Identification of Protein Synthesis Elongation Factor G as a 4.5 S RNA-binding Protein in Escherichia coli*

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Escherichia coli 4.5 S RNA is metabolically stable and abundant. It consists of 114 nucleotides, and it is structurally homologous to domain IV of mammalian signal recognition particle (SRP) RNA. In this study, we found two 4.5 S RNA-binding proteins in cell extracts by means of a gel mobility shift assay. One protein was identified as Ffh, which has been characterized as a 4.5 S RNA-binding protein. The other protein was separated from Ffh by two consecutive column chromatographic elutions and by monitoring the 4.5 S RNA binding activity. After the second chromatography, a dominant protein with an approximate molecular weight of 78,000 was associated with 4.5 S RNA binding activity. A sequence of the NH₂-terminal 19 residues of the 78-kDa protein was completely identical to that of the protein elongation factor G (EF-G) of E. coli, and further it cross-reacted with antiserum against E. coli EF-G. The results obtained using a synthetic oligo RNA corresponding to the 23 S rRNA defining the EF-G binding site indicated that 4.5 S RNA and 23 S rRNA are competitive in 4.5 S RNA binding and that a decanucleotide sequence conserved between them serves as a binding site for EF-G. Conservation of the SRP RNA binding activity of EF-G from Bacillus subtilis suggests that the binding of EF-G to SRP RNA is essential for its function.

Signal recognition particle (SRP)* is a cytosolic ribonucleoprotein that facilitates the targeting of presecretory proteins to the endoplasmic reticulum membrane (1–4). Mammalian SRP is composed of a 7 S RNA (7SL RNA, here referred to as SRP RNA) and six proteins (5, 6). Although SRP RNAs have been identified in all cells analyzed to date, including bacteria, archaee, and eukaryotes (7), their functions in vivo are little understood. SRP-like RNAs of eukaryotes consist of four domains (domains I–IV) based on the predicted structure of human SRP RNA. On the other hand, the length and secondary structure of eubacterial SRP RNAs vary. Almost all SRP RNA of eubacteria, including Escherichia coli 4.5 S RNA, can be folded into a single hairpin (8). This structure is considered to be homologous to domain IV of mammalian SRP RNA. In contrast, the secondary structure of SRP RNA from two Gram-positive bacteria, Bacillus subtilis (9, 10) and Clostridium perfringens (11), is strikingly similar to that of eukaryotic SRP RNA, although they lack domain III. Functional analyses of B. subtilis scRNA have indicated that additional domains (domains I and II) are needed for the formation of heat-resistant spores (12). Phylogenetic studies have revealed that the primary and secondary structures of domain IV are highly conserved throughout evolution (7, 8, 13). The evolutionary conservation of SRP RNA-like RNA suggests that its function must be essential. E. coli 4.5 S RNA, which is encoded by the fts gene (14), is 114 nucleotides long and essential for cell growth (15). The 4.5 S RNA is primarily transcribed as a precursor, and its maturation requires the tRNA processing activity, RNase P (16). Mature 4.5 S RNA is largely double-stranded. The primary and secondary structures of 4.5 S RNA are similar to those of the domain IV of the mammalian 7 S RNA. In E. coli, 4.5 S RNA binds to Ffh (P48) (17–19), which is homologous to the eukaryotic SRP54 protein, the SRP subunit that binds to signal sequences (20, 21). The molecular mass of E. coli Ffh is about 48 kDa and contains the distinct G and M domains (22, 23). Like mammalian SRP, the E. coli 4.5 S RNA-Ffh complex binds specifically to the signal sequence of presecretory proteins (24). By analogy to mammalian SRP, Ffh may serve as a key component in signal binding. Depletion of either Ffh or 4.5 S RNA affects translocation across the cytoplasmic membrane of several secreted proteins and results in the accumulation of precursors (17, 25). It has been suggested that the FtsY protein, encoded by the ftsY gene, participates in a possible SRP pathway in bacteria because of its homology with the α-subunit of the mammalian SRP receptor (26, 27). These results suggested that there was an SRP-SRP receptor-mediated transport system in bacteria (3, 28). However, pulse-chase experiments demonstrated that depletion of 4.5 S RNA affects the translocation of a limited set of secreted proteins (25). The defective process of a subset of secreted proteins cannot explain all of the indispensable functions of 4.5 S RNA in cell growth. Furthermore, about 25% of 4.5 S RNA can be precipitated by anti-P48 antiserum (17). Therefore, it is plausible that 4.5 S RNA has a function other than that as the ribonucleoprotein with Ffh. Concentration measurements showing that 4.5 S RNA is in a 30-fold molar excess to Ffh support this notion (29). It was initially proposed that 4.5 S RNA is directly involved in translation (15). In particular, in E. coli cells deficient in 4.5 S RNA, translationally defective ribosomes accumulated, and consequently the translational activity of 4.5 S RNA-depleted extract decreased (30). Initiation was particularly impaired in the depleted extract. Moreover, some mutations of EF-G, 23 S rRNA in the region defining the EF-G-binding site and of tRNA synthetases, reduce the requirement for 4.5 S RNA severalfold (31). By genetic and biochemical analysis of 4.5 S RNA-depleted cells, Brown (32) concluded that 4.5 S RNA acts immediately after ribosomal translocation.

To further investigate the function of 4.5 S RNA in translation, it is useful to identify and purify the 4.5 S RNA-binding protein from E. coli.

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‡The abbreviations used are: SRP, signal recognition particle; EF-G, elongation factor G; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); scRNA, small cytoplasmic RNA.
protein other than Ffh from E. coli cell extracts. Here, we report that EF-G binds to 4.5 S RNA and that 4.5 S RNA competes with 23 S rRNA in the region defining the EF-G-binding site.

**EXPERIMENTAL PROCEDURES**

Preparation of Cell Extracts from E. coli K12 Cells—E. coli K12 cells growing in L-broth containing 0.1% glucose were harvested 4 h after inoculation and suspended in buffer A (20 mM Tris-HCl (pH 7.8), 20 mM NH4Cl, 10 mM (CH3COO)2Mg, 5 mM 2-mercaptoethanol) at 4°C to volumes of 4 wet weight. The suspension was disrupted by sonication (3 min, 180 watts, 9 kHz), using a Koba Medical Application Co., Ltd. (Tokyo, Japan) 180 kubota model 200M (Kubota Medical Application Co., Ltd., Tokyo, Japan) at 4°C. The sonicate was centrifuged at 15,000 × g for 20 min at 4°C, followed by 260,000 × g for 2 h. Supernatants were fractionated with ammonium sulfate (38–68%). After centrifugation at 17,000 × g for 20 min, precipitates were suspended in buffer B (20 mM Tris-HCl (pH 7.8), 10 mM (CH3COO)2Mg, 5 mM 2-mercaptoethanol, 0.25 mM sucrose) and stored at –20°C until use.

In Vitro Synthesis of 32P-Labeled 4.5 S RNA of E. coli and Truncated Form of B. subtilis and C. perfringens scRNA Including Domain IV—The DNA fragments including domain IV of each RNA from E. coli, B. subtilis, and C. perfringens were placed under the control of the SP6 promoter. A 134-bp DNA fragment, encoding mature E. coli 4.5 S RNA, was amplified with the oligonucleotides, 5′-GAAGCAGTAGTTAGCTCAAGCC-3′ and 3′-GCCAGCT-3′. These six primers were designed to create HindIII and BamHI sites at the 5′ and 3′ ends of the resulting polymerase chain reaction products, respectively. Purified products were amplified with oligonucleotides, 5′-GAAGCAGTAGTTAGCTCAAGCC-3′ and 3′-GCCAGCT-3′. A 153-bp DNA fragment, corresponding to positions 96–230 of mature scRNA of C. perfringens, was amplified with oligonucleotides, 5′-GAAGCAGTAGTTAGCTCAAGCC-3′ and 3′-GCCAGCT-3′. These six primers were designed to create HindIII and BamHI sites at the 5′ and 3′ ends of the resulting polymerase chain reaction products, respectively. Purified products were first digested with both HindIII and BamHI, then inserted between the HindIII and BamHI sites in a HindIII–BamHI fragment of 134-bp (Promega, Madison, WI). The resulting plasmids were linearized by digestion with BamHI. The transcription reaction contained 40 mM Tris-HCl (pH 7.9), 6 mM MgCl2, 10 mM dithiothreitol, 2 mM spermidine, 50 µg/ml bovine serum albumin, 10 mM NaCl, 0.5 mM each of ATP, GTP, UTP, 2.5 µM CTP, 70 units of RNase inhibitor (Takara Shuzo Co., Ltd., Kyoto, Japan), 10 µCi of [α-32P]CTP (400 Ci/mmol, Amersham International, Buckinghamshire, UK), 1 pmol of linearized DNA fragment, and 35 units of T7 RNA polymerase (Takara Shuzo Co., Ltd., Kyoto, Japan). It was precipitated by ethanol twice. Purified RNAs were resolved in sterile, deionized water. The concentrations of radiolabeled RNAs were determined from an absorption spectrum at 260 nm.

**RESULTS**

E. coli Cells Express Two 4.5 S RNA-binding Proteins—To identify 4.5 S RNA-binding proteins in E. coli cell extract, an RNA mobility shift assay was conducted. Incubating E. coli cell extracts with 32P-labeled 4.5 S RNA, synthesized in vitro, led to the appearance of two complexes (complexes 1 and 2) that could be followed by nondenaturing PAGE (Fig. 1A). The amount of complex formed was dependent on the amount of protein added (lanes 2–7), and it was sensitive to proteinase K. This indicated that the complexes were composed of protein and 32P-labeled RNA. The binding specificity of these complexes was demonstrated by competition with unlabeled RNAs. The formation of the two complexes was significantly reduced by adding unlabeled 4.5 S RNA (Fig. 1B, lanes 1–5). When the unlabeled domain II region of B. subtilis scRNA, which is restricted to E. coli, was used as a competitor, the formation of complex 1 was not affected (Fig. 1B, lane 9). There is a difference between binding affinities of proteins consisting of complex 1 and 2 for 4.5 S RNA. A 65-fold molar excess of unlabeled 4.5 S RNA prevented the formation of complex 2 (Fig. 1B, lane 3), but diminished only 15% of complex 1. These data demonstrate that E. coli cells express at least two RNA-binding proteins with a different molecular mass and affinity for 4.5 S RNA.

Purification of 4.5 S RNA-binding Proteins from E. coli Cell Extract—After dialysis against buffer C containing 0.15 M KCl, the E. coli cell extract was applied to a DEAE Sephadex A-50 ion exchange column. The fractions were assayed for RNA binding activity by gel mobility shift analysis using an in vitro synthesized radioactive E. coli 4.5 S RNA transcript as the probe. As shown in Fig. 2, the two proteins that constituted complexes 1 and 2 were separable. Complex 1 consisted of protein(s) from fractions 28–40 (Fig. 2B). In addition, a small amount of a second complex (complex 2) with a lowered gel mobility was also present in fractions 28–40. When purified Ffh was used in the gel mobility shift analysis instead of the cell extract, a single-shift band appeared (Fig. 2B). The migration of this complex was identical to that of complex 2, indicating that Ffh protein is responsible for the formation of complex 2. To isolate the 4.5 S RNA-binding protein in complex 1, fractions 34–40 were combined and gel-fractionated. The fractions were also assayed for RNA binding activity, which was found between fractions 27 and 31 (Fig. 3A). When aliquots of these
Fractions were resolved by denaturing PAGE, the stained gel revealed a single predominant protein with an apparent molecular mass of about 78,000 (Fig. 3B). When the N-terminal region of the 78-kDa protein was sequenced, only the following was obtained, ARTTPAIRYNIGISAHID. A search of the non-redundant protein database of EMBL using the BLAST search program revealed that this 78-kDa protein is EF-G (Swiss Prot™ accession no. P02996). The identity of this 78-kDa protein with EF-G was confirmed by immunoblotting using rabbit anti- E. coli EF-G antiserum (Fig. 4). However, we could not exclude the possibility that a low level of contaminating protein in the fraction is responsible for the band shift. E. coli EF-G is a member of the GTPase protein superfamily, and the action of this factor is regulated by ribosomes (34). Moreover, the means of purifying EF-G from bacteria have been confirmed by monitoring ribosome-dependent GTP hydrolysis activity (35). To establish that EF-G indeed interacts with 4.5 S RNA, purified EF-G was derived by Dr. H. Noller (Thimann Laboratories, University of California at Santa Cruz) and analyzed by means of gel mobility shift. As shown in Fig. 5, with 32P-labeled 4.5 S RNA, a complex was formed with the purified EF-G, and the amount of shift band increased in proportion to the amount of purified EF-G added. Quantitative analysis shows that binding activity of the purified EF-G, based on the ribosome-dependent GTPase and given as femtomoles of 4.5 S RNA shifted per microgram of protein, is about 0.60. On the other hand, as shown in Fig. 3B, Sephadex G-100 chromatographic elution yielded only one major polypeptide (EF-G). Measurement of the protein contents in fraction number 29 revealed that the amount of protein used for the gel mobility shift assay in Fig. 3A is about 2 µg. The calculated binding activity of our purified EF-G (about 0.66) was nearly identical to that of EF-G purified based on the ribosome-dependent GTPase activity. The gel mobility shift band formed with purified EF-G migrates slightly faster than does complex 1 (Fig. 5, lanes 1 and 2). This might be due to a difference in the salt concentration, because the sample applied in lane 1 contained about 0.25M KCl (Fig. 2A). However, we cannot exclude the possibility that there are other proteins besides EF-G in complex 1 that help or modify the binding of EF-G to 4.5 S RNA. These results confirmed that EF-G is a novel 4.5 S RNA-binding protein. As described above, the action of EF-G is regulated by guanine nucleotide. We then examined whether or not adding GTP/GDP affects the binding activity of EF-G to 4.5 S RNA. Using 0.34 µg of purified EF-G, we performed a gel mobility shift assay as described under “Experimental Procedures” except that GTP/GDP were present at various concentrations. As shown in Fig. 6, the binding activity of EF-G was increased with the increasing amounts of GDP. At 2 mM, GDP increased the RNA-binding activity 2.5-fold. At more than 2 mM, since electrophoresis of shift band was disturbed, we could not quantify it. On the other hand, GTP did not increase the activity.

EF-G Interacts with Conserved Nucleotide Sequence between

**Fig. 2.** Partial purification of two 4.5 S RNA-binding proteins in E. coli cytoplasmic extract by ion exchange chromatography on a DEAE Sephadex A-50. Proteins extracted from E. coli cells were loaded onto a DEAE Sephadex A-50 column. Fractions of 3 ml were collected (A). Using 6 µl of each fraction, the 4.5 S RNA-binding activity was detected by a gel mobility shift assay using 32P-labeled RNA (B). Two RNA-protein mobility shift complexes (complexes 1 and 2) are indicated. Purified E. coli Ffh was analyzed as a control (E. coli Ffh).
the Domain IV of 4.5 S RNA and of 23 S rRNA—Two lines of evidence have shown that EF-G associates with a region of 23 S rRNA surrounding nucleotide 1068 (36, 37). Moreover, it is notable that the decanucleotide sequence from 1068 to 1077 of 23 S rRNA (5'-GAAGCAGCCA-3') is identical to the decanucleotide sequence from 58 to 67 of mature 4.5 S RNA (Fig. 7A), suggesting that EF-G recognizes the conserved structure, including this decanucleotide sequence, between 4.5 S RNA and 23 S rRNA. To examine this possibility, we synthesized the oligo-purified RNA (5'-AGGAUGUUGGCUUAGAAGCAGCAUCAU-3'), spanning residues 1054-1081 of 23 S rRNA, and used it as a competitor (Fig. 7B). The formation of complex 1 was significantly reduced by increasing the amount of unlabeled synthetic RNA, whereas it did not affect the formation of complex 2 (Fig. 7B).

B. subtilis EF-G also Binds to the Domain IV Region of Bacterial SRP RNAs—To establish whether or not EF-G generally contains SRP RNA binding activity, B. subtilis EF-G was purified as described for E. coli EF-G. Purified EF-G from B.

FIG. 3. Purification of protein(s) forming complex 1 by gel filtration over a Sephadex G-100 column. Eleven milligrams of the partially purified protein in pooled fractions 34–40 from the DEAE-Sephadex A-50 chromatography shown in Fig. 2 were concentrated and loaded onto a 1.5 x 54-cm Sephadex G-100 column. The fraction volumes were 1 ml. Using each 6 µl of fractioned samples, the 4.5 S RNA-binding activity was detected by a gel mobility shift assay (A). Proteins in 6 µl of each fraction were resolved by the electrophoresis on a 12.5% denaturing gel and stained with Coomassie Brilliant Blue (B). Proteins in the pooled fractions 34–40 shown in Fig. 2 were also analyzed (pooled fractions).

FIG. 4. Immunoblots of proteins fractionated from the Sephadex G-100 gel filtration. The fractions shown in Fig. 3 were resolved by electrophoresis on a 12.5% denaturing gel and blotted onto a polyvinylidene difluoride membrane. E. coli EF-G was detected by immunoblotting against anti-E. coli EF-G antiserum (a gift from Dr. Y. Kajiro, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama, Japan) followed by enhanced chemiluminescence. Purified EF-G was also analyzed (E. coli EF-G) as a control.

FIG. 5. Activity of 4.5 S RNA binding to the purified E. coli EF-G. After incubating 0.1 ng of 32P-labeled 4.5 S RNA with 0.5 (lane 2), 1 (lane 3), 3 (lane 4), and 6 µg (lane 5) of the purified EF-G (a gift from Dr. H. Noller, Thann Laboratories, University of California at Santa Cruz), samples were analyzed by a gel mobility shift assay. The position of the complex formed with EF-G is indicated. The controls were proteins in fraction 30 eluted from DEAE Sephadex A-50 (lane 1).

FIG. 6. Effects of GTP and GDP on the binding activity of EF-G to 4.5 S RNA. Prior to the gel mobility shift assay, 0.34 µg of the purified EF-G was incubated with various concentrations of the nucleotide at 10 °C for 30 min. The extent of 4.5 S RNA binding calculated by quantitative analysis of autoradiography is expressed as the percentage of 4.5 S RNA bound to EF-G in the absence of the nucleotide. Each plot was derived from the mean for three independent data sets.
subtilis was subjected to NH₂-terminal sequencing, which revealed only AREFLEKTRNIGIMAHIDA. This was completely identical to the deduced amino acid sequence from the nucleotide sequence of the B. subtilis EF-G gene.² To make a radioactive probe, the DNA fragments including only domain IV of B. subtilis and C. perfringens SRP RNAs were placed under the control of the SP6 promoter. Fig. 8 shows that B. subtilis EF-G interacted with not only B. subtilis scRNA but also with the corresponding regions of C. perfringens and E. coli SRP RNAs. These results are consistent with published data, showing that the function of bacterial SRP RNAs is interchangeable among species (38–41).

association of EF-G with 4.5 S RNA in E. coli Cells—To examine whether 4.5 S RNA can associate with EF-G in vivo, cell extracts were prepared as described under "Experimental Procedures," then immunoprecipitated with anti-E. coli EF-G antiserum. The potential 4.5 S RNA associated with the EF-G was extracted by phenol and examined by Northern hybridization. Fig. 9 shows one distinct band (lane 4) in the RNA associated with EF-G. This band did not appear in the presence of preimmune antiserum (lane 3). Moreover, ribonuclease digestion of the cell extracts abolished the band. Quantitation of the autoradiogram revealed that 10% of the total 4.5 S RNA associated with EF-G in vivo.

**Discussion**

EF-G promotes the translocation of peptidyl-tRNA and associated mRNA from the A to the P site after peptidyl transfer (42–45) and it interacts with ribosomes during protein synthesis. EF-G-dependent protection of the nucleotide positions A¹⁰⁶⁷ and A¹⁰⁶⁹ against chemical probes adds to increasing evidence that this region of 23 S rRNA is involved in EF-G-related interactions (37). We showed that EF-G directly binds to 4.5 S RNA. Moreover, we showed that the region of 4.5 S RNA, including the conserved decanucleotide sequence (5'-

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² H. Yoshikawa, K. Yasumoto, H. Lin, S. M. J. opp, Y. Ohashi, S. Kakinuma, K. Tanaka, F. Kawamura, H. Yoshikawa, and H. Taka-hashi, unpublished results.
GAAGCAGCCA-3'), competed with the 23 S rRNA region defining the EF-G-binding site. These data are consistent with the notion that the function of 4.5 S RNA in translation is associated with that of EF-G (31, 32). We considered the physical role(s) of 4.5 S RNA binding to EF-G. Based upon several observations, Brown (32) mentioned that 4.5 S RNA acts transiently at the ribosome rather than by being a stable component of a small subset of ribosomes and that 4.5 S RNA is associated with the ribosome following translation but prior to departure of EF-G. First, depletion of 4.5 S RNA confers fusidic acid resistance in E. coli (31). The antibiotic fusidic acid binds to the EF-G-ribose complex in combination with either GTP or GDP and stabilizes EF-G-GDP on the ribosome, preventing further elongation (46). Second, some mutations in genes for either EF-G or tRNA synthetase, and in 23 S rRNA encoding Ffh, EF-G and 4.5 S RNA in the gel mobility shift assay, overlap. Moreover, we could not detect ternary complex including Ffh, EF-G and 4.5 S RNA in the gel mobility shift assay, indicating that the two functions of 4.5 S RNA are separate.

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