. Hausner,T.P., Atmadja,J. and Nierhaus,K.H. (1987) Evidence that the G2661 region of 23S rRNA is located at the ribosomal binding sites of both elongation factors. *Biochimie*, **69**, 911–923.
4. Moazed,D., Robertson,J.M. and Noller,H.F. (1988) Interaction of elongation factors EF-G and EF-Tu with a conserved loop in 23S RNA. *Nature*, **334**, 362–364.
5. Munishkin,A. and Wool,I.G. (1997) The ribosome-in-pieces: binding of elongation factor EF-G to oligoribonucleotides that mimic the sarcin/ricin and thiostrepton domains of 23S ribosomal RNA. *Proc. Natl Acad. Sci. USA*, **94**, 12280–12284.
6. Thompson,J., Schmidt,F. and Cundliffe,E. (1982) Site of action of a ribosomal RNA methylase conferring resistance to thiostrepton. *J. Biol. Chem.*, **257**, 7915–7917.
7. Ryan,P.C., Lu,M. and Draper,D.E. (1991) Recognition of the highly conserved GTPase center of 23 S ribosomal RNA by ribosomal protein L11 and the antibiotic thiostrepton. *J. Mol. Biol.*, **221**, 1257–1268.
8. Thompson,J. and Cundliffe,E. (1991) The binding of thiostrepton to 23S ribosomal RNA. *Biochimie*, **73**, 1131–1135.
9. Thompson,J., Musters,W., Cundliffe,E. and Dahlberg,A.E. (1993) Replacement of the L11 binding region within *E.coli* 23S ribosomal RNA with its homologue from yeast: in vivo and in vitro analysis of hybrid ribosomes altered in the GTPase centre. *EMBO J.*, **12**, 1499–1504.
10. Rosendahl,G. and Douthwaite,S. (1994) The antibiotics micrococcin and thiostrepton interact directly with 23S rRNA nucleotides 1067A and 1095A. *Nucleic Acids Res.*, **22**, 357–363.
11. Xing,Y. and Draper,D.E. (1995) Stabilization of a ribosomal RNA tertiary structure by ribosomal protein L11. *J. Mol. Biol.*, **249**, 319–331.
12. Rodnina,M.V., Savelsbergh,A., Matassova,N.B., Katunin,V.I., Semenkov,Y.P. and Wintermeyer,W. (1999) Thiostrepton inhibits the turnover but not the GTPase of elongation factor G on the ribosome. *Proc. Natl Acad. Sci. USA*, **96**, 9586–9590.
13. Pettersson,I. (1979) Studies on the RNA and protein binding sites of the *E. coli* ribosomal protein L10. *Nucleic Acids Res.*, **6**, 2637–2646.
14. Beauclerk,A.A., Cundliffe,E. and Dijk,J. (1984) The binding site for ribosomal protein complex L8 within 23 s ribosomal RNA of *Escherichia coli*. *J. Biol. Chem.*, **259**, 6559–6563.
15. Egebjerg,J., Douthwaite,S.R., Liljas,A. and Garrett,R.A. (1990) Characterization of the binding sites of protein L11 and the L10.(L12)4 pentameric complex in the GTPase domain of 23 S ribosomal RNA from *Escherichia coli*. *J. Mol. Biol.*, **213**, 275–288.
16. Rosendahl,G. and Douthwaite,S. (1993) Ribosomal proteins L11 and L10.(L12)4 and the antibiotic thiostrepton interact with overlapping regions of the 23 S rRNA backbone in the ribosomal GTPase centre. *J. Mol. Biol.*, **234**, 1013–1020.
17. Gudkov,A.T., Gongadze,G.M., Bushuev,V.N. and Okon,M.S. (1982) Proton nuclear magnetic resonance study of the ribosomal protein L7/L12 in situ. *FEBS Lett.*, **138**, 229–232.
18. Cowgill,C.A., Nichols,B.G., Kenny,J.W., Butler,P., Bradbury,E.M. and Traut,R.R. (1984) Mobile domains in ribosomes revealed by proton nuclear magnetic resonance. *J. Biol. Chem.*, **259**, 15257–15263.
19. Hamman,B.D., Oleinikov,A.V., Jokhadze,G.G., Traut,R.R. and Jameson,D.M. (1996) Rotational and conformational dynamics of *Escherichia coli* ribosomal protein L7/L12. *Biochemistry*, **35**, 16672–16679.
20. Liljas,A. and Gudkov,A.T. (1987) The structure and dynamics of ribosomal protein L12. *Biochimie*, **69**, 1043–1047.
21. Traut,R.R., Lambert,J.M. and Kenny,J.W. (1983) Ribosomal protein L7/L12 cross-links to proteins in separate regions of the 50 S ribosomal subunit of *Escherichia coli*. *J. Biol. Chem.*, **258**, 14592–14598.
22. Traut,R.R., Dey,D., Bochkariov,D.E., Oleinikov,A.V., Jokhadze,G.G., Hamman,B. and Jameson,D. (1995) Location and domain structure of *Escherichia coli* ribosomal protein L7/L12: site specific cysteine crosslinking and attachment of fluorescent probes. *Biochem. Cell Biol.*, **73**, 949–958.
23. Montesano-Roditis,L., Glitz,D.G., Traut,R.R. and Stewart,P.L. (2001) Cryo-electron microscopic localization of protein L7/L12 within the *Escherichia coli* 70 S ribosome by difference mapping and Nanogold labeling. *J. Biol. Chem.*, **276**, 14117–14123.
24. Bocharov,E.V., Sobol,A.G., Pavlov,K.V., Korzhnev,D.M., Jaravine,V.A., Gudkov,A.T. and Arseniev,A.S. (2004) From structure and dynamics of protein L7/L12 to molecular switching in ribosome. *J. Biol. Chem.*, **279**, 17697–17706.
25. Mulder,F.A., Bouakaz,L., Lundell,A., Venkataramana,M., Liljas,A., Akke,M. and Sanyal,S. (2004) Conformation and dynamics of ribosomal stalk protein L12 in solution and on the ribosome. *Biochemistry*, **43**, 5930–5936.
26. Diaconu,M., Kothe,U., Schlünzen,F., Fischer,N., Harms,J.M., Tonevitsky,A.G., Stark,H., Rodnina,M.V. and Wahl,M.C. (2005) Structural basis for the function of the ribosomal L7/12 stalk in factor binding and GTPase activation. *Cell*, **121**, 991–1004.
27. Wool,I.G., Gluck,A. and Endo,Y. (1992) Ribotoxin recognition of ribosomal RNA and a proposal for the mechanism of translocation. *Trends Biochem. Sci.*, **17**, 266–269.
28. Endo,Y. and Wool,I.G. (1982) The site of action of alpha-sarcin on eukaryotic ribosomes. The sequence at the alpha-sarcin cleavage site in 28 S ribosomal ribonucleic acid. *J. Biol. Chem.*, **257**, 9054–9060.
29. Endo,Y. and Tsurugi,K. (1987) RNA N-glycosidase activity of ricin A-chain. Mechanism of action of the toxic lectin ricin on eukaryotic ribosomes. *J. Biol. Chem.*, **262**, 8128–8130.
30. Ban,N., Nissen,P., Hansen,J., Moore,P.B. and Steitz,T.A. (2000) The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. *Science*, **289**, 878–879.
31. Harms,J., Schluenzen,F., Zarivach,R., Bashan,A., Gat,S., Agmon,I., Bartels,H., Franceschi,F. and Yonath,A. (2001)

High resolution structure of the large ribosomal subunit from a mesophilic eubacterium. *Cell*, **107**, 679–688.
32. Schuwirth,B.S., Borovinskaya,M.A., Hau,C.W., Zhang,W., Vila-Sanjurjo,A., Holton,J.M. and Cate,J.H. (2005) Structures of the bacterial ribosome at 3.5 Å resolution. *Science*, **310**, 827–834.
33. Korostelev,A., Trakhanov,S., Laurberg,M. and Noller,H.F. (2006) Crystal structure of a 70S ribosome-tRNA complex reveals functional interactions and rearrangements. *Cell*, **126**, 1065–1077.
34. Agrawal,R.K., Linde,J., Sengupta,J., Nierhaus,K.H. and Frank,J. (2001) Localization of L11 protein on the ribosome and elucidation of its involvement in EF-G-dependent translocation. *J. Mol. Biol.*, **311**, 777–787.
35. Valle,M., Zavialov,A., Li,W., Stagg,S.M., Sengupta,J., Nielsen,R.C., Nissen,P., Harvey,S.C., Ehrenberg,M. *et al.* (2003) Incorporation of aminoacyl-tRNA into the ribosome as seen by cryo-electron microscopy. *Nat. Struct. Biol.*, **10**, 899–906.
36. Connell,S.R., Takemoto,C., Wilson,D.N., Wang,H., Murayama,K., Terada,T., Shirouzu,M., Rost,M., Schüler,M. *et al.* (2007) Structural basis for interaction of the ribosome with the switch regions of GTP-bound elongation factors. *Mol. Cell*, **25**, 751–764.
37. Gao,H., Sengupta,J., Valle,M., Korostelev,A., Eswar,N., Stagg,S.M., Van Roey,P., Agrawal,R.K., Harvey,S.C. *et al.* (2003) Study of the structural dynamics of the *E. coli* 70S ribosome using real-space refinement. *Cell*, **113**, 789–801.
38. Frank,J., Sengupta,J., Gao,H., Li,W., Valle,M., Zavialov,A. and Ehrenberg,M. (2005) The role of tRNA as a molecular spring in decoding, accommodation, and peptidyl transfer. *FEBS Lett.*, **579**, 959–962.
39. Powers,T. and Noller,H.F. (1990) Dominant lethal mutations in a conserved loop in 16S rRNA. *Proc. Natl Acad. Sci. USA*, **87**, 1042–1046.
40. Kunkel,T.A., Bebenek,K. and McClary,J. (1991) Efficient site-directed mutagenesis using uracil-containing DNA. *Methods Enzymol.*, **204**, 125–139.
41. Gay,P., Le Coq,D., Steinmetz,M., Berkelman,T. and Kado,C.I. (1985) Positive selection procedure for entrapment of insertion sequence elements in gram-negative bacteria. *J. Bacteriol.*, **164**, 918–921.
42. Sigmund,C.D., Ettayebi,M., Borden,A. and Morgan,E.A. (1988) Antibiotic resistance mutations in ribosomal RNA genes of *Escherichia coli*. *Methods Enzymol.*, **164**, 673–690.
43. Uchiumi,T., Hori,K., Nomura,T. and Hachimori,A. (1999) Replacement of L7/L12.L10 protein complex in *Escherichia coli* ribosomes with the eukaryotic counterpart changes the specificity of elongation factor binding. *J. Biol. Chem.*, **274**, 27578–27582.
44. Arai,K.I., Kawakita,M. and Kaziro,Y. (1972) Studies on polypeptide elongation factors from *Escherichia coli*. II. Purification of factors Tu-guanosine diphosphate, Ts, and Tu-Ts, and crystallization of Tu-guanosine diphosphate and Tu-Ts. *J. Biol. Chem.*, **247**, 7029–7037.
45. Nishimura,S., Harada,F., Narushima,U. and Seno,T. (1967) Purification of methionine-, valine-, phenylalanine- and tyrosine-specific tRNA from *Escherichia coli*. *Biochim. Biophys. Acta.*, 142133–142148.
46. Uchiumi,T., Honma,S., Endo,Y. and Hachimori,A. (2002) Ribosomal proteins at the stalk region modulate functional rRNA structures in the GTPase center. *J. Biol. Chem.*, **277**, 41401–41409.
47. Arai,K., Kawakita,M., Nakamura,S., Ishikawa,K. and Kaziro,Y. (1974) Studies on the polypeptide elongation factors form *E. coli*. VI. Characterization of sulfhydryl groups in EF-Tu and EF-Ts. *J. Biochem. (Tokyo)*, **76**, 523–534.
48. Moazed,D. and Noller,H.F. (1986) Transfer RNA shields specific nucleotides in 16S ribosomal RNA from attack by chemical probes. *Cell*, **47**, 985–994.
49. Moazed,D. and Noller,H.F. (1989) Intermediate states in the movement of transfer RNA in the ribosome. *Nature*, **342**, 142–148.
50. Sergiev,P.V., Lesnyak,D.V., Burakovsky,D.E., Kiparisov,S.V., Leonov,A.A., Bogdanov,A.A., Brimacombe,R. and Dontsova,O.A. (2005) Alteration in location of a conserved GTPase-associated center of the ribosome induced by mutagenesis influences the structure of peptidyltransferase center and activity of elongation factor G. *J. Biol. Chem.*, **280**, 31882–31889.
51. Frank,J. and Agrawal,R.K. (2000) A ratchet-like inter-subunit reorganization of the ribosome during translocation. *Nature*, **406**, 318–322.
52. Gao,H., Valle,M., Ehrenberg,M. and Frank,J. (2004) Dynamics of EF-G interaction with the ribosome explored by classification of a heterogeneous cryo-EM dataset. *J. Struct. Biol.*, **147**, 283–290.
53. Lill,R., Robertson,J.M. and Wintermeyer,W. (2000) Binding of the 3' terminus of tRNA to 23S rRNA in the ribosomal exit site actively promotes translocation. *EMBO J.*, **8**, 3933–3938.
54. Zavialov,A.V. and Ehrenberg,M. (2003) Peptidyl-tRNA regulates the GTPase activity of translation factors. *Cell*, **114**, 113–122.