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Important role of the tetraloop region of 4.5S RNA in SRP binding to its receptor FtsY

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ABSTRACT

Binding of *Escherichia coli* signal recognition particle (SRP) to its receptor, FtsY, requires the presence of 4.5S RNA, although FtsY alone does not interact with 4.5S RNA. In this study, we report that the exchange of the GGAA tetraloop sequence in domain IV of 4.5S RNA for UUCG abolishes SRP-FtsY interaction, as determined by gel retardation and membrane targeting experiments, whereas replacements with other GNRA-type tetraloops have no effect. A number of other base exchanges in the tetraloop sequence have minor or intermediate inhibitory effects. Base pair disruptions in the stem adjacent to the tetraloop or replacement of the closing C-G base pair with G-C partially restored function of the otherwise inactive UUCG mutant. Chemical probing by hydroxyl radical cleavage of 4.5S RNA variants show that replacing GGAA with UUCG in the tetraloop sequence leads to structural changes both within the tetraloop and in the adjacent stem; the latter change is reversed upon reverting the C-G closing base pair to G-C. These results show that the SRP-FtsY interaction is strongly influenced by the structure of the tetraloop region of SRP RNA, in particular the tetraloop stem, and suggest that both SRP RNA and Ffh undergo mutual structural adaptation to form SRP that is functional in the interaction with the receptor, FtsY.

Keywords: Ffh; prokaryotes; protein targeting; RNA structure; signal recognition particle

INTRODUCTION

The signal recognition particle (SRP) functions in cotranslational targeting of ribosomes synthesizing proteins with an N-terminal targeting signal to the membrane of the endoplasmic reticulum (ER) in eukaryotes and to the plasma membrane in prokaryotes (Walter & Johnson, 1994; Rapoport et al., 1996; de Gier et al., 1997). SRP recognizes an N-terminal targeting peptide emerging from the translating ribosomes, and, subsequently, the ribosome–SRP complex binds to the SRP receptor at the membrane. Compared to mammalian SRP (Walter & Blobel, 1983), bacterial SRP is of simpler composition and consists of one RNA (4.5S RNA) and one protein (Ffh), which share homologies with their respective eukaryotic functional counterparts, 7S RNA (Poritz et al., 1988) and SRP54 protein (Bernstein et al., 1989; Römisch et al., 1989). The majority of proteins secreted from bacteria appears to be exported posttranslationally by the SecB pathway, and the SRP pathway may be used for a few proteins only. However, the SRP pathway in *Escherichia coli* is essential for membrane insertion of several inner membrane proteins (Ulbrandt et al., 1997; Beck et al., 2000), and the SRP and SecB pathways use the same translocon for protein translocation (de Gier et al., 1998; Valent et al., 1998).

Ffh and the bacterial SRP receptor, FtsY, belong to the group of SRP-related GTPases. Their G domains contain an insertion, I box, in the effector loop, and an N-terminal four-helix N domain that is closely packed against the G domain (Freymann et al., 1997; Montoya et al., 1997). The M domain of Ffh contains the binding sites for the signal sequence and the SRP RNA (Keenan et al., 1998). The structure of the M domain of human SRP54 is similar (Clemons et al., 1999). SRP and FtsY were reported to moderately stimulate each other's

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GTPase activity (Powers & Walter, 1995), and a conformational change in the I box region of FtsY upon binding to SRP was demonstrated by fluorescence measurements (Jagath et al., 2000).

Eukaryotic SRP RNA (7S RNA) can be divided into four structural domains (I–IV) (Poritz et al., 1988) or eight helices (Larsen & Zwieb, 1991), of which the most conserved domain IV (or helix 8) is present in all SRP RNAs, including 4.5S RNA from *E. coli.* The structure of an RNA fragment comprising domain IV of *E. coli* 4.5S RNA has been determined by NMR (Schmitz et al., 1999a, 1999b). Genetic and biochemical analyses indicated that the conserved nucleotides present in the internal loop are important for Ffh binding (Wood et al., 1992; Lentzen et al., 1996). The crystal structure of the complex of domain IV RNA with an M domain fragment of Ffh shows that the protein interacts with both internal loops and that the structure of loop B in the complex differs from that of the free RNA (Batey et al., 2000).

The formation of the Ffh–FtsY complex strongly depends on the presence of 4.5S RNA (Kusters et al., 1995; Powers & Walter, 1995; Jagath et al., 2000), although the binary complex was reported to be formed, albeit slowly, in the absence of 4.5S RNA when an N-terminally truncated form of FtsY was used (Peluso et al., 2000). In the mammalian system, the SRP54-7S RNA complex is required for membrane targeting of ribosome-nascent chain complexes (Hauser et al., 1995). Mutations in the tetranucleotide loop and in the adjoining stem of *Schizosaccharomyces pombe* 7S RNA did not abolish function in vivo (Selinger et al., 1993). Here we report that similar base exchanges in the tetraloop and the adjoining stem in *E. coli* 4.5S RNA, which do not affect the affinity for Ffh, strongly influence the binding of SRP to its receptor FtsY, indicating that the structure of SRP RNA modulates the interaction of SRP with its receptor.

RESULTS

4.5S RNA mutants

E. coli 4.5S RNA has a highly base-paired secondary structure that is closed with a GGAA tetraloop hairpin adjacent to a stem of 4 bp (Fig. 1A). To study the role of the tetraloop region of 4.5S RNA in the interaction of SRP with its receptor, FtsY, 4.5S RNA mutants were constructed with various base substitutions in the tetraloop region (Fig. 1B). Furthermore, the GGAA tetraloop sequence was replaced with another tetraloop-forming sequence, UUCG, which forms a UNCG-type tetraloop. Other replacements were made by introducing all-pyrimidine sequences, UUUU and CUUC, which are found in domain IV hairpin loops of yeast and plant SRP RNAs (Althoff et al., 1994), respectively, and do not form a tetraloop structure. Finally, the stem adjoining the tetraloop was mutated by disrupting 1 or 2 bp and by reversing the C-G closing base pair to G-C.

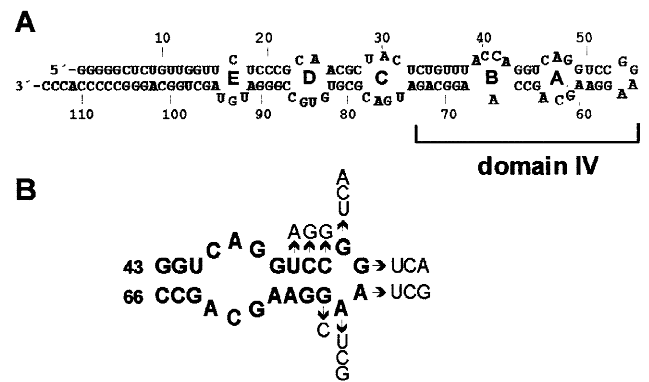

FIGURE 1. 4.5S RNA from *E. coli.* **A**: Secondary structure of 4.5S RNA. Internal loops are labeled A–E. **B:** Base exchanges introduced into the tetraloop region. Single or multiple mutations were introduced as indicated (arrows).

Ffh binding to 4.5S RNA mutants

The affinities of Ffh binding to 4.5S RNA and a number of 4.5S RNA mutants were measured by nitrocellulose filtration. The attainment of equilibrium was established by demonstrating that the same fraction of RNA was found in the complex when either the association of the free components or the dissociation of preformed complexes was measured (Fig. 2). From the titrations with wild-type 4.5S RNA and a number of mutants (see examples in Fig. 2), K_d values of 3–4 nM were obtained. Thus, the affinity of 4.5S RNA for Ffh was not signifi-

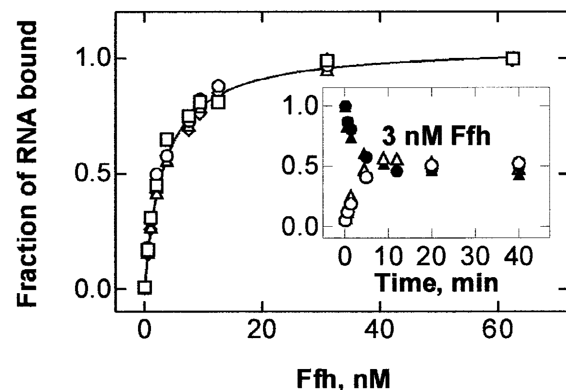

FIGURE 2. Nitrocellulose filter binding of 4.5S RNA mutants to Ffh. 5′-^{32}P-labeled 4.5S RNA (circles), 4.5S RNA(UUCG) (triangles), 4.5S RNA(CUUC) (squares), and 4.5S RNA(GUUCGC) (diamonds) (30 pM) were titrated with Ffh and complex formation determined by nitrocellulose filter binding. Saturation levels are normalized; actual levels varied from 65 to 85% of the input labeled RNA. Inset: Binding equilibrium assayed by association of 4.5S RNA (30 pM) and Ffh (3 nM) (open symbols) or by dissociation of the preformed complex (closed symbols) of Ffh with wild-type 4.5S RNA (circles) or 4.5S RNA(UUCG) (triangles) after dilution to the same concentrations.

cantly affected by the replacements of the wild-type GGAA tetraloop sequence with UUCG, UUUU, or CUUC. The same is probably true, at least qualitatively, for the other 4.5S RNA mutants, as shown by the results of the gel retardation and targeting assays presented below.

SRP-FtsY complex formation studied by gel retardation

The formation of the SRP-FtsY complex in the presence of GMPPNP could be studied by gel retardation (Kusters et al., 1995; Jagath et al., 2000). The results of the gel-shift experiments are summarized in Table 1; representative examples are shown in Figure 3. All 4.5S RNA variants studied formed a complex with Ffh, in keeping with the results of the titration experiments. However, SRP containing 4.5S RNA with UUCG or UUUU replacing the tetraloop sequence did not form a detectable complex with FtsY. Partial complex formation with FtsY was observed with CUUC (Fig. 3) and GAAU (not shown), indicating a weaker complex. SRP mutants with other variations in all four positions of the tetraloop sequence were not significantly impaired in complex formation, including a mutant in which GGAA was replaced with GAAA, which also forms a GNRA-type tetraloop structure. These results show that the nucleotide sequence in the domain IV hairpin loop of 4.5S RNA has no direct influence on the interaction of SRP with FtsY. Thus, the inability of SRP containing 4.5S RNA(UUCG) to form a detectable complex with FtsY most likely has to be attributed to structural alterations.

Another set of mutants was constructed by reversing the C-G base pair adjacent to the tetraloop to G-C. In the context of the GGAA tetraloop, reversal of the closing base pair had no effect on ternary complex formation with FtsY as studied by gel shift (data not shown). The same mutation in 4.5S RNA(UUCG) did not restore complex formation in the gel-shift assay (data not shown), although some activity was observed in the targeting assay (see below).

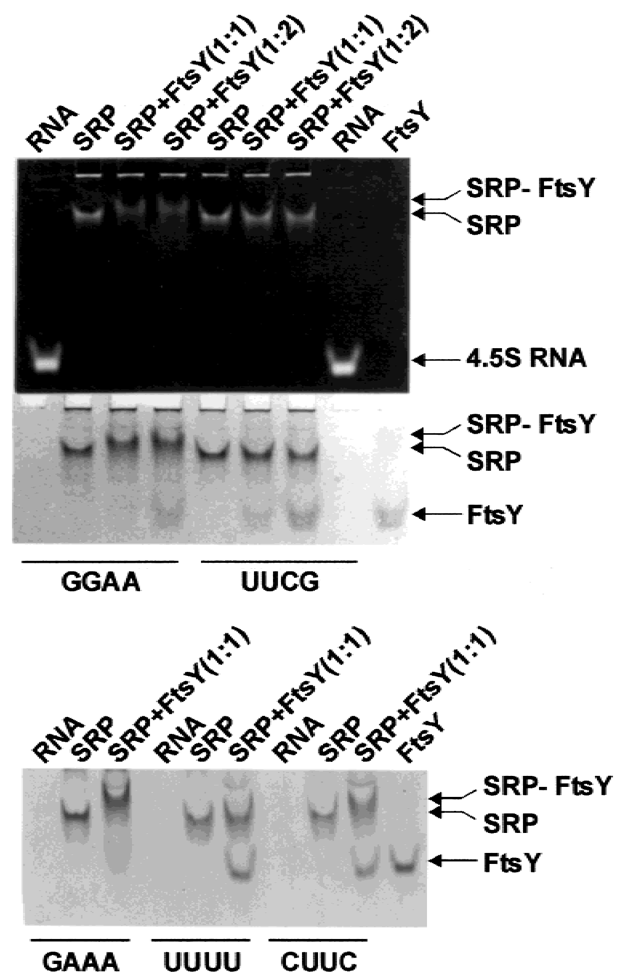

FIGURE 3. Interaction of SRP mutants with FtsY as analyzed by gel retardation. Top panel: RNA staining with ethidium bromide; lower panels: protein staining with Coomassie. Concentrations: 4.5S RNA: 2 μM; SRP: 2 μM; FtsY: 2 μM (1:1) or 4 μM (1:2).

TABLE 1. Influence of substitutions in the tetraloop region of 4.5S RNA domain IV on SRP-FtsY interaction.

Sequence GUCC(NNNN)GGAA	Gel Shift	Targeting Assay
Sequences forming tetraloops		
GGAA (wt)	+++	+++
GAAA[a]	+++	+++
UUCG	−	−
Sequences not forming tetraloops		
UUUU	−	+
CUUC	++	++
GUCG	nd	+++
UGAA[b]	+++	+++
GAAU	++	++

+++: high affinity, full complex formation/targeting with 1 μM FtsY; ++: intermediate affinity, partial complex formation/targeting with 1 μM FtsY; +: low affinity, partial complex formation/targeting with 10 μM FtsY; −: no complex formation/targeting at any condition.
wt: wild-type sequence; nd: not determined.
[a]Same activity with: GAGA, GGGA.
[b]Same activity with: AGAA, CGAA, GCAA, GUAA.

SRP interaction with ribosome nascent-chain complexes and membrane targeting

As a functional test of the interaction of SRP with ribosome nascent-chain complexes (RNCs) and with FtsY, we have used the protein targeting assay (Valent et al., 1998). RNCs were prepared in *E. coli* extracts by translating truncated mRNA coding for FtsQ, an integral inner membrane protein. SRP binding to RNCs is indicated by DSS crosslinking of the radioactively labeled nascent signal sequence to Ffh in SRP, and the productive interaction of the RNC-SRP complex with FtsY

is indicated by the disappearance of the crosslink in the presence of both FtsY and inverted vesicles of the *E. coli* plasma membrane containing the translocon (Valent et al., 1998).

In the control experiment with wild-type SRP, the nascent signal peptide was efficiently crosslinked to Ffh, as shown by the presence of the 62-kDa Ffh-NC crosslinked product, and the crosslinked product disappeared completely upon addition of FtsY, indicating successful targeting of RNCs to the translocon (Fig. 4); the results are summarized in Table 1. With SRP containing mutant 4.5S RNA(UUCG), as with the other mutants, crosslinking was quite efficient, indicating that binding of SRP to RNCs was undisturbed, but no release of nascent chain from SRP was observed upon addition of 1 μM FtsY (threefold excess over SRP). Also, in the case of the UUUU mutant, there was no efficient release of the nascent chain, and partial release was observed for the CUUC mutant. Single or double base substitutions at any position of the tetraloop sequence had no effect on the release of the nascent chain from SRP except for the GAAU mutant, where the release was somewhat less efficient.

Targeting assays were also performed at ten times higher concentration of FtsY (10 μM; 30-fold excess over SRP). Under these conditions, the extent of targeting was increased to full activity for 4.5S RNA with the CUUC and GAAU substitutions, and to partial activity with UUUU, whereas with UUCG, again no targeting was observed (Fig. 4; Table 1).

The crosslinking data show that none of the tetraloop mutations studied affected the binding of SRP to the nascent chain on the ribosome. The results of the targeting assays in the presence of FtsY, therefore, suggest that the exchange of the wild-type GGAA sequence of 4.5S RNA for UUCG decreases the affinity of SRP for FtsY to an undetectable level, and the exchange for CUUC, GAAU, and UUUU leads to intermediate affinities.

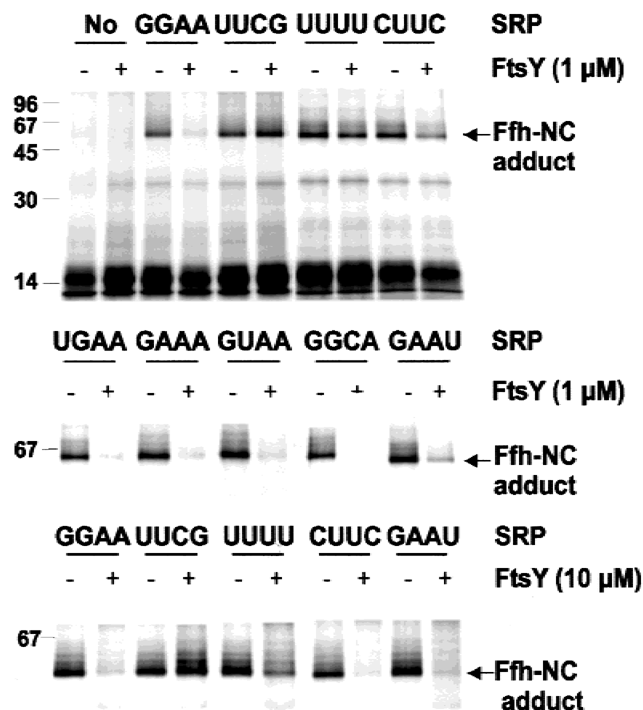

FIGURE 4. Release of RNCs from SRP by FtsY-dependent membrane targeting. SRP-RNCs containing 4.5S RNA with various tetraloop sequences were incubated with inverted vesicles and GMPPNP without and with 1 μM or 10 μM FtsY, as indicated. Samples were crosslinked with DSS and analyzed on 12% polyacrylamide gels in the presence of SDS (see Materials and Methods). Positions of marker proteins are indicated on the left (M_r/1000).

Alterations in the tetraloop stem partially restore activity

The thermal stability of tetraloop-containing hairpins is rather high, in particular when a UUCG tetraloop is closed with a C-G base pair (Antao et al., 1991; Antao & Tinoco, 1992), as in the UUCG mutant of 4.5S RNA studied here. It appeared possible, therefore, that the loss of FtsY interaction of SRP caused by the replacement of the wild-type GGAA tetraloop sequence with UUCG was due to the formation of a very stable structure in the tetraloop region. To examine the function of the stem adjacent to the tetraloop, the loop-closing C-G base pair was disrupted by the single-base exchanges C52G or G57C, or the stability was lowered by interchanging the loop-closing base pair from C-G to G-C. Furthermore, the following 2 bp in the stem were disrupted by simultaneously introducing the mutations U50A and C51G.

In the context of the GGAA tetraloop, all stem mutants exhibited wild-type activity in the targeting assay (Fig. 5) and showed strong SRP-FtsY complex formation in the gel-retardation assay (data not shown), indicating that neither the closing base pair nor the other base pairs of the stem are important for the interaction (Table 2). In the context of the UUCG tetraloop, disrupting the closing base pair by a single base exchange (G57C) in the adjoining stem partially restored the targeting activity, albeit only at a high concentration of FtsY (Fig. 5). The C-G to G-C transversion of the

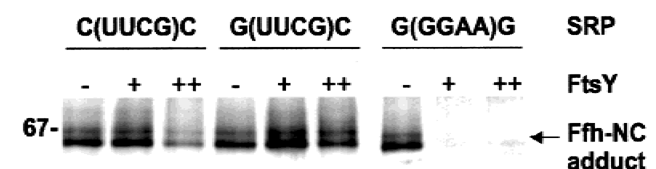

FIGURE 5. Influence of tetraloop stem mutations on targeting. Targeting reactions were carried out in the absence (−) and presence of 1 μM (+) or 10 μM (++) FtsY with SRP mutants with substitutions in the tetraloop stem of 4.5S RNA and 4.5S RNA(UUCG) (see Fig. 4). The position of a marker protein is indicated on the left (M_r/1000).

TABLE 2. Influence of mutations in the tetraloop stem of 4.5S RNA on SRP-FtsY interaction.

Sequence	Gel Shift	Targeting Assay
GUCC(GGAA)GGAA(wt)	+++	+++
GUCG(GGAA)GGAA	nd	+++
GUCG(GGAA)CGAA	+++	+++
GAGC(GGAA)GGAA	+++	+++
GUCC(UUCG)GGAA	−	−
GUCC(UUCG)CGAA	−	+
GUCG(UUCG)CGAA	−	(+)

+++: high affinity, full complex formation/targeting with 1 μM FtsY; +: low affinity, partial targeting with 10 μM FtsY; (+): very low affinity, slight targeting with 10 μM FtsY; −: no complex formation/targeting at any condition.
wt: wild-type sequence; nd: not determined.

closing base pair resulted in a slight recovery of targeting activity. The fact that the stem mutants of 4.5S RNA(UUCG) showed no targeting activity at a low concentration of FtsY and that the SRP-FtsY complex could not be detected by gel retardation even at a high concentration of FtsY indicates that the affinity of the interaction remained low.

Viability of *E. coli* cells expressing 4.5S RNA tetraloop mutants

To examine the function of the mutant alleles in vivo, the genes coding for precursor forms of 4.5S RNA containing various mutations were cloned into the plasmid pPre4.5Swt (see Materials and Methods) and transformed into *E. coli* FF283. In this strain, the chromosomal 4.5S RNA gene is under the control of the *tac* promoter. In the absence of IPTG, this strain fails to grow, unless the presence of plasmid-encoded 4.5S RNA restores growth to some extent (Struck et al., 1990). FF283 cells were transformed with plasmid constructs coding for either wild-type (pPre4.5S-GGAA) or mutant 4.5S RNA and plated on M9 agar plates (containing ampicillin). A single colony was inoculated in M9 medium containing glucose instead of lactose, and cells were grown at 30 °C. As shown in Figure 6, there was cell growth without inducer in the presence of wild-type plasmid (pPre4.5S-GGAA), whereas cells transformed with mutant plasmid (pPre4.5S-UUCG) did not grow. The remaining three mutant constructs restored little (pPre4.5S-UUUU and pPre4.5S-CUUC) or partial growth (pPre4.5S-GAAU).

These results show that the abilities of the variants of 4.5S RNA to support growth and to form SRP that is able to form a functional complex with FtsY are closely correlated. This suggests that the results on the SRP-FtsY interaction obtained in vitro also apply to the situation in vivo.

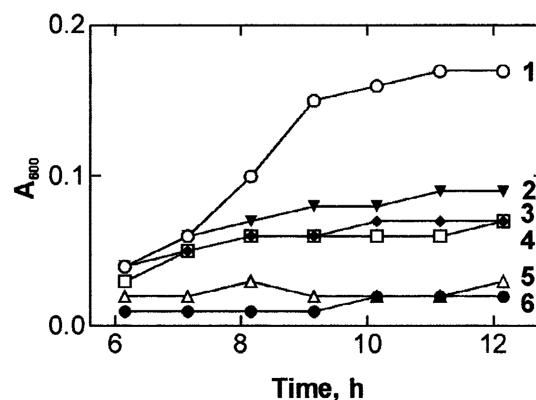

FIGURE 6. Growth of *E. coli* cells transformed with wild-type and mutant 4.5S RNA alleles. Single colonies of *E. coli* FF283 harboring wild-type (pPre4.5S-GGAA) or mutant pPre4.5S plasmid constructs were inoculated in the presence of ampicillin (see Materials and Methods). Cell growth at 30 °C was monitored by absorption at 600 nm; indicated times are hours after inoculation. 1: GGAA (wild type); 2: GAAU; 3: UUUU; 4: CUUC; 5: UUCG; 6: control without plasmid (no ampicillin added).

Chemical probing of 4.5S RNA

Chemical probing by both hydroxyl radical cleavage and methylation by dimethyl sulfate were used previously to study the structure of free and Ffh-bound 4.5S RNA (Lentzen et al., 1996). Here we have used chemical probing to reveal structural differences between three tetraloop variants of 4.5S RNA, that is, C(GGAA)G (wt); C(UUCG)G; G(UUCG)C, in the free state and in the complexes with Ffh in the absence or presence of FtsY. As shown in Figure 7, the pattern of hydroxyl radical cleavages changed significantly when the wild-type tetraloop sequence, GGAA, was replaced with UUCG. Extensive differences were observed in the tetraloop in that in the GGAA sequence, the first two positions were cleaved much more readily than the following two, whereas in the UUCG tetraloop, the last position was the most accessible for cleavage, with either a C-G or G-C closing base pair. Thus, the differ-

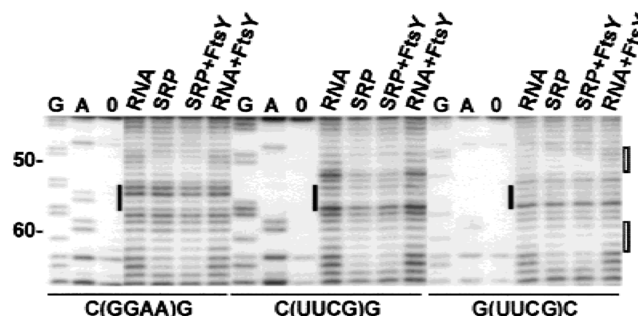

FIGURE 7. Hydroxyl radical probing of 4.5S RNA variants. G, A: sequencing lanes; 0: untreated RNA. The respective tetraloop sequences are indicated by closed bars, regions protected by Ffh binding by open bars.

ences in the structural details of the two types of tetraloops (Cheong et al., 1990; Heus & Pardi, 1991) are reflected in different cleavage patterns. The differences extend into the stem adjacent to the tetraloop, in that a much stronger cleavage was observed at position 51 in the tetraloop stem of the C(UUCG)G construct as compared to the wild type. Interestingly, this cleavage was no longer seen with G(UUCG)C where the closing C-G base pair was reverted to G-C, indicating that in the latter construct, the structure of the tetraloop stem was similar to the wild-type structure. Upon binding of Ffh, similar protection patterns were seen with the three 4.5S RNA constructs, indicating protections by Ffh of the 5′ side of the tetraloop stem (nt 49–51) and of the 3′ side of the adjacent symmetric internal loop A. The addition of FtsY did not introduce any change in the probing patterns, which is consistent with the notion that FtsY does not interact with the RNA directly.

The protection pattern of 4.5S RNA against DMS modification by Ffh binding, in particular the strong protection of A47 (Lentzen et al., 1996), was similar for the three constructs studied and did not change upon binding of FtsY (data not shown).

DISCUSSION

Most SRP RNAs, including mammalian and bacterial SRP, have GNRA-type tetraloops (GGAA or GAAA) closing the hairpin of domain IV, except for 7S RNA from *Saccharomyces cerevisiae*, where the sequence in the loop is UUUU, and 7S RNA from plants, where it is CUUC. The present work shows that the GGAA tetraloop sequence in *E. coli* 4.5S RNA can be replaced with a variety of other sequences, including ones which do not form a tetraloop structure, without any effect on either Ffh binding or the interaction of SRP with nascent-chain ribosomes or the SRP receptor, FtsY, or FtsY-dependent release of nascent-chain ribosomes from SRP in the targeting assay. Thus, neither the particular sequence nor the presence of a tetraloop structure in the apical region of domain IV seems to be essential for proper functioning of 4.5S RNA. There are, however, replacements that strongly diminish the interaction of SRP with FtsY, although they do not affect the affinity of Ffh binding to 4.5S RNA or the interaction of SRP with ribosome nascent-chain complexes. These replacements include CUUC or GAAU, which have intermediate effects, and UUUU, which has a stronger effect. The strongest effect, that is, no measurable SRP-FtsY interaction, is observed when the GGAA tetraloop is replaced with UUCG, which forms a UNCG-type tetraloop. The latter result was unexpected, because the replacement of the GAAA tetraloop sequence with UUCG in SRP RNA from the fission yeast, *S. pombe*, was compatible with SRP function in vivo, as was UUUC, whereas the CUUC replacement was deleterious (Selinger et al., 1993).

Melting studies on small RNA tetraloop hairpins by Antao and Tinoco (Antao et al., 1991; Antao & Tinoco, 1992) revealed that a UUCG tetraloop hairpin with a C-G loop-closing base pair is exceptionally stable compared to that of GGAA or GAAA tetraloops in the same context. This suggested that the impaired function of *E. coli* SRP containing the UUCG tetraloop may be due to the lack of conformational flexibility of the tetraloop region. Consistent with this contention is the finding that the function of UUCG-containing SRP in binding FtsY and in targeting was partially restored when the stability of the UUCG hairpin was lowered by either disrupting the first base pair of the adjoining stem or, slightly, by reversing it to G-C. Further support comes from NMR measurements on UUCG tetraloop hairpins, which show that substitution of a G-C for a C-G closing base pair increases the intrinsic flexibility of the tetraloop (Williams & Hall, 2000).

Similar alterations in the stem adjacent to the tetraloop in *S. pombe* SRP RNA caused growth defects that were attributed to conformational differences in the loop and adjoining stem regions suggested by enzymatic probing (Selinger et al., 1993). However, in *S. pombe* SRP, these effects could be due to an influence on SRP19 binding, as base-specific contacts in this stem region seem to be important for SRP19 binding, at least in human SRP (Zwieb, 1994). Because in the *E. coli* system, where there is no SRP19, such effects are not relevant, base exchanges in the stem adjacent to the tetraloop may have no apparent functional consequence in the wild-type background, but partially rescue FtsY binding to SRP containing 4.5S RNA(UUCG).

According to enzymatic probing data on the isolated domain IV of *S. pombe* SRP RNA (Selinger et al., 1993), substitutions of the tetraloop as well as base changes in the adjoining stem strongly influenced the structure not only within and around the tetraloop but also in the symmetric internal loop. The present hydroxyl radical probing data show that the substitution of the GGAA tetraloop for UUCG strongly influences the structure of the adjacent stem. In the structure of domain IV RNA determined by NMR (Schmitz et al., 1999a, 1999b), the tetraloop and the adjacent stem form a continuously stacked structure with the symmetric internal loop. It is conceivable that base changes in the tetraloop and the adjoining stem cause structural changes that extend into the symmetric internal loop. As both internal loops are involved in Ffh binding (Wood et al., 1992; Lentzen et al., 1996), base replacements in the tetraloop region may influence the structure of the complex by introducing structural changes at the Ffh binding site. The crystal structure of the complex of domain IV RNA with an M domain fragment of Ffh shows numerous interactions of the RNA-binding four-helix core in the symmetric internal loop A and further interactions in the asymmetric internal loop B (Batey et al., 2000). In the complex, the RNA adopts a structure that differs from

that of the free RNA. The major structural transition occurs in the asymmetric internal loop from which the four bases on the 5′ side (positions 39–42) that are stacked inside the internal loop in the free RNA are looped out upon binding of the M domain fragment, and interactions between asymmetric and symmetric internal loops, in part mediated by the protein, are formed (Batey et al., 2000).

Although complex formation of Ffh with FtsY depends on or is strongly influenced by 4.5S RNA, it is generally assumed that the interaction of FtsY with SRP takes place at Ffh, because an FtsY-4.5S RNA complex without Ffh could not be detected. Evidence for a direct Ffh–FtsY interaction has been reported for the proteins from *Mycoplasma mycoides*, as enhanced GTP hydrolysis was observed for the mixture of the two proteins, or for the mixture of FtsY with the NG domain of Ffh, in the absence of RNA (Macao et al., 1997). The formation of the binary complex of Ffh and N-terminally truncated FtsY in the absence of RNA was demonstrated recently, and the interaction was accelerated by 4.5S RNA (Peluso et al., 2000). According to the present results, the role of the RNA is both subtle and specific, in that SRP-FtsY complex formation strongly depends on structural details of the tetraloop region of 4.5S RNA that have no apparent influence on the affinity of the RNA for Ffh.

Our results suggest that the binding of 4.5S RNA to Ffh not only leads to a structural change of the RNA, as discussed above, but that it also changes the structure of Ffh, although no significant structural change of the M domain fragment is seen in the crystal structure of the M domain–RNA complex (Batey et al., 2000), compared with the M domain structure of *Thermus aquaticus* Ffh (Keenan et al., 1998). However, the possibility remains that Ffh–RNA complex formation results in structural changes in parts of the protein that were either not present in the fragment used for crystallization, that is, the NG domain and small parts of the M domain, or that were disordered in the crystal, that is, the finger loop. In conclusion, the present results suggest that structural details of the tetraloop region of 4.5S RNA, either of the tetraloop itself or of the adjoining stem, influence the structure of SRP such that the binding of FtsY is affected. This implies that both RNA and Ffh undergo mutual structural adaptation upon association to form SRP. The presumed structural change in Ffh induced by binding to 4.5S RNA probably extends beyond the M domain, which harbors the binding site for the RNA, as proteolysis data suggest that the structure of both M and NG domains of Ffh changes upon binding 4.5S RNA, indicating an influence of the RNA-binding M domain on the adjacent NG domain, as previously indicated by proteolysis experiments (Zheng & Gierasch, 1997). The interaction of Ffh in SRP with FtsY strongly depends on the presence of GTP (Kusters et al., 1995; Jagath et al., 2000), and it has been proposed that the conformational state of the G domain in response to the bound nucleotide is signaled to other domains and influences their function (Freymann et al., 1999). It is likely that the modulation of the interdomain communication in Ffh, by ligands such as GTP, 4.5S RNA, or the nascent signal peptide presented by the ribosome, is an important element of the regulation of SRP function. In this modulation, the SRP RNA appears to have a crucial role beyond that serving as a scaffold for Ffh binding.

MATERIALS AND METHODS

Materials

Restriction enzymes were obtained from MBI Fermentas and New England Biolabs, Pfu polymerase from Stratagene, and Ni-NTA-Agarose from Qiagen. Disuccinimidyl suberate (DSS) was from Pierce (Rockford, Illinois). Nikkol (Octa-ethylene glycol mono-*n*-dodecyl ether) was purchased from Nikko Chemicals, Japan.

E. coli strains and plasmids

E. coli strain NovaBlue (Novagen) was used for subcloning. BL21 (DE3) pLysS strain (Studier & Moffatt, 1986) was used for expressing FtsY and Ffh from plasmids pET9-FtsY(Trp343) (Jagath et al., 2000) and pET24-Ffh, respectively. Strain MC4100 was used to obtain translation lysate and inverted membrane vesicles. Strain Top10F′ was used as a host for plasmid pC4Meth108FtsQ (Valent et al., 1997).

4.5S RNA mutants

Base changes were introduced into 4.5S RNA by megaprimer PCR (Jagath et al., 2000), using plasmid pT7-4.5S as a template. Mutating the 4.5S RNA gene between nt 50–55 (TCCGGA) causes the loss of a *Kpn*2I site that was used for screening. The first PCR was carried out using the sense mutagenic primer and the antisense pUC forward primer with pT7-4.5S as a template, using Pfu polymerase. The product was used as megaprimer for the generation of mutant plasmid in the second PCR. The product was treated with *Dpn*I and transformed into NovaBlue cells. Mutants were screened by the absence of the *Kpn*2I site and verified by DNA sequencing. Wild-type and mutant plasmids were linearized with *Cfr*42I and used as templates for T7 polymerase transcription (Jagath et al., 1998).

Plasmid pPre4.5Swt (Wood et al., 1992) was used for the expression of 4.5S RNA in vivo. Sense (*Sal*I) and antisense (*Bam*HI) primers were designed with *Sal*I and *Bam*HI sites at the 5′ and 3′ ends, respectively. The sense primer's 5′ end was extended by 24 bases so that the PCR product encoded for the precursor form of 4.5S gene. The PCR was carried out with *Sal*I and *Bam*HI primers using pT7-4.5 wild-type plasmid as a template. The resulting PCR product was digested with *Sal*I and *Bam*HI and cloned into pPre4.5Swt to obtain pPre4.5S-GGAA. To introduce mutations into the pre4.5S gene, the PCR was carried out with *Sal*I and *Bam*HI primers

using pT7-4.5S mutant plasmids (pT7-4.5S-UUCG, pT7-4.5S-UUUU, pT7-4.5S-CUUC, and pT7-4.5S-GAAU). Mutant pre4.5S PCR products were inserted into pPre4.5S-GGAA at the *Sal*I and *Bam*HI sites to obtain pPre4.5S-UUCG, pPre4.5S-UUUU, pPre4.5S-CUUC, and pPre4.5S-GAAU, respectively.

Expression and purification of Ffh and FtsY

The sequence coding for Ffh was amplified by PCR using Pfu polymerase and pET21-Ffh(His-6) as template. The PCR product was digested with *Nde*I and *Hin*dIII and cloned into pET24a to obtain pET24-Ffh coding for Ffh with a C-terminal histidine tag. pET24-Ffh was transformed into *E. coli* BL21(DE3) pLysS. Cells were grown in LB medium containing kanamycin (30 μg/mL) and chloramphenicol (30 μg/mL) and induced with IPTG at O.D.$_{600}$ = 0.4. Cells were harvested and Ffh was purified as described (Jagath et al., 1998). FtsY was expressed and purified as described (Jagath et al., 2000).

Filter-binding assay

Ffh binding to 4.5S RNA variants was measured by nitrocellulose filtration in 50 mM HEPES, pH 7.5, 70 mM ammonium acetate, 30 mM potassium acetate, 7 mM magnesium acetate, 1 mM DTT, 0.02% of Brij 35, 0.1 mg/mL bovine serum albumin, and 0.1 mg/mL poly(U). 5′-[^{32}P]phosphate-labeled 4.5S RNA variant (30 pM; 3×10^6 dpm/pmol) was incubated with varying amounts of Ffh for 10 min at 37 °C. For dissociation experiments, complexes were formed at 20-fold higher concentrations, and dissociation was induced by dilution. Binding mixtures were filtrated through nitrocellulose filters (Sartorius), the filters washed with 4 mL of binding buffer, and the bound radioactivity determined by liquid scintillation counting. Titration curves were evaluated by nonlinear fitting (Table Curve, Jandel Scientific).

Gel-retardation assay

SRP and SRP-FtsY complex formation was monitored on nondenaturing 7% polyacrylamide gel electrophoresis (Jagath et al., 2000). 4.5S RNA or SRP was incubated with FtsY in buffer (25 mM triethanolamine acetate, pH 7.4, 25 mM potassium acetate, 2.5 mM magnesium acetate, 5% glycerol, 0.1% Nikkol) in the presence of 0.2 mM GMPPNP at 25 °C for 5 min.

Targeting assay

SRP binding to ribosome nascent chain complexes was measured by DSS crosslinking of SRP to the ^{35}S-labeled signal sequence of *E. coli* FtsQ and FtsY-dependent targeting to the translocon contained in inverted vesicles of the *E. coli* plasma membrane by the loss of SRP-crosslinked signal sequence (11, 29). Crosslinked material was analyzed on 12% polyacrylamide gels in the presence of SDS.

Chemical probing of 4.5S RNA

RNA cleavage by hydroxyl radicals generated by the Fe(II)-EDTA reaction with hydrogen peroxide and DMS modification were performed in buffer B essentially as previously described (Lentzen et al., 1996). Cleavage or modification patterns were obtained by reversed transcriptase sequencing using a 5′-^{32}P-labeled oligodeoxynucleotide primer complementary to the 3′ terminus of 4.5S RNA.

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