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Binding sites of the 19-kDa and 68/72-kDa signal recognition particle (SRP) proteins on SRP RNA as determined by protein–RNA "footprinting"

(α-sarcin/ribonucleoprotein/reconstitution/secretion/targeting)

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ABSTRACT **We have used the nuclease α-sarcin to map the binding sites of the 19-kDa and the 68/72-kDa proteins of signal recognition particle (SRP) on SRP RNA. We found that the regions of protection to nuclease afforded by the two proteins were distinct. p19 protected primarily the two tips in the RNA secondary structure. p68/72 protected a large region extending across the center of the particle and altered the nuclease pattern in the regions that p19 would bind, suggesting that these two proteins may be in close proximity in the particle. The protection afforded by the two proteins in combination was equal to the sum of the individual protections. We have not observed cooperativity in the binding of these two proteins as assessed by the protection assay; nor do we have any evidence that the structure becomes more compact as it assembles. The map derived from this "footprint" analysis places the signal recognition domain (p54 bound to the RNA via the 19-kDa protein) and the elongation arrest domain (associated with the *Alu* end of the particle) on opposite ends of the particle. Thus, it is possible that SRP recognizes signals by the direct interaction of p54 with the signal sequence at the nascent chain exit site and simultaneously blocks elongation by the entrance of p9/14 into the aminoacyl tRNA site 16 nm away.**

Signal recognition particle (SRP) is a small cytoplasmic ribonucleoprotein that functions in the targeting of secretory proteins to the membrane of the endoplasmic reticulum (for review, see ref. 1). To perform this function, SRP maintains a number of different activities: signal recognition, elongation arrest, and translocation promotion. Of these three activities, two (signal recognition and translocation promotion) are absolutely essential, while the third (elongation arrest), although not absolutely required for the translocation process *per se*, appears to increase the fidelity of the process (2). All three activities have been mapped to distinct protein domains by biochemical mutagenesis experiments (2–4). It is reasonable to assume that for the proteins to be active they must be oriented in the particle in a particular relative geometry. For this reason, we sought to define the sites of interaction of the SRP proteins on SRP RNA.

SRP is composed of six distinct polypeptides (72, 68, 54, 19, 14, and 9 kDa, respectively) and one molecule of RNA (7SL RNA, here referred to as SRP RNA) (5). The six polypeptides are organized into four proteins (2, 6), two of which are monomeric (referred to as p54 and p19) and two of which are heterodimeric (referred to as p68/72 and p9/14). The SRP RNA within SRP is highly resistant to micrococcal nuclease (3, 7), suggesting that most of it is involved in protein contact. Conversely, three of the four SRP proteins have been shown to bind independently to the RNA (2, 6).

Electron spectroscopic imaging has been used to localize the SRP RNA within the particle. The mass of the RNA was found to be concentrated at the two ends of the particle, suggesting that the RNA spans the length of SRP, forming an extended stem structure, which serves as a backbone for SRP assembly (8).

One function of the RNA in SRP is clearly to form a structural lattice. When SRP was disassembled into RNA and protein components, the proteins no longer sedimented together as a particle, but rather sedimented as monomers or dimers (6). Furthermore, these proteins, which in the presence of the RNA form a tightly bound particle that is stable up to 1 M salt, could be separated after disassembly into four distinct groups under nondenaturing conditions (2).

Micrococcal nuclease digestion of SRP yielded two distinct particles that could be separated by sucrose gradient sedimentation (7). p9/14 cosedimented with the *Alu*-like sequences of SRP RNA and the remaining proteins cosedimented with the S sequences of the RNA. In previous experiments (3), it was demonstrated that this latter subparticle, termed SRP(S), contained the signal recognition and translocation promoting activities of the particle and hence contained all the essential functions that enable SRP to mediate targeting. Thus, the elongation arrest domain has been mapped to the "*Alu* end" of the RNA, and the signal recognition and protein translocation functions have been mapped to the "S end."

α-Sarcin, a small basic protein purified from *Aspergillus giganteus*, is a RNase with sequence homology to RNase U2 (for review, see ref. 9), which cuts on the 3′ side of most adenines and guanines. More specifically, although there appears to be some preference for particular purines within an RNA molecule, that preference is not based on secondary structure (10). It was shown to be a useful enzyme for footprinting RNA by Huber and Wool (11), who succeeded in determining the binding sites for each of the ribosomal proteins (L5, L18, and L25) on *Escherichia coli* 5S rRNA by this method. We have used α-sarcin to map on the SRP RNA the proteins responsible for signal recognition (p19 together with p54) and translocation promotion (p68/72).

MATERIALS AND METHODS

Materials. Nuclease-free bovine serum albumin, proteinase K, and calf liver tRNA were purchased from Boehringer

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Abbreviation: SRP, signal recognition particle.

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Mannheim; RNase α-sarcin was a kind gift of Ira Wool (University of Chicago); T4 RNA ligase and RNases T1 and U2 were purchased from Pharmacia; [γ-^{32}P]ATP was purchased from ICN; T4 polynucleotide kinase was from United States Biochemical, Cleveland; dimethyl sulfoxide (glass-distilled and filtered through 0.5-μm filters) was from Aldrich. All other reagents were purchased as described(2).

Purification of SRP, SRP Proteins, and SRP RNA. These procedures are described in ref. 2.

Synthesis of [^{32}P]pCp. pCp was synthesized according to England *et al.* (12). 5′-Labeled [γ-^{32}P]ATP (1 mCi; 7000 Ci/mmol; 1 Ci = 37 GBq) was incubated for 1 hr at 37°C in 20 μl of 25 mM Tris·HCl, pH 8.0/5 mM $Mg(Cl)_2$/5 mM dithiothreitol/50 μg of nuclease-free bovine serum albumin per ml/2 mM 3′ CMP/250 units of T4 polynucleotide kinase per ml. The sample was then incubated for 15 min at 65°C before dilution to 50 μl for storage.

3′-Labeling of 7SL RNA. Labeling was performed essentially as described (12). SRP RNA (2 μg) in 2 μl was mixed with 2 μl of methyl sulfoxide, heated for 3 min at 65°C, and chilled in ice water. [^{32}P]pCp (100 μCi) was evaporated to dryness in a Savant Speed-Vac concentrator. The final volume of the ligation reaction was 10 μl and contained 50 mM Hepes (pH 7.5), 20 mM $MgCl_2$, 10 μg of nuclease-free bovine serum albumin per ml, 6 μM ATP, the methyl sulfoxide/RNA mixture, and 8 units of T4 RNA ligase. The ligation reaction mixture was incubated overnight at 4°C and was stopped by bringing the mixture to 1% $NaDodSO_4$/20 mM EDTA/50 mM Tris·HCl, pH 7.5/600 μg of calf liver tRNA per ml. Proteinase K was then added to 200 μg/ml and incubated at 37°C for 30 min. RNA was then purified by perchlorate extraction as described (5), or by phenol extraction followed by ethanol precipitation, and separated from tRNA on urea acrylamide gels.

Footprinting with α-Sarcin. 3′-Labeled SRP RNA (0.6 pmol) was mixed with purified SRP protein at various stoichiometries (a 2-fold and a 10-fold excess of protein to RNA is shown here) under previously described reconstitution conditions (2). One tube contained SRP protein compensating buffer rather than protein. The RNA concentration during the reconstitution ranged from 100 to 150 nM. After reconstitution, the sample was diluted to final buffer conditions of 20 mM Hepes, pH 7.5/100 mM KOAc/1 mM $Mg(OAc)_2$/10 μg of calf liver tRNA per ml/50 μg of nuclease-free bovine serum albumin per ml. The tRNA and bovine serum albumin were added prior to the final dilution, so that competitor would be present as soon as the sample was in low ionic strength.

α-Sarcin was then added to 0.2 or 2 μM and the samples were incubated for 15 min at 30°C. After digestion, the sample was diluted to 50 μl and 5 mM EDTA. Samples were extracted with hot (60°C) phenol, ethanol precipitated, and resuspended in 10 μl of formamide sample buffer [95% formamide/10 mM EDTA/0.1% (wt/vol) each xylene cyanol and bromphenol blue]. Samples were heated to 95°C for 5 min and chilled in ice water. Digestion products were displayed on a 5% acrylamide/50% urea wedge gel. Gels were rinsed for 20 min with 5% methanol/5% acetic acid and then dried at 80°C for 30 min. Bands were visualized by autoradiography.

Digestion with Nucleases T1 and U2. 3′-Labeled SRP RNA (0.3 pmol) was mixed with 1 μg of calf liver tRNA and evaporated to dryness. It was then resuspended in 2 μl of 25 mM NaOAc/1 mM EDTA/7 M urea, containing 10 or 100 units of T1 per ml, or 100 units of U2 per ml (13), and incubated for 15 min at 55°C. Loading buffer (2 μl) was then added, and samples were heated to 95°C, quick chilled, and loaded onto gels as described above.

Alkaline Hydrolysis. 3′-Labeled SRP RNA (0.3 pmol) was mixed with 1 μg of calf liver tRNA and evaporated to dryness. The RNA was resuspended in 2 μl of 50 mM NaOH/1 mM EDTA and boiled for 40 sec. Loading buffer (2

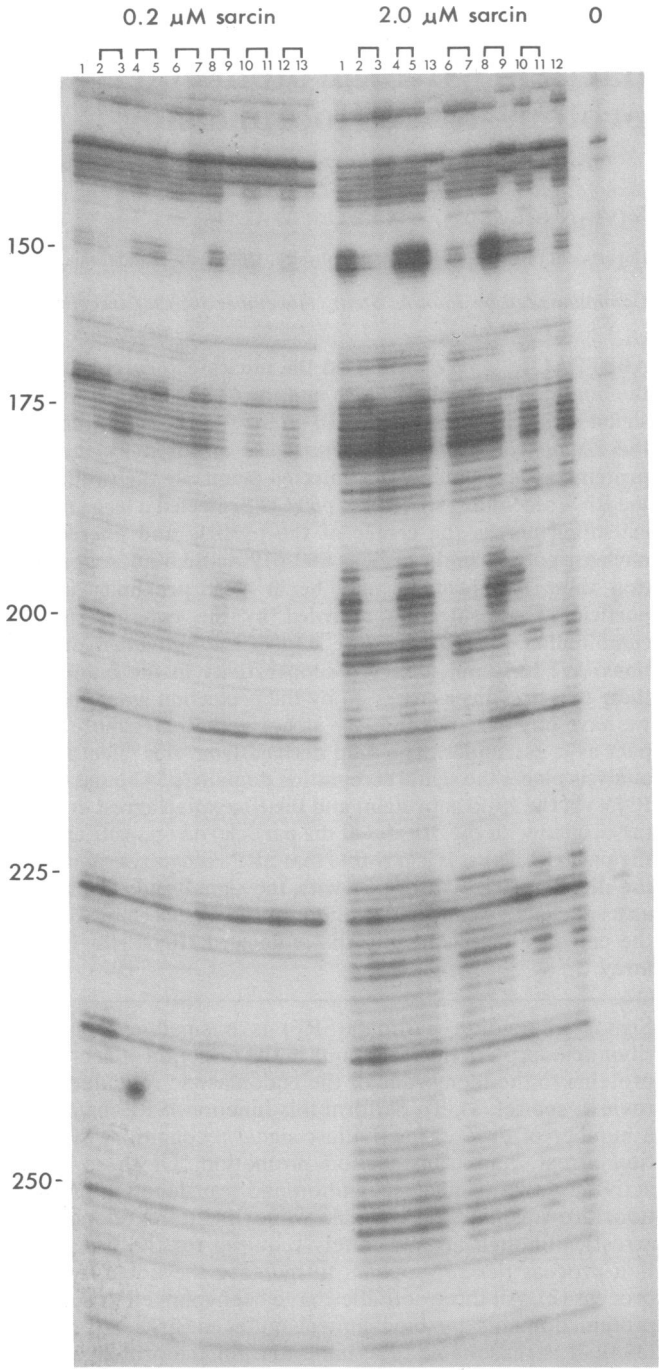

FIG. 1. Nuclease digestion pattern of SRP RNA in the absence and presence of SRP proteins. SRP proteins, singly or in combination, were reconstituted with 3′-labeled SRP RNA as described in *Materials and Methods*. The reconstitutes were then diluted and digested with 0.2 or 2 μM sarcin for 15 min at 30°C. The digested particle was phenol extracted, ethanol precipitated, and resolved on 7 M urea/6% acrylamide wedge gels. A representative gel is shown. Lanes: 1, naked RNA; 2, p19, 2× (2-fold excess over RNA); 3, p19, 10×; 4, p54, 2×; 5, p54, 10×; 6, p19 + p54, 2×; 7, p19 + p54, 10×; 8, p68/72, 2×; 9, p68/72, 10×; 10, p19 + p68/72, 2×; 11, p19 + p68/72, 10×; 12, p19 + p54 + p68/72, 2×; 13, p19 + p54 + p68/72, 10×; 0, uncut RNA. In other experiments, we saw alterations in the cutting pattern at nucleotides 192–209 and also in protection of that region by p19 (data not shown). This may reflect conformational changes in the RNA. Numbers on left represent the number of nucleotides from the 5′ end of the RNA.

μl) was added, and the sample was loaded directly onto the gel without further denaturation.

RESULTS

SRP RNA was labeled with [^{32}P]pCp using RNA ligase. After elution of the labeled RNA from a 7 M urea/6% acrylamide gel, the RNA was mixed with one or a combination of SRP proteins under conditions that promote reassembly of SRP (6). Proteins were added at either a 2- or a 10-fold molar excess of protein over RNA. Because α-sarcin is inhibited by both monovalent and divalent cations, we diluted the reconstitute in the presence of a 20-fold molar excess of competitor RNA (calf liver tRNA) and a 4- to 10-fold molar excess of competitor protein (nuclease-free bovine serum albumin) prior to digestion. In a separate experiment, other competitor RNAs (total oocyte RNA and total ribosomal RNA) gave identical results (data not shown).

We found that α-sarcin was remarkably inactive even at the relatively low concentrations of monovalent and divalent cations used in these experiments [100 mM KOAc, 1 mM $Mg(OAc)_2$], requiring an excess of nuclease over RNA to see a sequencing ladder (in Fig. 1, we show 0.2 and 2 μM sarcin on 0.02 μM RNA). This is consistent with the results found for 28S ribosomal RNA, in which the concentration required to cleave 50% of the RNA was 4.2 μM (10), and in that case even lower monovalent (50 mM KCl) and divalent (none) cation was used. However, the digestion at these high concentrations was sufficient to detect protection by the protein, as shown in Fig. 1.

Lane 1 shows the digestion pattern of naked SRP RNA in the region of the S sequences, which has been previously shown (7) to be the site of binding for p19 and p68/72. [The localization of these proteins to the S region of the particle has been confirmed by the absence of protection outside this region (data not shown).] The positions at which α-sarcin cleaves naked SRP RNA are shown as capital letters in Fig. 2*A*, and in the secondary structure diagram as double lines in Fig. 2 *B* and *C* (for secondary structure models, see refs. 3, 14, and 15).

The pattern of nucleolytic cuts in the presence of each of the proteins and of the proteins in combination is shown in Fig. 1. The protection that results from adding these proteins is depicted schematically in Fig. 2 *B* and *C*. p19 (Fig. 1, lanes 2 and 3) protects the RNA from digestion primarily at nucleotides 147–153 and 192–201 and lightly at nucleotides 207–209. The regions constitute the tips in the secondary structure of the RNA (Fig. 2*B*).

p54 does not protect the RNA on its own (Fig. 1, lanes 4 and 5), consistent with the result that p54 does not bind to the RNA, as assessed by sucrose gradient analysis. Because p54 has been shown to bind via p19 (6), we assembled these two proteins together on the RNA and found the footprint to be identical to that found for p19 alone (compare lanes 2 and 3 with lanes 6 and 7). Thus, p54 is not induced to bind RNA in the presence of p19, nor does it alter the binding of p19 to the RNA.

p68/72 protects the RNA at sites distinct from p19. The sites of protection by this protein are extensive (see Fig. 1, lanes 8 and 9 and Fig. 2*C*), although perhaps the most striking protection (because of the strong nucleolytic cleavage on naked RNA in this region) is at nucleotides 171–183. Note also that although p68/72 does not completely protect the sites protected by p19, it alters the pattern of digestion seen in those regions (giving an enhancement of digestion at C-196, for example, and an incomplete protection at nucleotides 147–153). This alteration in the cutting pattern suggests that p68/72 binds close to, but not directly on, the loop protected by p19.

The protection pattern seen when p68/72 and p19 are reassembled together on the RNA (Fig. 1, lanes 10 and 11) is the sum of the patterns seen when each of the proteins is bound individually. In particular, the regions that were altered in their digestion pattern by p68/72 but fully protected by p19 are fully protected when the proteins are added in combination. In this case, the only bands remaining in the S region are those found in the undigested RNA (lane 0). This is consistent with the results from nuclease digestion studies on intact SRP (3, 7), in which the entire S region was

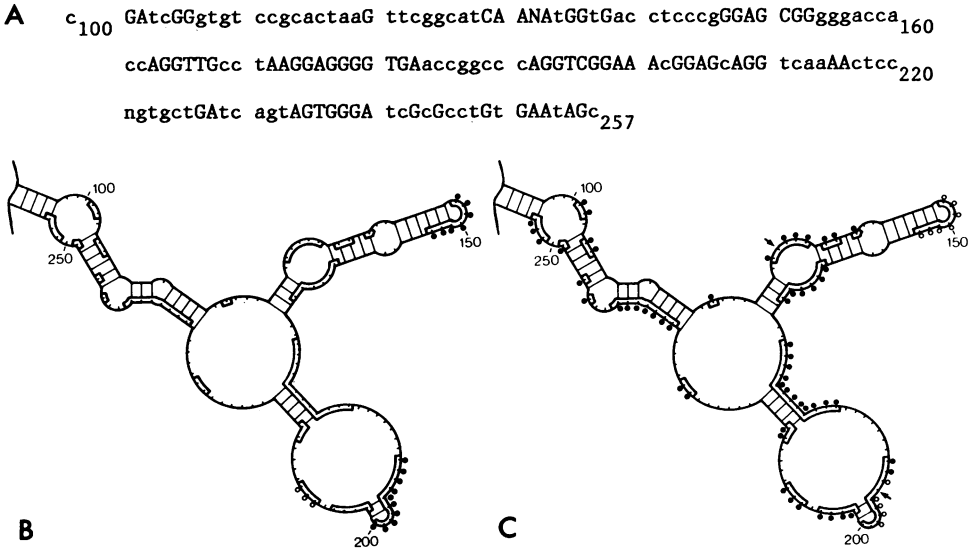

FIG. 2. Sites on canine SRP RNA that are susceptible to nuclease cleavage. (*A*) Nucleotides that were cut by α-sarcin are shown in capital letters. Canine SRP RNA was sequenced by primer extension with reverse transcriptase. N, ambiguous nucleotides. (*B* and *C*) Protection of SRP RNA by SRP proteins (p19, *B*; p68/72, *C*). Nucleotides that were cut by α-sarcin in the absence of added protein are shown as double lines in the secondary structure diagram. Nucleotides that are protected from nuclease cleavage by protein are indicated by circles along the secondary structure diagram. Solid circles indicate strong protection, open circles indicate weaker protection, and arrows indicate enhancement.

found to be nuclease resistant. [α-Sarcin has been observed to cut SRP only once, at approximately nucleotide 75 in the *Alu* stem (not shown).]

A higher concentration of p68/72 (a 10-fold excess) than of p19 (a 2-fold excess) was required to completely protect the RNA from nuclease. This result suggests that p19 has a higher affinity for the RNA than does p68/72, which may indicate that this protein serves as an initiator of assembly. However, when the two proteins were bound in combination, the affinity of p68/72 was not demonstrably increased; again, a 10-fold excess of protein was required to completely protect the RNA. Thus, we have no evidence as yet for any cooperativity of binding, which was suggested by previous reconstitution experiments (6). It is possible that the cooperativity of binding is brought in by another component (p9/14), that we have lost the cooperativity in the further purification of these proteins, or that we have simply not tested for cooperativity at a concentration of protein where the effect could be detected.

DISCUSSION

We have mapped by nuclease protection the binding sites of the 19-kDa and the 68/72-kDa proteins of SRP on the SRP RNA. We found that the protection of SRP RNA by p19 is very specific: only two regions, corresponding to the tips of the predicted secondary structure of the molecule, are altered in their digestion pattern. The region of protection includes the most highly conserved region of the molecule (14). When secondary structures are drawn for putative SRP RNAs from the two yeast species *Schizosaccharomyces pombe* and *Yarrowia lipolytica*, these sequences are conserved, in contrast to the overall lack of primary sequence homology in the rest of the molecule (21). Furthermore, canine p19 has been shown by footprint analysis to protect these conserved regions on *S. pombe* RNA (21). Thus, p19 binding may be sequence specific and it has already been possible in one case to identify the SRP RNA in an evolutionarily distant organism by its ability to bind canine p19.

In contrast, p68/72 protects a large region of the RNA, the conservation of which is more strongly seen in secondary rather than primary structure. It is possible that p68/72 binds primarily to double-stranded regions of the RNA and is positioned properly along the particle by the structure of the RNA rather than by its sequence. Other RNA binding proteins have been shown to recognize structure rather than sequence. For example, ricin A chain has recently been shown to recognize the three-dimensional structure of 28S ribosomal RNA (16).

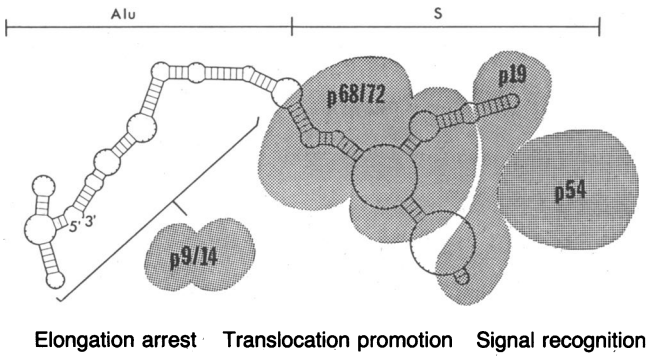

FIG. 3. Map of the relative geometry of the protein domains with respect to the RNA secondary structure. The *Alu* and S domains of SRP RNA are indicated. The relative positions of p68/72 and p19 on the SRP secondary structure as determined by footprinting analysis are indicated. Binding of p54 is shown to occur through p19. The precise binding site of p9/14 has not yet been determined; however, it is clear from previous nucleolytic dissection studies of SRP that p9/14 binds to and largely protects the *Alu* end of SRP RNA. Activities assigned to the protein domains are indicated.

The addition of p54 to the reassembly mixture had no effect on the pattern of nuclease protection. Thus, p54 appears to bind to the particle solely through protein–protein interactions. Interestingly, in the absence of RNA, no such protein–protein interaction is observed (6). The interaction of SRP RNA with p19 must therefore induce a structural change within the protein so that it can bind p54 with high affinity.

In electron microscopic analysis (8), SRP was found to be composed of three domains; it seems reasonable that these three domains correspond to the three sets of proteins that bind to the SRP RNA independently of one another—i.e., p68/72, p19 and p54 (see above), and p9/14. Electron spectroscopic imaging experiments (8) further showed that the RNA was extended in the particle and that the predicted secondary structure of the RNA may reflect features of the tertiary structure of the molecule within SRP, with the prominent stem in the secondary structure running along the length of the particle between the first and third domains. We have therefore positioned the three sets of polypeptides along the axis of the particle, assuming that the *Alu* end (which binds p9/14; refs. 3 and 7) of the RNA lies at one end of SRP, that the stem region (which binds p68/72) lies in the middle, and that the tips in the secondary structure (which bind p19) lie at the other end. Given the morphologically defined three-domain structure of SRP (see above), we think it likely that p54 is found together with p19 at the end of the particle opposite the *Alu* end.

These three sets of proteins are responsible for the three activities of SRP that can be analyzed *in vitro*. Specifically, p9/14 is required for elongation arrest (2), p68/72 for protein translocation (4), and p54 for signal recognition (4, 17, 18).

It is tempting to combine the structural information derived from electron microscopy with the footprintig analysis described here to position the three activities of SRP along its longitudinal axis (Fig. 3). This model places the elongation arrest domain and the signal recognition domain at opposite ends of the particle. SRP has been shown to be 24 nm long (19) and the aminoacyl tRNA site and the nascent chain exit site 16 nm apart (20). The positioning of the elongation arrest domain and the signal recognition domain at opposite ends of SRP is consistent with the model that SRP physically bridges the distance between the nascent chain exit site and the aminoacyl tRNA site, and that these protein domains on SRP perform their functions by a direct interaction with the two regions on the ribosome, as opposed to by some long-range conformational effect.

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