The L8 protein complex consisting of L7/L12 and L10 in *Escherichia coli* ribosomes is assembled on the conserved region of 23 S rRNA termed the GTPase-associated domain. We replaced the L8 complex in *E. coli* 50 S subunits with the rat counterpart P protein complex consisting of P1, P2, and P0. The L8 complex was removed from the ribosome with 50% ethanol, 10 mM MgCl$_2$, 0.5 M NH$_4$Cl, at 30 °C, and the rat P complex bound to the core particle. Binding of the P complex to the core was prevented by addition of RNA fragment covering the GTPase-associated domain of *E. coli* 23 S rRNA to which rat P complex bound strongly, suggesting a direct role of the RNA domain in this incorporation. The resultant hybrid ribosomes showed eukaryotic translocase elongation factor (EF)-2-dependent, but not prokaryotic EF-G-dependent, GTPase activity comparable with rat 80 S ribosomes. The EF-2-dependent activity was dependent upon the P complex binding and was inhibited by the antibiotic thiostrepton, a ligand for a portion of the GTPase-associated domain of prokaryotic ribosomes. This hybrid system clearly shows significance of binding of the P complex to the GTPase-associated RNA domain for interaction of EF-2 with the ribosome. The results also suggest that *E. coli* 23 S rRNA participates in the eukaryotic translocase-dependent GTPase activity in the hybrid system.

Binding of translocases, EF$_1$-G-GTP in prokaryotes and EF-2-GTP in eukaryotes, to a specific site of the ribosome causes GTP hydrolysis that drives translocation of peptidyl-tRNA from the A-site to the P-site during protein biosynthesis (1–3). The binding site of the prokaryotic EF-G has been identified on the universally conserved regions, the “GTPase-associated domain” surrounding residue 1067 and the “sarcin/ricin loop” of residues 2653–2667 in 23 S rRNA (4, 5). Interaction of the eukaryotic EF-2 with the equivalent domains of 28 S rRNA has also been suggested (6–8). Despite the highly conserved features of the two RNA domains which interact with the translocases EF-G and EF-2, these factors are not interchangeable between prokaryotic and eukaryotic translational systems.

Another component, long implicated in the ribosomal interaction with the translocase, is the stalk protein L7/L12 in *E. coli*. This protein together with L10 forms a stable pentameric complex, (L7/L12)$_2$(L7/L12)L10, termed L8 (12), and this complex binds to the GTPase-associated domain of 23 S rRNA through its interaction with L10 (13, 14). The eukaryotic counterpart of the prokaryotic L8 is the “P complex” consisting of homodimers of P1 and P2 and monomeric P0 (15, 16). Involvement of these constituent proteins in the activity of eukaryotic EF-2 has been demonstrated in vitro (17, 18). An essential role of protein P0 for assembly of the complex into yeast ribosomes and for cell viability has also been shown in vivo (19, 20). Rat P complex reconstituted from isolated P1, P2, and P0 specifically binds to the GTPase-associated domain of 28 S rRNA, probably through P0 protein (21, 22). This binding site is nearly equivalent to that for the *Escherichia coli* L8 complex in 23 S rRNA, as determined by footprinting (23, 24). In contrast with this similar RNA recognition feature of the stalk protein complexes, amino acid sequence homology of each protein constituent is very low between prokaryotic and eukaryotic counterparts (25, 26). It is therefore presumed that the evolutionary divergence between prokaryotic L8 complex and eukaryotic P complex may parallel the lack of sequence similarity at the regions of EF-G and EF-2 interacting with their respective pentameric complexes.

We here attempt to replace L8 complex in *E. coli* ribosomes with the rat counterpart. The L8 complex is specifically detached from the GTPase-associated RNA domain within the ribosome in ethanol/NH$_4$Cl (12, 27), and subsequently the rat P complex is incorporated into the core ribosome. This replacement of the stalk protein complex changes the specificity of translocase interaction from prokaryotic EF-G to eukaryotic EF-2. The results demonstrate the functional significance of the ribosomal stalk protein complex and its binding to the GTPase-associated RNA domain for the ribosome-translocase interaction. The results also provide information about conserved features of rRNA involved in the translocase binding.

**MATERIALS AND METHODS**

Ribosomes and Core Ribosomes Lacking L8 Complex—*E. coli* Q13 cells (10 g) were ground with 20 g of alumina, and ribosomes were extracted with 20 ml of buffer A containing 10 mM MgCl$_2$, 20 mM NH$_4$Cl, 5 mM 2-mercaptoethanol, and 20 mM Tris-HCl, pH 7.5. The lysate was centrifuged twice for 30 min at 20,000 × g. The supernatant was layered on 1.2 M sucrose cushion in Buffer A and ultracentrifuged for 14 h at 45,000 rpm (160,000 × g) in a Hitachi P500A2 rotor at 4 °C. The ribosome pellet was suspended in 20 ml of Buffer B containing 10 mM MgCl$_2$, 0.5 M NH$_4$Cl, 5 mM 2-mercaptoethanol, and 20 mM Tris-HCl, pH 7.5. Ribosomes were pelleted by ultracentrifugation for 3 h at 45,000 rpm with the same rotor. The salt wash was repeated two more times, and the ribosome pellet was resuspended in buffer A and stored at −80 °C.

The L8 complex in *E. coli* 70 S ribosomes was removed according to...
FIG. 1. Extraction of L10-L7/L12 (L8) complex from E. coli 70 S ribosomes. The ribosomal proteins from 0.4 A_{260} units of intact 70 S ribosomes (lane 1), the core ribosomes treated in 0.5 M NHCl, 50% ethanol at 30 °C (lane 2), and the proteins (1.5 μg) released by the NHCl, ethanol extraction (see "Materials and Methods") were analyzed by 16.5% SDS-polyacrylamide gel electrophoresis. The gel was stained with Coomassie Brilliant Blue.

Hamel et al. (27) with some modifications, as follows. The salt-washed 70 S ribosomes (200 A_{260} units) in 2 ml of the extraction buffer consisting of 20 mM MgCl₂, 1 mM NHCl, 10 mM 2-mercaptoethanol, and 40 mM Tris-HCl, pH 7.5, were preincubated at 30 °C for 5 min. The solution was mixed with 1 ml of ethanol (prewarmed at 30 °C) with stirring at 30 °C. After 10 min, another 1 ml of ethanol was added, and stirring was continued for 5 min at 30 °C. The solution was centrifuged at 25,000 × g for 10 min, and the ribosomal pellet was dissolved in 2 ml of the extraction buffer. The same extraction was repeated once. Isolation of the resulting core ribosomes and split proteins was as described (27).

Preparation of Rat P Complex—The mammalian ribosomal P complex was reconstituted in vitro by mixing proteins P0, P1, and P2 isolated from rat liver ribosomes, as described previously (21). Formation of the complex was confirmed by 6% polyacrylamide gel electrophoresis under nondenaturing conditions (22).

Plasmid Construction and in Vitro RNA Synthesis—The DNA fragment comprising residues 1029–1127 (GTPase-associated domain) of the 23 S rRNA was amplified using the polymerase chain reaction (30) and inserted into HindIII and XbaI sites of an expression vector, pSP718 (Roche Molecular Biochemicals). The RNA fragment was synthesized with SP-6 RNA polymerase and isolated, as described previously (31).

Gel Retardation Assays—The [γ-32P]RNA fragment (5 pmol) was reannealed by incubation at 40 °C for 20 min in 5 μl of solution containing 20 mM MgCl₂, 300 mM KCl, 20 mM Tris-HCl, pH 7.5. After addition of protein samples, the mixture was incubated further at 30 °C for 10 min. RNA-protein binding was examined by electrophoresis in 2% polyacrylamide gel electrophoresis (28) (data not shown). Besides major proteins L7/L12 and L10, trace amounts of L1, L11, and S2 were also released from 70 S ribosomes by the extraction.

Preparation of Rat P Complex—The mammalian ribosomal P complex was reconstituted in vitro by mixing proteins P0, P1, and P2 isolated from rat liver ribosomes, as described previously (21). Formation of the complex was confirmed by 6% polyacrylamide gel electrophoresis under nondenaturing conditions (22).

Translocase-dependent GTPase Activity—The E. coli core ribosomes (2.5 pmol) were incubated with or without the rat P complex, as indicated in the figure legends, in 10 μl of a buffer containing 5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, pH 8.0, and 100 μg of EF-2 and 3 nmol [γ-32P]GTP (60–100 cpm/pmol). In the case of the prokaryotic EF-G-dependent GTP hydrolysis, the reaction was started by mixing the same buffer solution containing 0.5 μg of EF-G, and 30 nmol of [γ-32P]GTP (6–10 cpm/pmol). To expand lower activities dependent on EF-G, we used 3 nmol of [γ-32P]GTP with higher specific radioactivity instead of 30 nmol used for the prokaryotic EF-G-dependent activities. No marked difference of EF-G-dependent activity of rat 80 S ribosomes or hybrid ribosomes was observed between the two concentrations of GTP used (not shown). The reaction proceeded for 10 min at 37 °C and was stopped by adding 0.1 ml of solution consisting of 0.02 N silicotungstic acid and 0.02 N H₂SO₄. The solution was mixed with 0.1 ml of 5 mM sodium phosphate, pH 7.0, and 5% ammonium molybdate in 4 N H₂SO₄. The inorganic phosphate liberated was then extracted with 0.4 ml of isobutyl alcohol-benzene (1:1) (32) and counted in an Aloka liquid scintillation counter, LSC-1000. The data represented in Figs. 4–7 are means of 2–4 assays.

The Translocase EF-2 and EF-G—The mammalian translocase EF-2 was purified from pig liver, as described previously (33). E. coli EF-G was purified as described by Kaziro et al. (32).

RESULTS

The eukaryotic ribosomal P complex is believed to be the counterpart of prokaryotic L8 complex that constitutes the ribosomal stalk. We have recomposed the P complex from isolated rat proteins P0, P1, and P2 and shown its binding site in 28 S rRNA to be nearly equivalent to that for E. coli L8 complex in 23 S rRNA (22). We tested cross-binding of the rat P complex to the E. coli GTPase-associated domain by gel retardation using an RNA fragment comprising residues 1029–1127 (Fig. 2A). The P complex bound to the E. coli RNA domain (Fig. 2B, lane 3). Interestingly, the stability of this RNA-protein complex was higher than that with the cognate protein L8 (lane 2). This binding ability of the P complex for E. coli RNA was comparable with that for rat RNA (see Ref. 22).

To test whether the rat P complex binds to E. coli core ribosomes lacking the L8 complex (Fig. 1), the core ribosomes were incubated with the P complex and then analyzed by sucrose density gradient centrifugation and immunoblotting with anti-P monoclonal antibody reactive with P0, P1, and P2. The P complex bound to 70 S ribosomes and 50 S subunits, but not to 30 S subunits (Fig. 3A). These bindings were prevented by adding an excess amount of the RNA fragment encompassing the GTPase-associated domain (Fig. 3B). The results suggest that the rat P complex was incorporated into the E. coli ribosome through its binding to the GTPase-associated domain.
We termed the resultant particles “E. coli-rat hybrid ribosomes.” In this experiment, a significant portion of the core 70 S ribosomes was dissociated into 50 and 30 S subunits by ultracentrifugation in 15 mM MgCl₂ (upper panels), a condition that normally gives only 70 S couples. This dissociation occurred, irrespective of binding of P complex (see upper panels of Fig. 3, A and B), suggesting that removal of L8 complex from the 50 S subunits lowers their affinity for 30 S subunits.

The hybrid ribosomes as well as E. coli 70 S and rat 80 S ribosomes were tested for interaction with the translocase proteins by measuring their GTPase activity dependent on prokaryotic EF-G (Fig. 4A) and eukaryotic EF-2 (Fig. 4B). Intact E. coli (70S) and rat ribosomes (80S) had activities specific to prokaryotic EF-G and eukaryotic EF-2, respectively. The activity of the former was about 10-fold higher than the latter. The hybrid ribosomes (70S-P) showed eukaryotic EF-2-dependent activity (Fig. 4B) but not prokaryotic EF-G-dependent activity (Fig. 4A). The level of EF-2-dependent activity of the hybrid ribosome was comparable with that of rat 80 S ribosomes (Fig. 4B) but lower than that of intact E. coli ribosomes dependent on EF-G (Fig. 4A). The induction of EF-2-dependent activity of E. coli core ribosomes was apparently because of addition of the P complex. The P complex alone, however, was inactive (Fig. 5).

Induction of EF-2-dependent activity by mixing the E. coli core ribosomes and rat P complex was prevented by adding the RNA fragment encompassing the GTPase-associated domain, a competitor for the binding site of P complex (Fig. 6). This result is consistent with a failure of the hybrid formation by adding the RNA competitor (Fig. 3B) and suggests that binding of the P complex to the GTPase-associated domain is crucial for EF-2-dependent GTPase activity.

The antibiotic thiostrepton binds to the GTPase-associated domain of E. coli ribosomes and inhibits GTPase activity dependent on prokaryotic EF-G (35). This drug was used to test whether the RNA domain of E. coli ribosomes participates in EF-2-dependent GTPase activity in the hybrid ribosome. As shown in Fig. 7, the EF-2-dependent activity of the hybrid ribosome, unlike that of the rat 80 S ribosome, was inhibited by thiostrepton, although its inhibition efficiency was not as high as for the intact E. coli ribosome. This result suggests that the same RNA domain of E. coli 23 S rRNA is involved in the eukaryotic EF-2-dependent GTPase activity of the hybrid ribosome as well as in the prokaryotic EF-G dependent activity of the native 70 S ribosome.
L11, the protein responsible for efficient thiostrepton binding (35, 38), is absent from a part of ribosome population. However, this is unlikely, because addition of excess L11 at the time of the hybrid formation gave no marked effect on the thiostrepton resistance (data not shown). It is more likely that the binding of the heterologous P complex affects the RNA conformation of the thiostrepton/L11 binding site. If this is the case, the slight conformational change of the RNA domain may take part in the conversion of the translocase accessibility from prokaryotic EF-G to eukaryotic EF-2. We infer that the stalk protein complex may play a role not only in interacting directly with the translocase, but also in modulating the functional conformation of rRNA.

Studies by three dimensional cryo-electron microscopy (39) and directed hydroxyl radical probing (40) suggest that EF-G interacts with several sites of the ribosome including the region at the base of the stalk. It is implied that major structural features of the translocase binding site are conserved between eukaryotic and prokaryotic ribosomes except for the stalk protein complex. In fact, two highly conserved regions, the GT-Pase-associated domain and the sarcin/ricin loop of E. coli 23S rRNA have been identified as the EF-G binding sites by chemical footprinting analysis (4). The equivalent GTPase-associated domain (7) and the sarcin/ricin loop2 of rat 28S rRNA are also protected by binding of eukaryotic EF-2. A portion of the GTPase domain including residues 1056–1103 of E. coli 23S rRNA can be replaced with the homologous sequence of yeast 26S rRNA without significant loss of the EF-G-dependent GTPase activity of the E. coli ribosome (41). Directed hydroxyl radical probing analysis by Wilson and Noller has clarified proximity relationships between EF-G and several other rRNA elements whose secondary structures are conserved between prokaryotes and eukaryotes (40). Therefore, we infer that the stalk protein complex and several conserved rRNA regions are the main determinants of the translocase-dependent function in the ribosome.

The x-ray crystallographic studies have defined five domains of EF-G (42, 43). Domains I and II are homologous to those of EF-Tu, and domains III, IV, and V mimic the size and shape of the tRNA portion of the EF-Tu-aminoacyl-tRNA-GTP ternary complex (44). The cryo-electron microscopic study on the ribosome-EF-G complex has shown that domains I and V are located at the base of the stalk and also that there is an “arc-like” connection between the stalk and the G’ subdomain within domain I of EF-G (39). It seems to be likely that the kingdom-dependent specificity of factor-ribosome interaction rests on the appropriate matching of the stalk protein complex with the G’ domain of translocase.

2 T. Uchiumi, unpublished results.
E. coli-Rat Hybrid Ribosomes

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