Ribosomal Proteins at the Stalk Region Modulate Functional rRNA Structures in the GTPase Center*

Received for publication, July 24, 2002
Published, JBC Papers in Press, August 26, 2002, DOI 10.1074/jbc.M207424200

Toshio Uchiumi‡§, Sachiko Honma†, Yaeta Endo¶, and Akira Hachimori‡

From the §Institute of High Polymer Research, Faculty of Textile Science and Technology, Shinshu University, Ueda 386-8567, Japan and the †Department of Applied Chemistry, Faculty of Engineering, Ehime University, Matsuyama 790-8577, Japan

Replacement of the L10/L7/L12 protein complex and L11 in Escherichia coli ribosomes with the respective rat counterparts P0/P1/P2 and eukaryotic L12 causes conversion of ribosomal specificity for elongation factors from prokaryotic elongation factor (EF)-Tu/EF-G to eukaryotic EF (eEF)-1α/eEF-2. Here we have investigated the effects of protein replacement on the structure and function of two rRNA domains around positions 1070 and 2660 (sarcin/ricin loop) of 23 S rRNA. Protein replacement at the 1070 region in E. coli 50 S subunits was demonstrated by chemical probing analysis. Binding of rat proteins to the 1070 region caused increased accessibility of the 2660 and 1070 regions to ligands for eukaryotic ribosomes: the ribotoxin pepocin for the 2660 region (E. coli numbering), anti-28 S autoantibody for the 1070 region, and eEF-2 for both regions. Moreover, binding of the E. coli L10/L7/L12 complex and L11 to the 1070 region was shown to be responsible for E. coli ribosomal accessibility to another ribotoxin, gypsofilin. Ribosomal proteins at the 1070 region appear to modulate the structures and functions of the 2660 and 1070 RNA regions in slightly different modes in prokaryotes and eukaryotes.

Translation elongation is stimulated by actions of the two elongation factors EF-Tu and EF-G on a common site in the ribosome, termed the “GTPase center,” in a GTP-dependent manner. A picture of the ribosomal GTPase center has come from the facts that several ribosomal functions dependent on the GTP-binding factors are hampered by a single molecule of the antibiotic thiostrepton (reviewed in Ref. 1) or by the actions of ribotoxins α-sarcin and ricin, which cause single covalent modification into rRNA (reviewed in Ref. 2). The target sites of thiostrepton and ribotoxins have been determined on the highly conserved regions around positions 1070 and 2660 (Escherichia coli numbering is used throughout) of 23 S rRNA, respectively (1–5). Moreover, the 2660 region, termed the sarcin/ricin loop, has been identified as the site of interaction with both EF-Tu and EF-G (6–8) and the 1070 domain as the site with EF-G (7, 8) by biochemical approaches. These two RNA regions appear to play cooperatively a crucial role in the mechanism of translation elongation at the GTPase center.

The ribosome also contains an important protein component that participates in the GTPase-related functions: the acidic stalk protein termed L7/L12 in prokaryotes (9). There are two homodimers of L7/L12 anchored to another protein, L10 (10, 11). Selective deletion of this protein disrupts ribosomal functions dependent on elongation factors (12, 13). Cryoelectron microscopic studies have shown that EF-Tu and EF-G bind to the base region of the L7/L12 stalk in the ribosome (14–16). The L10/L7/L12 complex, together with another protein, L11, binds to the 1070 RNA domain of 23 S rRNA by direct interaction of L10 with the RNA (17–19). Therefore, it is conceivable that the function of L7/L12 is closely correlated with the 1070 RNA domain through L10. However, it is less easy to understand the molecular basis of functional correlation between L7/L12 and the 2660 RNA region. Hydroxyl radical probing has shown the close proximity of the sarcin/ricin loop to L11 bound to the 1070 region (20). Furthermore, current crystallographic and cryoelectron microscopic studies have clarified that the 1070 region and the sarcin/ricin loop are adjacent to each other in the ribosomal large subunit (21–23), although the exact structures of L7/L12, L10, and L11 have not been resolved by x-ray crystallography at 2.4-Å resolution (21). Considering the flexible nature of the L7/L12 region in the ribosome (24, 25), there may be a dynamic network of RNA-protein, RNA-RNA, and protein-protein interactions involving L10/L7/L12, L11, and the 1070 and 2660 RNA regions in the GTPase center.

Despite the highly conserved structures of the 1070 and 2660 RNA regions (26–28), the GTPase center of eukaryotic ribosomes differs from the prokaryotic one in the following aspects. 1) The eukaryotic GTPase center functions only with eukaryotic elongation factor (eEF)-1α and eEF-2, not with the prokaryotic equivalents EF-Tu and EF-G (29–31). 2) The rate of GTPase turnover in the eEF-2/80 S ribosome system is ~10-fold slower than in the prokaryotic EF-G/70 S ribosome system (32). 3) Some ribotoxins, including ricin, which depurinates the A2660 base, are highly specific for eukaryotic ribosomes (33). 4) An anti-28 S human autoantibody strongly binds to the eukaryotic 1070 RNA domain, but not to the prokaryotic domain (34). 5) The antibiotic thiostrepton is specific only for the prokaryotic 1070 RNA region associated with the prokaryotic protein L11 (1). These characteristics of the eukaryotic GTPase center are apparently ascribed at least in part to eukaryotic ribosomal proteins and their interactions with the rRNA.

The eukaryotic counterparts of prokaryotic L7/L12, L10, and L11 bound to the 1070 RNA region are believed to be P1/P2, P0, and eL12, respectively (35). Formation of a P0-P1/P2 complex
has been demonstrated in various eukaryotic ribosomes: human (36), Artemia salina (37), rat (38), and yeast (39, 40). We previously replaced E. coli L10/L7/L12 and L11 constituting the stalk of the 50 S subunits with rat P0/P1/P2 and eL12 and showed, by this replacement, the ribosome-activated functional interactions with eEF-1α and eEF-2 instead of prokaryotic EF-Tu and EF-G (see Fig. 1) (31). This implies that the ribosome-dependent interactions to the 1070 RNA domain are responsible for the kingdom-specific function of the GTPase center. This functional conversion is of interest in connection with the influence of the proteins on rRNA domains in the GTPase center. Here we show that the protein-dependent functional switch is accompanied by cooperative changes in some structural features of the 1070 and 2660 RNA regions of 23 S rRNA. From this study, we suggest an additional role of ribosomal proteins at the stalk region as modulators of the rRNA function in the GTPase center.

MATERIALS AND METHODS

E. coli Ribosomal Subunits and 50 S Core Particles—The L11-deficient mutant strain AM68 of Escherichia coli (41) was a gift from Dr. E. R. Dabbs (University of the Witwatersrand). The Q13 strain of E. coli was provided by Dr. A. Wada (Osaka Medical College). Ribosomes and the ribosomal subunits were prepared from both E. coli strains as described previously (31). The 50 S subunits deficient in L7/L12 protein (core I; see Fig. 1) were prepared from the Q13 50 S subunits according to Hamel et al. (12). The 50 S subunits deficient in L11 (core II) were from the AM68 strain. The 50 S particles deficient in both the L10/L7/L12 complex and L11 (core III) were prepared by extraction of core II in a solution containing 50% ethanol and 0.5 mM NH₄Cl at 0 °C as described previously (31). The 50 S core subunits and the released L7/L12 complex were recovered as described (12).

Ribosomal Proteins and Their Binding to E. coli Core Ribosomes—The rat P0/P1/P2 protein complex and eL12 were prepared as described previously (38). E. coli L11 was purified as described previously (42). In a typical experiment of rat protein binding to E. coli core particles, 20 pmol of 50 S core III subunits were incubated with 4 μg of P0/P1/P2 complex and 0.8 μg of eL12 in a solution (50 μl) containing 10 mM MgCl₂, 75 mM NH₄Cl, 0.2 mM dithiothreitol, and 50 mM potassium cacodylate, pH 7.2 (or 50 mM Tris-HCl, pH 7.5), at 37 °C for 5 min. In some experiments, E. coli L10/L7/L12 and L11 were added instead of the rat proteins. Incorporation of rat proteins into the E. coli core particles was confirmed on native agarose/acylamide composite gel (43) as described previously (31).

Chemical Probing with Dimethyl Sulfate—Ribosome samples (20 pmol) in 50 μl of 10 mM MgCl₂, 75 mM NH₄Cl, 0.2 mM dithiothreitol, and 50 mM potassium cacodylate, pH 7.2, were mixed with 1 μl of dimethyl sulfate (DMS; 1:4 dilution in ethanol) and incubated at 37 °C for 10 min. A portion of rRNA extracted from each sample with phenol was analyzed by primer extension and gel electrophoresis as described by Moazed and Noller (44). The primers used were 5'-GCTTCGGTGCAT-3' and 5'-GCTGGGCAAGTTTCGT-3' for the 2906 domain, complementary to residues 1139–1158 and 2758–2777 of 23 S rRNA, respectively.

Anti-28 S Antibody—Lupus serum containing a high titer of anti-28 S antibody (34), specific for the GTPase-associated RNA domain, was provided by Dr. Keith Elkon (Cornell University Medical College). The IgG was purified with a protein A-agarose column (Bio-Rad). To remove the low titer of antibodies to ribosomal proteins present in the serum, the isolated IgG was applied to a column of Affi-Gel 10 (Bio-Rad) coupled with rat total ribosomal proteins (38). The Fab fragments were prepared by papain digestion of the IgG (38). The undigested IgG and the Fc fragment were removed with protein A-agarose.

In Vitro RNA Synthesis and Gel Retardation—The DNA fragments containing 1030–1124 of E. coli 23 S RNA and the corresponding region of rat 28 S RNA were amplified by PCR (45) and inserted into the HindIII and XbaI sites of pSPST8 (Roche Molecular Biochemicals). The RNA fragments were synthesized using the plasmid DNAs and SP6 RNA polymerase as described previously (46). The [32P]RNA fragments (10 pmol) in 10 μl of solution containing 10 mM MgCl₂, 50 mM KCl, and 40 mM Tris-HCl, pH 7.6, were hybridized to 50 μl of L10/L7/L12 complex or rat P0/P1/P2 and 0.3 μg of E. coli L11 or rat eL12 and incubated at 30 °C for 5 min. The samples were then mixed with 12 μg of anti-28 S Fab fragments and incubated at 30 °C for 5 min. The RNA-protein complexes were separated on native 6% polyacrylamide gel as described previously (38).

Ribosomes and Their Treatments—The type 1 ribotoxin pepocin, which is highly specific to eukaryotic ribosomes, was purified from pumpkin, Cucurbita pepo (47). Another type 1 toxin, gypsophilin, which acts on prokaryotic as well as eukaryotic ribosomes, was purified from Gypsophila elegans (48). Ribosome samples (10 pmol) were incubated with pepocin or gypsophilin in 50 μl of 5 mM MgCl₂, 25 mM KCl, and 50 mM Tris-HCl, pH 7.6, at 37 °C for 20 min. Ribosomal RNA was extracted with phenol, and a portion of each sample was used as the template for primer extension as described above. Depurination at position 2660 by toxins was detected as a stop signal of primer extension by gel electrophoresis, followed by autoradiography. Levels of sensitivity for toxins were estimated by the intensity of radioactivity of the stop signals.

Assays for Ribosomal Activity—The eEF-2-dependent GTPase activity of the hybrid ribosomes was assayed in 20 μl of solution containing 2.5 pmol of hybrid 50 S subunits, 7.5 pmol of 30 S subunits (optimum for the activity), 3 μmol of [γ-32P]GTP (40–50 pmol/μmol), 0.5 μg of eEF-2, 7.5 mM MgCl₂, 25 mM NH₄Cl, 12.5 mM KCl, 0.2 mM dithiothreitol, and 50 mM Tris-HCl, pH 7.5, which was incubated at 37 °C for 10 min. The inorganic phosphate liberated was counted as described previously (32). The eEF-1α/eEF-2-dependent polyphenylalanine synthesis was performed in 100 μl of solution containing 5 pmol of hybrid 50 S subunits, 25 pmol of 30 S subunits (optimum for the activity), 10 μg of poly(U), 80 μg of E. coli tRNA pre-charged with 40 pmol of [14C]phenylalanine (400cpm/μmol), 9 mM MgCl₂, 80 mM NH₄Cl, 5 mM KCl, 50 mM Tris-HCl, pH 7.5, 0.2 mM dithiothreitol, 1 μg of eEF-2, and 4 μg of eEF-1α, which was incubated at 37 °C for 10 min. The polyprokaryotic EF-Tu/EF-G-dependent reaction was carried out under the same conditions, except that the reaction mixture contained 1 μg of EF-G and 4 μg of EF-Tu instead of eEF-1α and eEF-2. The polymerized radioactivity was counted as described by Möller et al. (13).

RESULTS

Replacement of L10/L7/L12 and L11 with Rat Counterparts on the 1070 RNA Domain—The L10/L7/L12 and L11 at their binding sites on the domain located around position 1070 of 23 S rRNA, we analyzed the RNA region by footprinting using DMS as a chemical probe (Fig. 2). Upon removing L11 and more notably upon removing both L10 and L11 and L7/L12, the reactivity of bases C1072, A1084, A1085, A1086, A1089, C1102, and A1103 with DMS was enhanced (Fig. 2A, lanes 3 and 4). The reactivity of A1088 was markedly enhanced by deletion of only L11, whereas A1046 was reactive in the absence of both L10/L7/L12 and L11. Addition of either rat P0/P1/P2 (lane 5) or eL12 (lane 7) to the 50 S core subunit protected the chemical modifications of all these bases, except A1046, A1089, and A1086. The modification of A1088 was protected only by eL12, whereas that of A1046 was protected by P0/P1/P2 and enhanced by eL12. A1070 was also enhanced by eL12. The rat proteins had no effect on modification of A1046.

We also tested the effect of the protein replacement at the 1070 RNA region on chemical modifications around position 2660 of 23 S rRNA. No effect was detected by chemical probing in the region including residues 2640–2770 (data not shown).

Protein Replacement Affects a Structural Feature of the 1070 RNA Domain—To examine the effect of the rat proteins on the 1070 domain of E. coli 23 S rRNA, we used an RNA probe, anti-28 S antibody from human autoimmune serum (34, 52), which recognizes a conformational epitope of the eukaryotic 1070 RNA domain (38). It has been also shown that the reac-
tivity of anti-28 S antibody with E. coli 50 S subunits is very low compared with that with HeLa and rat 60 S subunits (34). As shown in Fig. 3A, the anti-28 S Fab fragment did not bind to an RNA fragment covering the 1070 region of E. coli 23 S rRNA upon gel retardation (Fig. 3A, lane 2), although it bound strongly to the equivalent fragment of rat 28 S rRNA (38). By contrast, E. coli ribosomal proteins L10–L7/L12 (lanes 3 and 4) and L11 (lanes 5 and 6) bound to the E. coli RNA fragment. This binding of E. coli proteins had no effect on anti-28 S antibody binding (lanes 4 and 6). However, cross-binding of the rat P0-P1/P2 complex to the E. coli RNA (lane 7) resulted in accessibility of the RNA to the anti-28 S Fab fragment, as shown in a supershift (lane 8). Binding of rat eL12 (lane 9) also allowed the RNA weak access to the anti-28 S Fab fragment (lane 10).

Anti-28 S antibody bound to the hybrid ribosomes containing rat P0-P1/P2 and eL12 and inhibited the GTPase activity dependent on eEF-2 (Fig. 3B). Because anti-28 S antibody was prepared from polyclonal human IgG (see “Materials and Methods”), we performed an antibody absorption test to confirm that this inhibition was due to anti-28 S antibody, and not to some other antibody. The anti-28 S antibody sample was preincubated with the rat RNA fragment covering the 1070 region of 28 S rRNA to which anti-28 S antibody strongly bound (38). During this preincubation, the ribosomal GTPase activity was rescued from inhibition by the antibody (Fig. 3B). The results indicate that the inhibition of the activity results from binding of anti-28 S antibody to the 1070 RNA domain in the hybrid ribosome.

Effect of Protein Replacement at the 1070 Domain on the Sarcin/Ricin Loop—We also examined whether the protein replacement at the 1070 RNA domain affects the 2660 region, the sarcin/ricin loop, of 23 S rRNA. As a probe of the structure and function of the 2660 region, we used the type 1 ribotoxin pepocin, which efficiently depurinates the A2660 base of mammalian ribosomes and is 0.0001-fold less effective in E. coli ribosomes (47). We assayed the pepocin-dependent depurination of A2660 by primer extension (Fig. 4A). Depurination was detected neither in the E. coli 50 S subunit (lane 2) nor in the naked 23 S rRNA (lane 7) under conditions including 0.3 μM pepocin, an amount more than adequate to depurinate base 2660 of rat 60 S subunits (data not shown). Pepocin had no effect on 50 S core III subunits deficient in L10–L7/L12 and L11 (lane 3). Addition of rat P0-P1/P2 alone (lane 4) and eL12 alone (lane 5) to core III had no effect on pepocin accessibility. However, addition of these rat proteins together to core III caused sensitivity to pepocin (lane 6), indicating that binding of both the rat proteins to the 1070 RNA domain makes the E. coli 2660 RNA domain accessible to pepocin.

To demonstrate a contribution of the 2660 RNA domain to the function of the hybrid ribosomes, we tested the effects of pepocin treatment on the induced functions of the hybrid ribosomes. The activities of eEF-2-dependent GTPase (Fig. 4B) and eEF-1α/eEF-2-dependent polyphenylalanine synthesis (Fig. 4C) of the hybrid ribosomes were inhibited by the toxin treatment (0.3–1.2 μM). The same pepocin treatment had no effect on the activities of E. coli ribosomes dependent on prokaryotic EF-Tu and EF-G. Therefore, the 2660 RNA region affected by the rat proteins is apparently functional with eukaryotic elongation factors in the hybrid ribosome.

Binding of eEF-2 to the 1070 and 2660 RNA Regions in the Hybrid Ribosome—To examine the interaction of eEF-2 with the 1070 and 2660 RNA regions in the hybrid ribosomes, DMS footprinting analysis was performed (Fig. 5). Chemical modifications of bases around the 2660 (Fig. 5A) and 1070 (Fig. 5B) regions were analyzed by primer extension and gel analysis. Addition of eEF-2 and the non-cleavable GTP analog GMP-PNP to the hybrid ribosomes in which L10–L7/L12
and L11 were replaced with rat P0/P2 and eL12 resulted in protection of bases G2659, A2660, and A2665 against the modification (Fig. 5, lane 3). No protection was detected when GTP was substituted for GMP-PNP (lane 2) and when the intact E. coli ribosomes were used instead of the hybrid ribosomes (lanes 4–6). eEF-2/GMP-PNP protection was also observed at A1067 in the domain to which the rat proteins bound (Fig. 5B, lane 3), but not at the same base in the intact E. coli ribosomes (lane 5). The results are summarized in Fig. 5C.

L10/L7/L12 and L11 Affect the Sarcin/Ricin Loop of 23 S rRNA—Our results described above indicate a strong correlation between rat ribosomal proteins at the 1070 RNA domain and the functional state of the 2660 RNA region. These results pose the question of whether or not the E. coli counterparts L10/L7/L12 and L11 also influence the state of the 2660 RNA region in the 50 S subunit. To examine this point, we used another type 1 ribotoxin, gypsophilin, as a probe for the structure of the 2660 region in E. coli ribosomes. Unlike pepocin, gypsophilin is reactive with E. coli ribosomes at low concentrations; depurination of the A2660 base in 50 S subunits was detected at >1 mM toxin concentrations by primer extension (data not shown).

We used 4 nM gypsophilin to test the effects of L10/L7/L12 and L11 on ribosomal accessibility to the toxin. Levels of depurination of A2660 were estimated by the relative intensity of the bands corresponding to stop signals of primer extension (Fig. 6). Upon removing L7/L12 (core I) (Fig. 6A, lane 4) or L11 (core II) (lane 3), the level of depurination by gypsophilin decreased by 30% compared with that in the intact 50 S subunit (lane 2). Upon removing both L10/L7/L12 and L11 together (core III) (lane 5), depurination decreased by >80%, a level close to that in the naked 23 S rRNA (lane 6). Conversely, upon addition of isolated L10 (Fig. 6B, lane 3), the L10/L7/L12 complex (lane 4), or L10 and L11 (lane 5) to core III (lane 2), the depurination levels increased. Addition of the L10/L7/L12 complex together with L11 resulted in complete recovery of sensitivity to gypsophilin (lane 6). This core III ribosome reconstituted with L10/L7/L12 and L11 showed the activity of polyphenylalanine synthesis with EF-Tu and EF-G, and the level of activity was higher than 85% of the intact 50 S subunit (data not shown). It should be noted that all of the E. coli core and reconstituted samples showed no sensibility to pepocin up to 1.2 μM.
DISCUSSION

There is a "strong barrier" in the ribosome-translation factor interaction between prokaryotes and eukaryotes; prokaryotic 70 S ribosomes do not function in protein synthesis with the eukaryotic translation elongation factors, and eukaryotic 80 S ribosomes are inactive with the prokaryotic factors (29, 30). We recently changed this specificity by engineering E. coli ribosomes. Upon replacing the L10/L7/L12 complex in the 50 S subunits with the rat homolog P0/P1/P2, ribosomes became specific for eukaryotic factors and had polypeptide synthetic activity. Further replacement of E. coli L11 with the rat homolog eL12 enhanced polypeptide synthetic activity (31). These ribosomal proteins therefore seem to be crucially important for the ribosome-factor interaction. It is likely that direct interaction between the ribosomal proteins and elongation factors is involved in the kingdom-dependent specificity. In this study, we have provided evidence suggesting an additional contribution of the ribosomal proteins to the ribosome-factor specificity. The prokaryotic and eukaryotic ribosomal proteins appear to affect the structure and function of the sarcin/ricin loop around position 2660 as well as the 1070 region of 23 S rRNA in
slightly different ways. We infer that these protein-dependent modulations of rRNA domains are essential for ribosomal function in both eukaryotes and prokaryotes.

Effect of the Ribosomal Proteins on the 1070 RNA Region—The *E. coli* L10/L7/L12 complex and L11 bind to overlapping regions on the 1070 RNA domain of 23 S rRNA, as demonstrated by extensive chemical and RNase footprinting analyses (19, 53). Binding of L11 stabilizes a tertiary structure of the 1070 domain (54). However, the effect of L10-L7/L12 binding on RNA structure is not well understood, although the protein-de-
participate in the RNA-RNA interaction, not in the direct binding to L11, in the crystal structure of the RNA-L11 complex (55, 56). Therefore, both the L10-L7/L12 complex and L11 appear to be involved in the same tertiary packing of the RNA structure. This common effect of L10-L7/L12 and L11 on the RNA structure may explain their cooperative RNA binding (57).

It is of importance that either the rat P0-P1/P2 complex or eL12 protects the common 6 bases that are implicated in the RNA-RNA packing as described above. Both rat proteins seem to bind to the 1070 RNA domain in the 50 S core III subunit and to refold the tertiary structure, involving at least the common 6 bases instead of the E. coli ribosomal proteins. Furthermore, rat eL12 strongly protects A\textsuperscript{1088}. This is apparently due to recovery of the tertiary interaction between A\textsuperscript{1088} and A\textsuperscript{1109} that is important for stabilization of the RNA domain (54). The present results are in line with previous findings that binding sites for the rat P0-P1/P2 complex and eL12 are located in the regions of 28 S rRNA nearly equivalent to those for E. coli L10-L7/L12 and L11 in 23 S rRNA, respectively (38), and that the rat proteins cross-bind to an RNA fragment covering the 1070 domain of E. coli 23 S rRNA (32, 42).

Considering that the amino acid sequences of rat P0, P1/P2, and eL12 show only very low similarity to the respective E. coli counterparts L10 L7/L12, and L11 (35) and that the P0-P1/P2 complex binds to the 1070 RNA domain more tightly than the E. coli counterparts (Fig. 3A), it is likely that the binding of rat proteins has a different effect on the 1070 RNA domain than the of E. coli proteins. Enhancement of DMS modification of bases A\textsuperscript{1070} and A\textsuperscript{1089} (Fig. 2) may reflect characteristics of binding of rat eL12. Binding of the rat proteins to the 1070 RNA domain in 50 S subunits makes the ribosome insensitive to the antibiotic thiostrepton (31). This effect may reflect an alteration of the tertiary structure of the 1070 RNA domain by the rat proteins. However, because strong binding of thiostrepton seems to be due to direct interaction between thiostrepton and E. coli L11 (58), this drug may be not suitable as a conformational probe for the 1070 RNA domain. Here we used another ligand, anti-28 S autoantibody, which recognizes a conformational epitope of the 1070 RNA region of eukaryotic ribosomes. Strong binding of anti-28 S antibody to the E. coli 1070 RNA domain in the presence of rat P0-P1/P2 and its weak binding in the presence of eL12 (Fig. 3A) imply that both rat proteins affect the RNA structure, but that P0-P1/P2 makes a greater contribution to formation of the RNA epitope than eL12. It is unlikely that our results are caused by antibody binding to rat proteins for the following reasons. First, the antibody sample had no detectable reactivity with ribosomal proteins upon immunoblotting (data not shown); second, inhibition of the ribosomal activity by the antibody was completely prevented by pretreatment of the antibody sample with a rat RNA fragment covering the 1070 domain of 28 S rRNA (Fig. 3B); and third, addition of anti-P Fab fragments (anti-P is the most likely contaminant) to the RNA-P0-P1/P2 complex resulted in smearing of the shifted band (data not shown), unlike the clear band as shown in Fig. 3A (lane 8). From these lines of evidence, we infer that binding of rat P0-P1/P2 and eL12 to the E. coli 1070 RNA domain adjusts the tertiary structure to a eukaryote type, which is slightly different from the prokaryote type stabilized by E. coli L10-L7/L12 and L11.

It was unexpected that removal of L10-L7/L12 and L11 from the 50 S subunit had only a small effect on the DMS modification around an internal loop region comprising residues 1044–1050 and 1109–1101 that has been known as a major binding site for the E. coli L10-L7/L12 complex (19, 53). Only A\textsuperscript{1046} became accessible to DMS. As shown by crystallography (21), this region is stabilized by base pairs G\textsuperscript{1047}-A\textsuperscript{1111}, A\textsuperscript{1054}-G\textsuperscript{1110},

![Image](http://www.jbc.org/)

**Fig. 6.** Binding of E. coli ribosomal protein components to the 1070 RNA domain affects accessibility of the sarcin/ricin loop to the ribotoxin gypsophilin. A, the intact 50 S subunit (lane 2), core II (lane 3), core I (lane 4), core III (lane 5), and naked 23 S rRNA (lane 6) were incubated with 4 μM gypsophilin at 37 °C for 20 min. A portion of the rRNA extracted from each sample or from the intact 50 S subunit not treated with gypsophilin (lane 1) was assayed by primer extension and gel electrophoresis. Depurination levels were estimated by counting the radioactivity of the bands at position 2660 (bottom of the gel). The level of depurination in the intact 50 S subunit was taken as 100%. B, the intact 50 S subunit (lane 1), core III (lane 2), core III + L10 (lane 3), core III + L10-L7/L12 (lane 4), core III + L10 + L11 (lane 5), and core III + L10-L7/L12 + L11 (lane 6) were incubated with gypsophilin and analyzed described for A.
A1048-G1112, and A1505-C1109. These interactions appear to be maintained even after removal of L10-L7/L12 and L11 from the 50 S subunit. The other ribosomal constituents of the 50 S subunit seem to contribute to stabilization of this region, as discussed previously by Egebjerg et al. (19). Rat P0-P1/P2 and E. coli L10-L7/L12 presumably bind to the backbone structure around this region (38, 53) and affect the RNA tertiary structure as described above.

Effect of the Ribosomal Proteins on the 2660 RNA Region as Probed with Ribotoxins—The ribotoxin ricin recognizes a unique RNA conformation including a GAGA sequence around the 2660 region of 23 S/28 S rRNA (47, 48). We have tested the effect of the rat P0-P1/P2 complex and eL12 on toxin accessibility. As a probe for the 2660 loop, we used the ricin-like ribotoxins pepocin and gypsophilin, both of which depurinate A2660 in the large subunit rRNA (47, 48). Because pepocin acts preferentially on mammalian ribosomes; we did not detect depurination of A2660 in E. coli ribosomes with up to 1.2 μM pepocin. However, replacement of L10-L7/L12 and L11 in E. coli 50 S subunits with the rat P0-P1/P2 complex and eL12 caused ribosomal sensitivity to pepocin (Fig. 4) and to ricin. It is noteworthy that both of the rat ribosomal proteins were required for this effect. These observations suggest that tight and cooperative binding of the P0-P1/P2 complex with eL12 to the 1070 RNA domain results in the pepocin-sensitive state of A2660. It is unlikely that pepocin binds directly to the rat proteins and therefore to the hybrid ribosome because the ribosome carrying either rat protein alone showed no sensitivity to the toxin. Likewise, the insensitivity of E. coli ribosomes to pepocin is unlikely due to steric protection against pepocin by L10-L7/L12 and L11 because removal of these proteins did not induce sensitivity (Fig. 4). Because gypsophilin depurinates the A2660 base of E. coli 50 S subunits with lower efficiency than in rat 60 S subunits, this toxin was used to test the influence of the E. coli proteins on gypsophilin sensitivity. The depurination of A2660 depended on the presence of L10-L7/L12 and L11 (Fig. 6), implying that the RNA binding of these proteins is closely related to the sensitivity of E. coli ribosomes to gypsophilin. The evidence from these experiments with ribotoxins suggests that the ribosomal proteins bound to the 1070 RNA domain induce slightly different conformational states of the 2660 region in prokaryotes and eukaryotes.

It has been shown that protein L6 binds to helix 97 (46, 61) and that L6, together with L3, footprints bases A2657, A2662, C2666, and C2667 at the sarcin/ricin loop (46). Moreover, these proteins are adjacent to the sarcin/ricin loop in the crystal structure of the 50 S subunit (21). Therefore, L3 and L6 may affect the structural feature of the sarcin/ricin loop and participate in the RNA modulation by L10-L7/L12 and L11. L6-mediated modulation of the sarcin/ricin loop by L10-L7/L12 seems to be possible because the L10-L7/L12-binding site in the 1070 RNA domain makes contacts with helix 97, to which L6 binds directly, through C1049-G2751 base pairing (21). An action of L10-L7/L12 may be linked to L6 mediated by this RNA-RNA interaction.

Cooperation of the 1070 and 2660 RNA Regions in Ribosomal Function—A close correlation between the 1070 and 2660 RNA regions in E. coli ribosomes has been suggested by observations that binding of thiostrepton to the 1070 RNA region prevents the conformational cleavage of the 2660 loop region by α-sarcin (62) and that the N-terminal region of L11 taking part in strong thiostrepton binding (58) and also in EF-G binding (16) is located in close proximity to the 2660 loop region, as suggested by hydroxyl radical probing analysis (20). Moreover, the 1070 and 2660 RNA regions of 23 S rRNA have been demonstrated to be binding sites for the prokaryotic translocase EF-G in E. coli ribosomes. Our present footprinting study shows that EF-2 protects the 4 bases in the two RNA regions of the hybrid ribosome in the presence of GMP-PNP (Fig. 5), but not in the native E. coli ribosome. This protection is therefore dependent on replacement of E. coli L10-L7/L12 and L11 with rat P0-P1/P2 and eL12. These rat proteins seem to change the structural states of both RNA regions as discussed above and to make them functionally accessible to EF-2. Involvement of the two RNA regions in ribosomal function is also supported by the findings that the depurination of A2660 (Fig. 4) and anti-28 S antibody binding to the 1070 RNA domain (Fig. 3) inhibit the activity of hybrid ribosomes. The dual effects of the proteins on the 1070 and 2660 RNA regions strongly suggest that there is a close connection between the two RNA regions and ribosomal proteins bound to the 1070 region in the factor-dependent ribosomal functions. Information about these two RNA regions and the proteins that modulate their conformation and interactions will contribute to understanding the basic mechanism of translocation.

Acknowledgments—We thank Dr. Keith Elkon for providing anti-28 S lupus serum, Dr. Ryo Morishita (Ehime University) for helpful advice on conditions for ribotoxin treatments, and Dr. Robert R. Traut for reading the manuscript.

REFERENCES


17. Pettersson, I. (1978) in Nucleic Acid Res. 6, 2637–2646


2 T. Uchiumi, unpublished data.
Ribosomal Proteins at the Stalk Region Modulate Functional rRNA Structures in the GTPase Center
Toshio Uchiumi, Sachiko Honma, Yaeta Endo and Akira Hachimori

doi: 10.1074/jbc.M207424200 originally published online August 26, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M207424200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 59 references, 27 of which can be accessed free at http://www.jbc.org/content/277/44/41401.full.html#ref-list-1