Binding of Mammalian Ribosomal Protein Complex P0-P1-P2 and Protein L12 to the GTPase-associated Domain of 28 S Ribosomal RNA and Effect on the Accessibility to Anti-28 S RNA Autoantibody*

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We have investigated binding of rat ribosomal proteins to the “GTPase domain” of 28 S rRNA and its effect on accessibility to the anti-28 S autoantibody, which recognizes a unique tertiary structure of this RNA domain. Ribosomal protein L12 and P protein complex (P complex) consisting of P0, P1, and P2 both bound to the GTPase domain of rat 28 S rRNA in a buffer containing Mg2+. Chemical footprinting analysis of their binding sites revealed that the P complex mainly protected a conserved internal loop region comprising residues 1855–1861 and 1887–1889, whereas L12 protected an adjacent helix region encompassing residues 1867–1878 and 1887–1899. These sites are close to but distinct from the binding site for anti-28 S antibody determined previously. The bindings of P complex and L12 increased the anti-28 S accessibility, as revealed by gel retardation and quantitative immunoprecipitation analyses. In a Mg2+-eliminated condition, the RNA failed to bind to either anti-28 S or L12 but assembled into a complex under their coexistence. However, the RNA retained a property of binding to the P complex even in the absence of Mg2+, and this binding conferred high anti-28 S accessibility. These results indicated that the bindings of the P complex and L12 to their respective sites influenced the GTPase domain to increase the accessibility to anti-28 S. A possible RNA conformation adjusted by the protein bindings is discussed.

Despite extensive evidence for functional importance of rRNA molecules within the ribosome, it has been difficult to demonstrate biological activities of protein-free rRNA under physiological salt condition. This difficulty appears to be due to involvement of ribosomal proteins that induce and stabilize the higher order structure of rRNA (reviewed by Noller, 1991). Detailed knowledge of rRNA-protein binding and its effect on the RNA conformation is, therefore, important to elucidate the molecular basis of rRNA function. The “GTPase domain” within domain II of Escherichia coli 23 S rRNA is one of the best characterized portions (reviewed by Cundliffe, 1986; Egebjerg et al., 1990; Ryan et al., 1991; Rosendahl and Douthwaite, 1993). This RNA region has been identified as a site of interaction with elongation factor G (Sköld, 1983; Moazed et al., 1988) and with the antibiotic thiostrepton, an inhibitor of elongation factor-dependent processes in protein synthesis (Thompson et al., 1982; Egebjerg et al., 1989). Ribosomal protein L11 and the acidic protein complex L10(L12)2 cooperatively bind to this RNA domain (Dijk et al., 1979; Beaucerl et al., 1984) and construct a functional site of the 50 S subunit. L11 may participate in induction of a functionally important RNA conformation (Xing and Draper, 1995). On the other hand, the L10(L12)4 complex appears to affect the RNA conformation through cooperative binding with L11 (Rosendahl and Douthwaite, 1993).

The GTPase domain of eukaryotic 28 S rRNA has been also shown to interact with elongation factor 2 (Uchiumi and Kominami, 1994). However, its interaction with eukaryotic ribosomal proteins has been poorly characterized (Raué et al., 1990). We have presented an in vitro binding system for studying interaction between rat ribosomal proteins and the GTPase domain of 28 S rRNA (Uchiumi and Kominami, 1992). Rat proteins P1, P2, and P0 are structurally and functionally related to E. coli proteins L12 and L10, respectively (reviewed by Wool et al., 1996), and the complex P0(P1)2(P2)2 (P complex)1 is believed to be a counterpart of E. coli L10(L12)4 (Uchiumi et al., 1987). Furthermore, rat protein L12 is an equivalent of E. coli L11 (Suzuki et al., 1990). These rat proteins show specific binding to the GTPase domain of 28 S rRNA, and the affinity is higher than that of E. coli counterparts for 23 S rRNA (Uchiumi et al., 1995)2. It is therefore interesting to ask whether these mammalian proteins can induce a change in the RNA conformation to confer eukaryotic ribosomal characteristics.

Human autoantibodies have been successfully used to elucidate important cellular processes such as RNA splicing (Padgett et al., 1983), rRNA synthesis (Reimer et al., 1987), and protein synthesis (Uchiumi et al., 1991) and thus regarded as “reporter molecules” for functionally engaging structures within eukaryotic cellular macromolecules (Tan, 1989). We have used an anti-28 S autoantibody from lupus patients as a useful ligand for the GTPase domain of 28 S rRNA. This antibody binds to the RNA domain within mammalian ribosomes and inhibits the elongation factor-dependent GTPase activity (Uchiumi et al., 1991). Anti-28 S also binds to protein-free 28 S rRNA in the presence of Mg2+ (Uchiumi and Kominami, 1994). This implies that this antibody recognizes a conformational epitope of the RNA stabilized with Mg2+.

In the present article we characterize binding sites of mammalian ribosomal P protein complex and L12 within the GTPase domain of 28 S rRNA by using chemical probes. We also show that individual bindings of these ribosomal proteins affect the anti-28 S accessibility of the RNA. Our results sup-

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port a view that ribosomal proteins are involved in induction of the functionally important conformation of the GTPase domain.

MATERIALS AND METHODS

Ribosomal Proteins and Ribosomal RNA

Total ribosomal protein was obtained by extraction of rat liver ribosomes in 66% acetic acid, 33 mM MgCl$_2$ (Ogata and Terao, 1979). Preparation of the acidic ribosomal protein mixture P1/P2 and purification of proteins P0 and L12 were performed as described previously (Uchiumi and Kominami, 1992). A protein complex composed of P0, P1, and P2 (P complex) was formed by mixing these isolated proteins in the presence of 6 M urea, followed by dialysis against a renaturing buffer containing 300 mM KCl, 5 mM 2-mercaptoethanol, and 20 mM Hepes/KOH, pH 7.5 (Uchiumi and Kominami, 1992). Ribosomal RNAs were extracted from rat 80 S ribosomes with phenol. The 28 S rRNA was isolated by sucrose gradient centrifugation.

Anti-28 S IgG and Fab Fragments

The anti-28 S IgG was collected with a protein A-agarose column (Bio-Rad) from patients' sera provided from K. Elkon (Cornell University Medical College) and T. Sato (Niigata University School of Medicine). The IgG sample was applied to a column of Affi-Gel 10 (Bio-Rad) coupled with total ribosomal protein to remove a low titer of antibodies to proteins present in the serum. The eluted IgG showed no reactivity with any ribosomal proteins on immunoblotting. The Fab fragments were prepared using papain (Porter, 1959). The Fab fragment and undigested IgG were removed with protein A-agarose.

Chemical Modification and Primer Extension

Dimethyl Sulfate (DMS) and Kethoxal (KE) Modification—Ten pmol of 28 S rRNA was preincubated at 37 °C for 10 min in 50 μl of solution containing 5 mM MgCl$_2$, 300 mM KCl, 50 mM potassium cacodylate, pH 7.2. After addition of the P complex (4 μg) or L12 (1 μg), the mixture was incubated at 30 °C for 5 min. Chemical modification was started by addition of DMS (1 μl, 1:4 dilution in ethanol) or KE (2.5 μl, 37 mg/ml in water), followed by incubation at 30 °C for 15 min.

Carbodiimide (CMCT) Modification—Preincubation of the 28 S rRNA sample was in 30 μl of solution containing 5 mM MgCl$_2$, 300 mM KCl, 50 mM potassium cacodylate, pH 7.2. After addition of the P complex (4 μg) or L12 (1 μg), the mixture was incubated at 30 °C for 5 min. Chemical modification was started by adding CMCT (20 μl, 42 mg/ml in modification buffer), followed by incubation at 30 °C for 15 min.

Ethynitrosourea (ENU) Modification—Preincubation of the 28 S rRNA sample was performed in 50 μl of solution containing 5 mM MgCl$_2$, 150 mM KCl, 50 mM potassium cacodylate, pH 7.2. Modification was started by addition of ENU (2.5 μl, 234 mg/ml in ethanol), followed by incubation at 30 °C for 3 h. The other conditions in CMCT and ENU modification were as those in DMS and KE modification. The modified RNA was recovered as described by Moazed and Noller (1986). For the ENU-modified sample, another incubation at 50 °C for 10 min in 20 μl of 1 mM EDTA, 100 mM Tris-HCl, pH 9.0, was followed to split the ribophosphate backbone at the alkylated positions (Vlassov et al., 1987). Primer extension was performed according to the method of Moazed and Noller (1986) using a primer, 5′-GTATGGGC-CGGACGCTCCAG-3′ complementary to residues 1949–1968 of rat 28 S rRNA (Chan et al., 1983).

In Vitro RNA Synthesis

The DNA fragment containing residues 1841–1939 of rat 28 S rRNA was amplified by the polymerase chain reaction (Sakai et al., 1988) and inserted into HindIII and XbaI sites of an expression vector, pSP718 (Boehringer Mannheim). The plasmid DNA was linearized with XbaI and transcribed with SP-6 RNA polymerase in the presence of [32P]UTP (Uchiumi et al., 1991). The transcript was purified by gel filtration on a Sephadex G-50 column (Pharmacia Biotech Inc.) and renatured by incubation at 40 °C for 20 min in a buffer containing 20 mM MgCl$_2$, 350 mM KCl, 20 mM Tris-HCl, pH 7.5.

Gel Retardation Assay

The labeled RNA fragment (5 pmol) was incubated at 30 °C for 10 min in 10 μl of binding buffer containing 5 mM MgCl$_2$, 300 mM KCl, 20 mM Tris-HCl, pH 7.5, in the presence of P complex, L12, and anti-28 S Fab, as indicated in the figure legends. Electrophoresis was carried out using a 6% polyacrylamide nondenaturing gel (acylamide/bisacylamide ratio, 40:1) in 5 mM MgCl$_2$, 50 mM KCl, and 50 mM Tris-HCl, pH 8.0, which had been prerun for 30 min at 80 V and 4 °C. Samples were electrophoresed for 5 h at constant voltage with buffer recirculation. For binding analysis in the absence of MgCl$_2$, the same gel analysis was performed, except that 5 mM EDTA was added to the binding buffer and electrode buffer instead of 5 mM MgCl$_2$.

Immunoprecipitation

The labeled RNA fragment (5 pmol) was incubated at 30 °C for 10 min in 100 μl of the binding buffer containing 5 mM MgCl$_2$, 300 mM KCl, 20 mM Tris-HCl, pH 7.5, either in the presence of P complex and L12 or their absence. After addition of increasing amounts of anti-28 S IgG, incubation was continued for another 5 min. Each sample was then mixed with 100 μl of the same binding buffer containing a sufficient amount of protein A-Sepharose (Pharmacia) to adsorb total input IgG. The solution was incubated with gentle mixing at 4 °C for 1 h. The antibody-bound beads were washed three times with 500 μl of the binding buffer, and then the radioactivity was counted by a Beckman LS6000IC analyzer.

RESULTS

In Vitro Assembly of the P Complex—Protein P0 purified in the presence of urea became insoluble on removal of urea in the renaturation buffer. It remained, however, soluble without urea if the two acidic proteins P1 and P2 were present (Uchiumi and Kominami, 1992). In their mixture, P0, P1, and P2 proteins were expected to form a complex, because the mixture showed a strong activity to bind to the GTPase domain of 28 S rRNA. We have tentatively named it the "P complex" as a mammalian counterpart of the E. coli L10/L12$_2$ complex. This complex in the mixture was subjected to electrophoresis on a native polyacrylamide gel (Fig. 1A), providing a distinct band by Coomassie Brilliant Blue staining (Fig. 1A, lane 3). Neither P0 (Fig. 1A, lane 1) nor P1 and P2 (Fig. 1A, lane 2) gave such a band. To know whether this consisted of the three P proteins, P0, P1, and P2, protein components were isolated from this band, subjected to SDS-polyacrylamide gel electrophoresis, and then followed by immunoblots with anti-P antibody. This antibody recognizes a common carboxyl-terminal epitope of the three P proteins (Elkon et al., 1986). As shown in Fig. 1B, anti-P visualized the three P proteins, indicating successful in vitro formation of the complex of P0, P1, and P2. This P complex sample was used in the following RNA binding analysis.

Chemical Probing of Binding Sites for P Complex and L12—
Another ribosomal protein, L12 (a rat homologue of E. coli L11), also binds to the GTPase domain of 28 S rRNA (Uchiumi and Kominami, 1992). To map the binding sites of the P complex and L12 within the RNA domain, we used chemical footprinting as described by Moazed and Noller (1986). Three probes used for bases were DMS, KE, and CMCT. As shown in Fig. 2, the P complex binding completely protected bases A-1859, G-1921 and A-1922, and partially protected A-1876 and A-1887 from DMS modification (compare lane 4 with lane 3). Modification of U-1856 and G-1858 with CMCT were also observed in helix regions (see Fig. 5C). To demonstrate this accessibility, we used gel retardation analysis with a transcript covering residues 1841–1939 of rat 28 S rRNA (Fig. 6). Bindings of the P complex (Fig. 6, lane 2), L12 (Fig. 6, lane 3), and both the P complex and L12 (Fig. 6, lane 4) to the RNA fragment were detected as bands on the gel. Each band was further shifted by adding anti-28 S Fab fragments (Fig. 6, lanes 6–8). This indicated that anti-28 S bound not only to the protein-free RNA fragment (Fig. 6, lane 5) but also to RNA that had been bound with those proteins.

To know the effects of ribosomal protein bindings on anti-28 S accessibility, we used an immunoprecipitation assay (Fig. 7). Five pmol of 32P-labeled RNA fragment (3500 cpm) and the same amount of P complex-bound RNA, L12-bound RNA, and P complex- and L12-bound RNA were immunoprecipitated with increasing amounts of anti-28 S IgG, and their radioactivity was counted. The maximum radioactivity of the precipitate was 3300 cpm in all the samples. The values of IgG amounts that precipitated one-half of the maximum radioactivity of the precipitate were determined from the binding curves for individual samples (Fig. 7). In the protein-free RNA, the IgG amount for the regions protected by the P complex and L12, anti-28 S is expected to be accessible to these RNA-ribosomal protein complexes having assembled in vitro. To demonstrate this accessibility, we used gel retardation analysis with a transcript covering residues 1841–1939 of rat 28 S rRNA (Fig. 6). Bindings of the P complex (Fig. 6, lane 2), L12 (Fig. 6, lane 3), and both the P complex and L12 (Fig. 6, lane 4) to the RNA fragment were detected as bands on the gel. Each band was further shifted by adding anti-28 S Fab fragments (Fig. 6, lanes 6–8). This indicated that anti-28 S bound not only to the protein-free RNA fragment (Fig. 6, lane 5) but also to RNA that had been bound with those proteins.
respectively. Although the stimulation of anti-28 S binding was small, the results were reproducible in three assays.

The experiments described above were performed in the presence of Mg$^{2+}$, which seems to be required for the basal tertiary structure of the RNA domain (Uchiumi and Kominami, 1994). Gel retardation analysis under Mg$^{2+}$-eliminated conditions (Fig. 8) showed that the RNA had no detectable binding ability for anti-28 S (Fig. 8, lane 2) and L12 (Fig. 8, lane 4). However, a weak signal of the RNA-protein complex was detected by adding L12 and anti-28 S together (Fig. 8, lane 6), suggesting cooperative binding of L12 and anti-28 S to the GTPase domain. Even in this condition, the RNA retained a significant binding capability to the P complex (Fig. 8, lane 3). Further addition of anti-28 S Fab fragments to this RNA-protein complex caused a supershift (Fig. 8, lane 5), indicating a role of the P complex in enhancing anti-28 S binding. We infer that the P complex binding to the loop region make the adjacent region highly accessible to anti-28 S, presumably by inducing such a conformation as stabilized with Mg$^{2+}$ (Fig. 9). However, molecular details of this induction mechanism remain to be elucidated.

DISCUSSION

Protein Binding Sites in the GTPase Domain of Rat 28 S rRNA—In the present study, we have defined binding sites of the rat ribosomal P complex and L12 protein in the GTPase domain of 28 S rRNA using chemical probes (Fig. 5): the main P complex site lies within the internal loop of residues 1855–1861 and 1920–1922, whereas the L12 site lies within an adjacent helix region encompassing residues 1867–1878 and 1887–1899. The binding site of the anti-28 S autoantibody has been investigated previously (Uchiumi and Kominami, 1994). Footprinting analysis using chemical probes shows that the antibody protects bases corresponding to U-1877, G-1878, G-1879, and A-1909 located in two loop regions of residues 1876–1884 and 1904–1909 of rat 28 S rRNA. From this finding, we postulate that the two loops fold close together and construct the anti-28 S binding site (Fig. 9). The present study shows that L12 protects mainly the backbone phosphates rather than bases in the helix region. These footprinting data clearly distinguish the L12 binding site from the anti-28 S site, although both L12 and anti-28 S produce almost identical RNA fragments protected against RNase T1 digestion (Uchiumi et al., 1991; Uchiumi and Kominami, 1992). It can be presumed that L12 recognizes such a tertiary structure of the RNA region from one side and anti-28 S does from the other side, and both protect the same area from RNase digestion.

The isolated P0 protein shows an ability, although it is low, to interact with the RNA despite its poor solubility, whereas P1
and P2 proteins have no detectable RNA binding capability (Uchiumi and Kominami, 1992). These P proteins form a complex in vitro, named P complex, which has high ability to bind to the GTPase domain, probably through P0 moiety. The P complex mainly protects bases in the internal loop adjacent to the region for L12 and anti-28 S, suggesting that this protein recognizes base sequences in this loop region. This result is consistent with our previous observations as follows. The P complex shows no detectable binding ability to an RNA fragment containing only residues 1859–1921 lacking the loop region. Besides, P complex binding is abolished by G-1858-A to CU base replacements within this loop region (Uchiumi and Kominami, 1992).

The Protein-dependent Enhancement of Anti-28 S Accessibility of the GTPase Domain—In this study, we have also shown that the bindings of ribosomal proteins to the GTPase domain increase an affinity to anti-28 S antibody, which recognizes an RNA conformation of this domain (Uchiumi and Kominami, 1994). Although this enhancement is small (2–4 fold) under Mg\(^{2+}\)-containing conditions, the results are reproducible. It should be mentioned that this enhancement was estimated by quantitative immunoprecipitation, not conventional equilibrium dialysis or filter binding assays.

There are two possible interpretations for the protein-dependent enhancement of anti-28 S accessibility: (a) it derives from a protein-induced conformational change or stabilization of the RNA; and (b) it is due to direct interaction of the antibody with part of the bound protein(s), which contributes to the epitope. Recently, Xing and Draper (1996) investigated cooperativity between E. coli L11 (a homologue of rat L12) and thiostrepton in RNA binding and suggested that it is due to direct interaction between these two ligands. The data favor the latter possibility. However, the former interpretation seems to be more likely because of the following reasons. First, the en-
hancement of anti-28 S binding to the GTPase domain also occurred by *E. coli* L11 proteins, which is unlikely to participate in direct interaction with human autoantibody. Second, the antibody and the antibiotic thiostrepton are substantially different ligands, although both bind to a similar region of the GTPase RNA domain. Therefore, the binding feature of anti-28 S does not necessarily coincide with that of thiostrepton. In fact, compared with a large stimulation of thiostrepton binding by *E. coli* L11, the protein-dependent enhancement of anti-28 S binding is very small, implying the presence of distinct mechanisms between them. Third, no direct interaction was detected by immunoblotting and native gel analyses between the antibody and P complex-L12 proteins (not shown).

Assuming that the protein-dependent enhancement of anti-28 S binding is attributed to a change or stabilization of RNA conformation, it is likely that binding of rat L12 to one side of the L12-anti-28 S region (residues 1867–1914) increases the affinity of the other side to anti-28 S, and that binding of the P complex to the internal loop region of residues 1855–1861 and 1920–1922 increases accessibility of the L12-anti-28 S region to anti-28 S. Although the extent of the protein-dependent enhancement is small, this may be an important indication of a protein-induced fine tuning of RNA conformation.

**Conserved Protein Binding Features of the GTPase Domain**—

The present results are supported by data on the *E. coli* ribosome. *E. coli* protein L11, a homologue of rat L12, binds to the GTPase domain of 23 S rRNA (Schmidt et al., 1981) and protects 17 backbone riboses within the area of residues 1058–1085 against hydroxyl free radicals (Rosendahl and Douthwaite, 1993). This area corresponds to the region of residues 1869–1896 of 28 S rRNA. Sixteen phosphates in this area are also protected by the rat L12 protein against ethylnitrosourea, a reagent for backbone phosphates (this study). These protection data suggest that the L11-type protein recognizes the conserved tertiary structure of the RNA backbone. Binding of the antibiotic thiostrepton to the GTPase domain is markedly enhanced by the L11 binding. Thiostrepton thereby protects bases located in two loop regions of residues 1065–1073 and 1093–1098 of 23 S rRNA (Egebjerg et al., 1989), corresponding to loops of residues 1876–1884 and 1904–1909 in rat 28 S rRNA including the anti-28 S binding site. From this result, Egebjerg et al. (1990) proposed a model for the tertiary configuration of the L11-thiostrepton binding region in which the two loops protected by thiostrepton lie in close proximity. L11 and thiostrepton are presumed to stabilize such an RNA tertiary structure (Thompson et al., 1979; Draper et al., 1995; Xing and Draper, 1995). This is comparable with the fact that rat L12 and anti-28 S recognize a similar tertiary structure of the eukaryotic GTPase domain.

The *E. coli*-L8 complex (a counterpart of the rat P complex) binds to the GTPase domain of 23 S rRNA through L10 moiety (Pettersson et al., 1976; Pettersson, 1979; Beauclerk et al., 1984) and protects mainly the internal loop region comprising residues 1044–1050 and 1109–1111 against chemical reagents and nuclease (Egebjerg et al., 1990; Rosendahl and Douthwaite, 1993). This loop region is highly conserved; 7 of 10 bases are preserved between *E. coli* and rat. The corresponding loop region of rat 28 S rRNA is also protected by the P complex (this study). The binding of the *E. coli*-L8 complex appears to affect the adjacent L11-thiostrepton region through cooperative binding of L11 (Rosendahl and Douthwaite, 1993). However, it has not been investigated extensively whether the L8 complex itself plays a role in conformational modulation of the RNA. In this respect, it is interesting that the L8 complex binding increases reactivity of the ribose at position 1068 of the thiostrepton binding site (Rosendahl and Douthwaite, 1993). This result may reflect a conformational change in the L11-thiostrepton region by the L8 binding.

Comparison of results from the present study with data on the *E. coli* system leads to a conclusion that the feature of interaction between the GTPase domain and ribosomal proteins is well preserved from *E. coli* to rat. This is supported by cross-binding of *E. coli* L11 to the mammalian rRNA (El-Baradie et al., 1987; Uchiumi et al., 1995) and bindings of rat L12...
(Uchiumi et al., 1995) and the P complex3 to the E. coli rRNA. This conserved feature points to a considerable importance of the protein binding and its influence on the RNA conformation for the ribosome function.

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