Escherichia coli 4.5 S RNA is a structural homologue of signal recognition particle (SRP)\(^*\) in mammalian cells (1). Larsen et al. (2) proposed that the 300 nucleotides of mammalian 7 S-like RNA contain 8 helices (numbered 1 to 8). Based on the predicted secondary structure, almost all 7 S-like RNAs of eukaryotes and archaeabacteria are about 300 nucleotides long and contain 8 helices. In contrast to the structural integrity of these 7 S-like RNAs, eubacterial 7 S-like RNAs differ in size and have relatively little sequence identity (29%) outside of the conserved domain. However, eubacterial 7 S-like RNAs have an identical 22-nucleotide sequence within helix 8. A phylogenetic study has revealed differences in the length and secondary structure of 7 S-like RNAs between Gram-positive and Gram-negative bacteria. Almost all Gram-negative bacteria, including E. coli and Pseudomonas aeruginosa 7 S-like RNAs are around 120 nucleotides long and can be folded into a single hairpin, corresponding to helix 8 of mammalian 7 S RNA. The 7 S-like RNAs of Gram-positive bacteria, including Bacillus subtilis (3, 4) and Clostridium perfringens (5) consist of ~270 nucleotides, and the predicted secondary structure is strikingly similar to that eukaryotic 7 S-like RNA, although they lack helix 7. In eubacterial cells, 7 S-like RNA and Ffh protein, which is a homologue of SRP54, constitute a ribonucleoprotein particle and are involved in protein secretion (6–8).

On the other hand, we showed that 4.5 S RNA can bind to the protein elongation factor, EF-G, indicating that 4.5 S RNA is also involved in translation (9, 10). Indeed, 4.5 S RNA depletion causes a significant loss of translation (11–13), and suppressors of the 4.5 S RNA requirement reside in genes encoding components of the translation system, such as EF-G, 23 S rRNA, and tRNA synthetases (14, 15). In addition, 4.5 S RNA depletion leads to an increase in the amount of EF-G associated with ribosomes, suggesting that 4.5 S RNA is concerned with the mode by which EF-G associates with ribosomes (16). Therefore, we proposed that 4.5 S RNA is a bifunctional molecule that functions in translation and protein secretion by binding each protein. Because a ternary complex consisting of 4.5 S RNA, Ffh, and EF-G was undetectable in electrophoretic mobility shift assays, the binding site for both proteins overlaps in 4.5 S RNA. Wood et al. (17) demonstrated that nucleotides within the single-stranded region in helix 8 are important both for Ffh binding to the RNA and for optimal function of the RNA in vivo. This bulged structure is phylogenetically conserved among the 7 S-like RNA family (18). In contrast to accumulating information about Ffh binding, that structural features of 4.5 S RNA that are recognized by EF-G have not been investigated. The decanucleotide sequences from 1068 to 1077 of 23 S RNA (5'-GAAGCAGCCA-3') and from 58–67 of mature 4.5 S RNA are identical (9, 15, 16). The corresponding region of 23 S RNA is considered important for one of the EF-G binding sites (19–21). However, the decanucleotide alone is not sufficient for binding EF-G, suggesting that more higher level of structure of conserved bulge is also important for protein binding (9, 16). We investigated the RNA sequence and secondary structures affecting EF-G binding as follows. We performed deletion analysis of the 4.5 S RNA sequences, systematically altered bases and secondary structures, and assessed the consequences of these changes in vitro and in vivo. An NMR structural study of
E. coli 4.5 S RNA

FIG. 1. Predicted secondary structure and nucleotide sequence of E. coli 4.5 S RNA. The numbering corresponds to full-length E. coli 4.5 S RNA. Internal bulges are lettered A to E. A thick line marks the decanucleotide sequence identical in 4.5 S and 23 S RNAs.

a mutant 4.5 S RNA (A47U) suggested that the bulged structure is important for protein binding.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—Escherichia coli strain S1192 (Hfr relA spoT1 lacUV5 f conclusive was a gift from S. Brown (Department of Molecular Biology, University of Copenhagen, Denmark) (14). The 5′ portion of the normal chromosomal 4.5 S RNA gene in this strain has been replaced by a kanamycin-resistant determinant. The chromosome contains a second bacterial ffs gene that is expressed from the inducible Pta promoter as a part of a recombinant vector. A vector used to prepare the 32P-labeled RNA was pSP64 (Promega, Madison, WI). Plasmid pKK223-3 (-op) is a derivative of pKK223 (Amersham Pharmacia Biotech, Uppsala, Sweden), which contains the promoter sequence of the tacs promoter without the putative represor-binding site (16). Therefore, this plasmid can allow the gene cloned under the tac promoter to constitutively express in E. coli cells. The 4.5 S RNA genes with or without mutations were synthesized by annealing two chemically synthetic oligo DNAs encoding each mutant and introducing them as the BamHI/PstI sites of pKK223-3 (-op) or between the BamHI/HindIII sites of pSP64.

In Vitro Synthesis of 32P-labeled 4.5 S RNAs with or without Mutations—The RNA templates for in vitro transcription were generated by BamHI digestion of plasmid DNAs of the pSP64 series encoding wild-type or mutant 4.5 S RNA. 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, UTP, GTP, 25 µM CTP, 70 units of RNase inhibitor (Takara Shuzo Co., Ltd., Kyoto, Japan), 10 µl of [α-32P]CTP (400 Ci/mmol, Amersham Pharmacia Biotech, International, Little Chalfont, Buckinghamshire, UK), 1 pmol of linearized DNA fragment, and 35 units of SP6 RNA polymerase (Takara Shuzo). After reaction, RNA was precipitated with ethanol twice. Purified RNAs were resolved in sterile and deionized water. The concentrations of radiolabeled RNAs were determined from the specific activity of [α-32P]CTP incorporation into the transcripts. Prior to use, RNAs were renatured by incubation for 15 min at 65 °C followed by slow cooling to room temperature.

Production and Purification of Recombinant EF-G with a Tag Consisting of Six Consecutive Histidine Residues at the Carboxyl Terminal—E. coli EF-G mutant (EF-G2 (1–91)), with a deletion of all upstream sequence up to and including the second GTP-binding sequence element, was expressed and purified as described by Suzuma et al. (10). To express EF-G2 (1–91), E. coli M15 harboring both pREP4 (Qia- gen, Chatsworth, CA), and the plasmid encoding mutant EF-G was cultured on LB plates in the presence of 50 µg/ml of ampicillin. A single colony was inoculated into 20 ml of 2XTY medium containing 100 µg/ml ampicillin and 10 µg/ml kanamycin and then incubated for 16 h at 37 °C. A 10-ml inoculum of the overnight cultures was added to 1 liter of 2XTY medium containing 100 µg/ml kanamycin and 0.5 µg/ml of ampicillin. A single colony remaining on the filters were determined using a liquid scintillation counter (LS5000TA, Beckman).

RNA Synthesis and Purification for NMR Study—The 49-nucleotide fragment corresponding to the region from 29 to 77 of wild-type 4.5 S RNA (SRP49) was made by annealing two synthetic oligonucleotides. The oligonucleotides were designed to attach the T7 phage promoter sequence at the 5′ end of RNA coding region. The 49-nucleotide DNA fragment encoding a mutant 4.5 S RNA where A at position 47 of wild-type 4.5 S RNA is replaced by U was also constructed (A47U). The SRP49 and the mutant (A47U) were synthesized using an Ampliscribe T7 High Yield Transcription Kit (Epicentre Technologies Corporation). We prepared 32N-labeled RNA using 5N-NTPs (95.0 atom %, Nippon Sanso, Tokyo, Japan). After transcription, RNAs were purified by 15% polyacrylamide gel electrophoresis under denaturing conditions with 7 µm urea. The RNAs were eluted from gel in 0.3 M sodium acetate, precipitated with ethanol, and desalted by ultrafiltration using Centricon-3 (exclusion molecular weight 3,000) (Amicon).

NMR Experiments—The purified RNAs were incubated for 5 min at 95 °C and then cooled on ice. Samples containing 0.2–0.6 mM RNA were
minimum requirements of E. coli 4.5 S RNA for protein binding

FIG. 3. Protein binding activity of wild-type 4.5 S RNA and deletion mutants. A, sequences and possible secondary structures of E. coli 4.5 S RNA and deletion mutants. Predicted secondary structures of mutants were generated based on that of wild-type 4.5 S RNA. The numbering corresponds to full-length E. coli 4.5 S RNA. Internal bulges are lettered A–E. Boxed sequences of 5 and 10 nucleotides were added at both the 5’ and 3’ termini, respectively, to stabilize the terminal stem structure. B, RNA retention curves from filter binding assay. Binding activities of wild-type and mutant 4.5 S RNA were monitored by filter assays as described under “Materials and Methods.” Radioactivity retained on the filter was plotted against the concentration of EF-G added.

dissolved in 200 μl of 10 mM sodium phosphate buffer (pH 6.3) in 90% 
H2O, 10% D2O, and NMR spectra were measured using a DRX-500 
spectrometer (Bruker) at probe temperatures of 10–50 °C with asym- 
nmetrical NMR microtubes (Shigemi Co., Ltd., Tokyo, Japan). Solvent 
signals were suppressed by the jump-and-return pulse (22) for all 
experiments with pulse intervals of 65 μs. For one-dimensional mea- 
surements, the spectral width was 25 ppm, data points of 32 K were 
used, and the number of scans was 128. Prior to Fourier transforma- 
tion, line broadening of 3 Hz was applied. Two-dimensional NOESY 
experiments (23) were acquired with a mixing time of 150 ms. 512 free 
induction decays of 2 K data points were collected using the States-
TTPPI (time proportional phase incrementation) method (23); the 
number of scans was 256. Prior to Fourier transformation, the 1H-2H 
squared sine bell function for the t2 dimension and the 1H-2H squared sine bell function for the t1 dimension were applied, and zero-filling was applied to acquire real 2K × 1K spectra. For two-dimensional 15N-1H HMBC experiments, the interpulse delay was 4 ms, and 15N was decoupled according to the CARP (global optimized alternating-phase rectangular pulses) scheme (24) with a 90° pulse of 150 μs. Free induction decays (16 scans each) of 4 K data points in the t2 dimension were collected for 64 
data points in the t1 dimension in the phase-sensitive mode using the States-TTPPI method (23). The spectra of 2K × 256 data points were 
obtained by zero filling the t1 dimension and the 1H-2H squared sine bell function for both the t1 and t2 dimensions followed by Fourier trans- 
formation. The spectral width for 15N was 100 ppm.

FIG. 4. Growth restoration of E. coli 4.5 S RNA conditional mutants S1192 by introducing 4.5 S RNA derivatives. The growth of S1192-harbouring plasmid pK2223-3 (–op) expressing wild type (WT) (●) and mutants 1 (▲), 2 (▼), 3 (■), 4 (□), and 5 (●) was monitored by measuring absorbance at 660 nm.

RESULTS

Binding Activity of 4.5 S RNA to EF-G—E. coli 4.5 S RNA can bind both Ffh and EF-G. We examined the dose dependence of Ffh and EF-G Δ(1–91) binding upon full size 4.5 S RNA using a filter 
binding assay as described by Suzuma et al. (10). The binding affinity values (M0.5) for Ffh and EF-G, defined as the concentration 
that gave half-maximal binding, were 0.15 and 1.5 μM, respectively, 
indicating that the affinity of EF-G for 4.5 S RNA was lower than 
that of Ffh by approximately one order of magnitude.

Effects of Point Mutations in Conserved Bulge Structures on Protein Binding—Fig. 1 shows that E. coli 4.5 S RNA can be 
folded into a single hairpin. This structure is considered ho- 
mologous to domain IV of mammalian SRP RNA (7 SL RNA). 
In addition to the secondary structure, several nucleotides are 
highly conserved. These lie with the tetrancleotide loop and 
its adjacent single-stranded bulged regions. No other regions 
are universally conserved in the primary sequence of 7 SL-like 
RNAs. Moreover, among bacterial SRP RNAs, the numbers and 
structures of single-stranded bulges are conserved (25). A mu- 
tational study demonstrated that nucleotides within the bulge 
regions are important for Ffh binding to the RNA (26, 27). To 
determine the role of the bulge(s) and to identify which nucle- 
obides are essential for EF-G binding, we performed site-di-
rected mutagenesis studies on each bulge region. The ability of 
EF-G to bind the mutant 4.5 S RNAs was examined using a 
nitrocellulose filtration assay (Fig. 2). Within bulge A, point 
mutations at positions 47, 60, 61, 62, and 63 completely abol- 
ished binding, whereas those at G48U and G49C did not. A 
mutation from A to U at position 67, which lies within bulge B, 
also completely abolished the ability to bind EF-G, whereas 
those at G48U and G49C did not. A point mutation from A to U at position 67, which lies within bulge B, 
also completely abolished the ability to bind EF-G, whereas 
point mutations within the opposed bulged residues at posi- 
tions 39, 40, 41, and 42 did not. In contrast, all point mutations 
induced within bulges C, D, and E did not affect binding to 
EF-G. These results suggest that the nucleotide region that 
includes bulges A and B is necessary for EF-G binding.

Systematic Deletion of 4.5 S RNA to Produce the Minimal 
Binding Site for EF-G—As a prerequisite to structural studies 
of the RNA required for protein binding, a minimal RNA site 
for specific protein binding had to be developed. This was 
accomplished by the systematic deletion of entire bulges or 
portions of helices from wild-type 4.5 S RNA. Genes encoding 
bulges were constructed using annealing two complementary 
single-stranded DNAs. Each deletion mutant contained a com- 
plementary sequence at the 5’ and 3’ termini (5’-GGGGG-3’ 
and CCCCCACC-3’) to minimize the potential for alternative 
secondary structure formation as a result of deletion (Fig. 3A).

Mutants 1 and 2, in which bulges E or both D and E were 
deleted, bound to EF-G with wild-type affinity (Fig. 3B). An- 
other mutant with three deleted bulges (C, D, and E) also 
bound to EF-G with wild-type affinity. Mutant 3 consisted of 

FIG. 4. Growth restoration of E. coli 4.5 S RNA conditional mutants S1192 by introducing 4.5 S RNA derivatives.
bulges A and B in addition to the conserved domain IV, demonstrating that the structure of mutant 3 contains all the elements required for interaction with EF-G. To assess the importance of bulges A and B, we constructed mutants 4 and 5 that contained either bulge A or B in addition to the stem-loop structure. Fig. 3 shows that bulge A by itself has and can maintain appreciable binding affinity, whereas mutant 5 completely lost the activity. These results indicate that bulge A constitutes a minimal site in 4.5 S RNA required for binding to EF-G.

Function of the Deletion Mutant of 4.5 S RNA in Vivo—To examine the ability of the constructed deletion mutants shown in Fig. 3 to restore the growth of *E. coli* S1192 in the absence of IPTG, wild-type and deletion mutant 4.5 S RNA genes were inserted into plasmid pKK223–3 (-op) and introduced into S1192. The introduced genes were under the control of the tac promoter with a deleted operator sequence in all constructs. Therefore, the expression of 4.5 S RNA on the plasmid was independent of IPTG. The single copy of the 4.5 S RNA gene in the chromosome of S1192 is regulated by the *lac* repressor and requires IPTG for growth except when harboring a plasmid that provides 4.5 S RNA function. Therefore, growth in the absence of IPTG would be a good indicator of mutant function. The growth was maintained by a strain harboring wild-type 4.5 S RNA (Fig. 4) but was abolished by a plasmid without an insert in the absence of IPTG (data not shown), indicating that the phenotype of plasmid-borne 4.5 S RNA can be assessed using this *in vivo* system. The growth of transformants expressing mutants 1 and 2 was identical to that of wild-type 4.5 S RNA (Fig. 4). In contrast, mutants 4 and 5 no longer supported the growth of S1192 (Fig. 4). Mutant 3, which has decreased binding activity to EF-G, can restore the growth of S1192 but to a level of only half that of wild-type 4.5 S RNA.

These results indicate a close relationship between the protein binding and growth restoration activity of 4.5 S RNA-depleted cells.

Comparison of NMR Spectra of SRP49 and A47U Mutant—Fig. 5a shows two-dimensional $^{15}$N–$^1$H HMQC spectra (imino proton region) of the SRP49 (black) and A47U (red) at 25 °C. The imino proton resonances of G and U were distinguished by their corresponding $^{15}$N chemical shifts. The resonances were assigned by analyzing two-dimensional NOESY spectra as follows (data not shown). The imino proton resonances of three SRP49 G:U pairs U36:G70, U37:G39, and U45:G64 were identified by their unique chemical shifts and strong intra-base pair NOEs. The imino proton resonance of G53 in the GGAA tetraloop was also identified by its unique chemical shift (25, 28). Starting from these unique resonances, resonances were sequentially assigned by NOE connectivity as U36/G70-G4-G3-G2-G1, U45/G64-G44, and G53-G57-G58. The assignments of imino protons of SRP49 in this study were consistent with earlier NMR findings of SRP RNA (25, 28, 29). The imino proton resonances of three SRP49 G:U pairs U36:G70, U37:G39, G43, G49, U50, G54, G61, and G69 could not be observed or assigned because of broadening of the resonances, suggesting that these imino protons are not involved in hydrogen bonding. These results are summarized as the secondary structure shown in Fig. 5b. The spectra of SRP49 and A47U are almost identical except for three resonances (13.54, 13.25, and 13.02 ppm, Fig. 5a, red triangle) and the disappearance of the two resonances (11.85 and 11.34 ppm, Fig. 5a, black triangle). From the analysis of two-dimensional NOESY spectra, most of the imino proton resonances were assigned as described above (data not shown). The two missing resonances were assigned to a U45:G64 pair, because this pair is closest to the substituted residue (A47U) among the observed G:U pairs. This assignment was consistent with the fact that the resonances at 11.90 and 11.43 ppm were assigned to the U36:G70 pair based on the NOE connectivity of U36/G70-G4-G3-G2-G1. Although the new resonances could not be assigned sequentially because of the lack of NOEs, the resonance at 13.54 ppm was identified as an A:U pair because of the presence of a typical NOE between the imino proton of U and the H2 proton of A (data not shown). Because the U45:G64 pair disintegrated and a new A:U pair formed in the A47U mutant, we proposed the secondary structure shown in Fig. 5c, in which U45 bulges outward. The proposed secondary structure is similar to that of SRP49 except for the proposed EF-G binding region, which is consistent with the fact the spectra of SRP49 and the A47U mutant were almost identical except for five resonances. Accordingly, we suggest that the conformation around U47 in the A47U mutant differed from that of the corresponding region in SRP49 and that other regions were similar between the A47U mutant and SRP49. Thus, the loss of
EF-G binding activity in A47U may result from destruction of the active conformation around A47.

**DISCUSSION**

Our previous study suggested that 4.5 S RNA is dual function and participates in both translation and secretion by interacting with EF-G and Ffh, respectively (9, 16). Here we examined the region required for EF-G binding by introducing substitutions and deletions with the 4.5 S RNA coding region of the ffs gene. The effects of sequence elements and the secondary structure of phylogenetically conserved bulged regions were measured by mutagenic alteration of 4.5 S RNA. Changing any of the bulges C, D, or E did not significantly affect the binding activity. Moreover, even in bulge A or B, changes in nucleotide positions 39, 40, 41, 42, 48, and 49 did not reveal a key role in EF-G protein binding. In contrast, nucleotides 60, 61, 62, 63, and 64 in the conserved decanucleotide sequence were important for efficient binding because some mutations at this site almost completely abolished the binding. This result is consistent with our data showing that wild-type 4.5 S RNA can compete with 23 S RNA for binding to EF-G, but mutants with base substitutions in the decanucleotides cannot (9, 16). On the other hand, Wood et al. (17) demonstrated that point mutations within the bulged residues at positions 47, 48, 49, 61, and 62 completely abolish binding to Ffh, whereas mutations at positions 60 and 63 do not affect the binding. Taken together, these data suggest that nucleotides within single-stranded regions in bulges A and B are important for binding to both proteins and that the nucleotides essential for binding to each protein are overlapping but not identical. Our present data using deletion mutants indicated that mutant 2, consisting of 49 nucleotides (SRP49), is sufficient for protein binding and can restore the growth of 4.5 S RNA conditional mutant in the absence of IPTG. This 4.5 S RNA is the shortest among known SRP 7 S-like RNAs. Defining regions for EF-G binding would be useful in generating a minimal structure that would allow structural analysis by NMR. The structures of 24-, 28-, and 43-nucleotide fragments of 4.5 S RNA have been studied using multidimensional NMR (25, 29, 30). The crystal structure of the complex between the RNA-binding domain of Ffh (M domain) and the fragment of 4.5 S RNA has recently been determined at a resolution of 1.8 Å (31). This result demonstrates that in the symmetric loop (bulge A), stacking of five consecutive non-canonical base pairs generates a unique helical structure with a shallow minor groove. This groove in the symmetrical internal loop A (bulge A, in this study) is recognized by the α-helix-turn-helix (HTH) motif contained within the five α-helices of the Ffh M domain. Moreover, this groove was occupied by the hydrophobic segment of a neighboring M domain. In vitro chemical protection of 4.5 S RNA by Ffh showed that the protein mainly protects the 5′ side of domain IV, with highly conserved nucleotides A39, A47, G48, and G49 being the most protected (27). The nucleotides that are essential for EF-G binding were located opposite those important for Ffh binding. Therefore, remarkable conformational changes must occur in the binding region of the A47U mutant. Fig. 6 shows that at least three standard base pairs are stretched. Moreover, the amino groups of C46 and C62 were involved in base pairing, and the protruding phosphate backbone that may interact with proteins as a hydrogen bond acceptor in SRP RNA is different between the SRP49 and A47U mutant models. Thus, single changes can cause large conformational changes that may have brought about the loss of Ffh and EF-G binding affinity in the A47U mutant. Replacement of A47 with cytosine did not affect the binding activity (data not shown), suggesting that A47 is important for maintaining non-canonical helices rather than because it is recognized by proteins.

Here, we have defined the minimal structure of 4.5 S RNA for binding EF-G. Taken together with previous data reported by Wood et al. (17), these data suggest that Ffh and EF-G proteins recognize overlapping domains in the 4.5 S RNA. As mentioned above, an identical decanucleotide sequence is found in the EF-G binding sites of both 4.5 S and 23 S rRNA. Jovine et al. (34) indicated that the last five decanucleotide residues have almost identical conformations (root mean square deviation = 0.96 Å). With this structure resemblance, 4.5 S RNA can acquire the ability to compete with 23 S rRNA. Moreover, it is plausible that these sequences in both 4.5 S and 23 S rRNA act as a first binding site for EF-G. The biological function of the interaction between 4.5 S RNA and EF-G during translation remains to be determined. On the basis of all previous data including the structural comparison, we proposed that 4.5 S RNA might facilitate EF-G-GDP to be expelled from the ribosome by competing with 23 S rRNA for EF-G binding. However, depletion of 4.5 S RNA leads to the rapid inhibition of translation, and this loss of translation by depletion of 4.5 S RNA extends to the whole protein (13). Under normal conditions 4.5 S RNA is not a stable component of the ribosome, but once cells are treated with fusidic acid or viomycin, 4.5 S RNA comigrates with 70 S ribosome in a sucrose gradient. Under these conditions, distribution of Ffh protein was unchanged. Moreover, our quantitative analysis demonstrated that almost all Ffh protein in a cell makes a stable complex, and the amounts of this complex are unchanged through cell growth (10). Thus, the function of 4.5 S RNA in translation may be separable from that in protein secretion. Therefore, even if EF-G and Ffh can compete for 4.5 S RNA binding, this might not contribute to the function of 4.5 S RNA in translation.

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Minimal Functional Structure of *Escherichia coli* 4.5 S RNA Required for Binding to Elongation Factor G

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