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Binding Sites of the 9- and 14-Kilodalton Heterodimeric Protein Subunit of the Signal Recognition Particle (SRP) Are Contained Exclusively in the *Alu* Domain of SRP RNA and Contain a Sequence Motif That Is Conserved in Evolution

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The mammalian signal recognition particle (SRP) is a small cytoplasmic ribonucleoprotein required for the cotranslational targeting of secretory proteins to the endoplasmic reticulum membrane. The heterodimeric protein subunit SRP9/14 was previously shown to be essential for SRP to cause pausing in the elongation of secretory protein translation. RNase protection and filter binding experiments have shown that binding of SRP9/14 to SRP RNA depends solely on sequences located in a domain of SRP RNA that is strongly homologous to the *Alu* family of repetitive DNA sequences. In addition, the use of hydroxyl radicals, as RNA-cleaving reagents, has revealed four distinct regions in this domain that are in close contact with SRP9/14. Surprisingly, the nucleotide sequence in one of these contact sites, predicted to be mostly single stranded, was found to be extremely conserved in SRP RNAs of evolutionarily distant organisms ranging from eubacteria and archaebacteria to yeasts and higher eucaryotic cells. This finding suggests that SRP9/14 homologs may also exist in these organisms, where they possibly contribute to the regulation of protein synthesis similar to that observed for mammalian SRP in vitro.

The signal recognition particle (SRP) is a critical component of the biosynthetic pathway of most secretory proteins in mammalian cells (51). SRP was shown in vitro to bind specifically to signal sequences emerging from actively translating ribosomes (19, 20). This interaction causes a pausing in the elongation of the protein (23, 28, 53, 56). The SRP-ribosome complex is then targeted to the endoplasmic reticulum by the interaction with the SRP receptor (docking protein) (10, 28), normal speed of protein synthesis is resumed, and the ribosome becomes engaged with other membrane proteins which facilitate translocation of the nascent protein across the membrane.

SRP consists of six polypeptides and one molecule of RNA of 300 nucleotides (7SL RNA or SRP RNA). Functional activities have been assigned to different domains of the particle (39–41). The central domain of SRP (S domain) contains four protein subunits, SRP72, SRP68, SRP54, and SRP19, and the central portion of SRP RNA. The S domain is essential and sufficient for the signal recognition and targeting activities of SRP.

The second domain of SRP (*Alu* domain) contains the two smallest SRP subunits, SRP9 and SRP14, and the sequences comprising about 100 nucleotides from the 5′ end and 50 nucleotides from the 3′ end of SRP RNA. These sequences are very homologous to the mammalian *Alu* family of repetitive sequences; in fact, phylogentic analyses indicate that SRP RNA is the evolutionary progenitor to the repetitive *Alu* sequences (49). The physiological function, if any, of the repetitive *Alu* DNA is unknown. As part of SRP, the *Alu* domain is required for elongation arrest, as SRP particles lacking this domain are deficient in this function.

cDNAs encoding SRP9 and SRP14 have recently been isolated and have been used to study the RNA binding characteristics of these proteins (43, 44). It was shown that neither of the two proteins alone could specifically bind SRP RNA; rather, the presence of both proteins was required to form a stable RNA-protein complex. These proteins were shown to form a stable heterodimeric complex in the absence of SRP RNA. Thus, the presence of both proteins is required for the assembly of the functional *Alu* domain. In this study, we have examined the structural components of SRP RNA that are involved in binding the heterodimeric protein SRP9/14.

MATERIALS AND METHODS

Materials. Sources of materials were as follows: SP6 RNA polymerase and plasmid SP64, Promega Biotec; bovine serum albumin, proteinase K calf liver, and *Escherichia coli* tRNA, Boehringer Mannheim Biochemicals; T4 RNA ligase and RNase T_1, Pharmacia LKB Biotechnology Inc.; [γ-^{32}P]ATP, ICN; [α-^{32}P]ATP and [α-^{32}P]GTP, Amersham Corp.; restriction enzymes, T4 polynucleotide kinase, and ligase, New England BioLabs, and avian myeloblastosis virus reverse transcriptase, Life Sciences. All other reagents were obtained from Sigma Chemical Co. T4 DNA polymerase, gene 45 protein, and 44/62 proteins were the kind gifts of Jack Barry and Bruce Alberts (University of California, San Francisco); T7 RNA polymerase was kindly prepared by Pablo Garcia (University of California, San Francisco).

Construction of the SRP RNA gene and derivatives. The SRP RNA gene preceded by the T7 RNA polymerase promoter was synthesized by using overlapping oligonucleotides (Fig. 1). The combination of the two first and the two last oligonucleotides created sticky ends specific for the restriction enzymes *Eco*RI and *Xba*I, respectively. The

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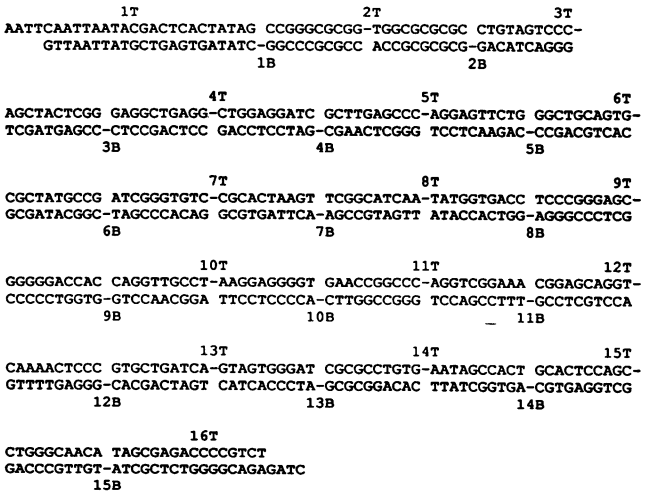


FIG. 1. Sequence of the synthetic SRP RNA gene. The SRP RNA gene preceded by the T7 RNA polymerase promoter was synthesized from the oligonucleotides shown. Sequence blocks joined by a dash represent one oligonucleotide. The oligonucleotides are numbered 1T to 16T and 1B to 15B (T, top-strand oligonucleotides; B, bottom-strand oligonucleotides). The last nucleotide of 1T is the first nucleotide of SRP RNA. The sequence of the oligonucleotide 61B was GCGATACGGCGCGCGGACAC.

oligonucleotides contained in the 5′ and in the 3′ half of the SRP RNA gene were annealed and ligated in two separate reactions and then ligated with each other. This stepwise ligation protocol resulted in an increased yield of SRP RNA gene recombinants in the final ligation reaction. To this end, 100 pmol of each of the top- and bottom-strand oligonucleotides 1T to 8T and 1B to 7B, and 9T to 16T and 8B to 15B, were combined in separate pools and phosphorylated with 5 nmol of ATP and 16 U of T4 polynucleotide kinase for 1 h at 37°C in kinase buffer (50 mM Tris-HCl [pH 7.5], 10 mM magnesium chloride, 5 mM dithiothreitol, 1 mM spermidine, 1 mM EDTA). The reaction was stopped by precipitating the oligonucleotides in 2 M ammonium acetate with a twofold excess of isopropanol. The oligonucleotides were resuspended in 30 μl of 10 mM Tris (pH 7.5)–1 mM EDTA (TE), heated to 65°C, and annealed by cooling slowly to room temperature (30 min). The first ligation was done in ligase buffer (50 mM Tris-HCl [pH 8], 10 mM magnesium chloride, 1 mM spermidine, 20 mM dithiothreitol), 0.5 mM ATP, and 10 U of T4 ligase per μl for 15 min at room temperature and stopped by precipitation. The two ligation reaction mixtures were combined, religated for 1 h at room temperature, and precipitated. The ligated material was then digested for 2 h with 100 U of each of the enzymes *Eco*RI and *Xba*I. The digested DNA was fractionated on a 2% low-melting-point agarose gel. DNA in the size range of 320 nucleotides (the expected size of the SRP RNA gene fragment) was ligated into the *Eco*RI- and *Xba*I-cut pSP64 plasmid vector. Usually this ligation reaction contained a 10-fold molar excess of the insert. The inserts of the plasmids obtained from the recombinants (p7Sswt) were sequenced by the chain termination method (38). The sequence of the synthetic SRP RNA derived from this clone is identical to the sequence of canine SRP RNA (41a), except at position 53, where the A residue was replaced by U as found in human SRP RNA (48, 49). The plasmid containing exclusively the *Alu* portion of SRP RNA preceded by the T7 RNA polymerase promoter (p7Salu) was synthesized as outlined above, using the oligonucleotides 1T to 6T, 14T to 16T, 1B to 5B, 14B, 15B, and 61B, which joins 6T to 14T (Fig. 1), in a single annealing and ligation reaction.

Preparation of SRP RNAs and derivatives. Plasmids p7Sswt and p7Salu were linearized with the restriction enzyme *Xba*I and transcribed with T7 RNA polymerase to generate synthetic SRP RNA and the *Alu* portion of it, respectively. For synthesis of the 60-nucleotide-long RNA fragment comprising the 5′ end of SRP RNA, plasmid p7Sswt was linearized with *Sau*3A. In vitro transcription with T7 RNA polymerase was performed in a 100-μl reaction as described previously (29), using 4 μg of DNA template, 0.75 mM each nucleoside triphosphate (NTP), and 10 mM magnesium acetate. The transcription reaction was extracted with phenol-chloroform (1:1), precipitated, and dissolved in 20 μl of sample buffer containing 95% formamide, 10 mM Na_2EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol, and the RNA was fractionated on a denaturing 6% polyacrylamide gel. The RNA was eluted, extracted with phenol, dissolved in water, and stored at −70°C. Yeast U4 small nuclear RNA was synthesized from plasmid pSPU4B (a kind gift of Doug Black, Whitehead Institute, Cambridge, Mass.), using SP6 RNA polymerase (27).

Drosophila melanogaster and *Schizosaccharomyces pombe* SRP RNAs were purified from DEAE fractions enriched in SRP (32). Canine SRP RNA was extracted from purified canine pancreatic SRP (52). The various RNA species were labeled at their 3′ ends with [^{32}P]pCp and RNA ligase (7). The ligase reactions were fractionated on an 8 M urea–6% polyacrylamide gel. Labeled RNA species of the appropriate sizes were excised, extracted with phenol, and precipitated twice with ethanol before use in the reconstitution experiments.

Purification of SRP proteins; reconstitution and assay of complete or partial SRPs. The purification of SRP proteins and the reconstitution of SRP from proteins and RNA were performed as described previously (40). Binding of SRP RNA or its derivatives to SRP9/14 was performed in SRP buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-KOH [pH 7.5], 5 mM magnesium chloride, 1 mM dithiothreitol, 0.01% Nikkol) containing variable concentrations of potassium acetate (the usual concentration was 500 mM potassium acetate). Protein-to-RNA ratios were as indicated for each experiment. The elongation arrest activity was determined as described previously (44) except that the translation reaction mixture contained preprolactin and in vitro-synthesized sea urchin cyclin mRNA transcribed from plasmid CYCΔ90 (31) instead of globin mRNA. The concentrations of the reconstituted SRP samples were based on the concentrations of SRP RNAs that were added to the reconstitution reactions. The concentration of the canine SRP RNA was determined by measurement of A_{260}. The concentration of the in vitro-synthesized SRP RNA was determined by hybridization using a ^{32}P-labeled oligonucleotide complementary to the SRP RNA as a probe and pancreatic SRP RNA as a standard.

Hydroxyl radical cutting reaction. The binding of SRP RNA to SRP9/14 was performed as described above at a final salt concentration of 500 mM potassium acetate. In parallel samples, which served as a negative control, SRP9/14 was replaced by the same amount of bovine serum albumin. After binding, 1-μl fractions of each of the samples (containing 0.5 pmol of RNA) were added to 20 μl of SRP buffer containing 5 mM dithiothreitol. An aliquot of 1 μl of each of

the following four solutions was combined in a drop at the side of the Eppendorf tube in the given order: (i) 25 mM ferrous ammonium sulfate [$Fe(NH_4)_2(SO_4)_2$], (ii) 50 mM EDTA, (iii) 125 mM sodium ascorbate, and (iv) 2.5% hydrogen peroxide. The drop was spun into the samples, which were incubated for 3 min on ice. The reaction was stopped by the addition of 25 μl of 0.1 M thiourea, 10 μl of 3 M sodium acetate, 1 μg of *E. coli* tRNA, and 40 μl of TE, followed by phenol-chloroform extraction and precipitation with 3 volumes of 95% ethanol. The RNA was dissolved in 20 μl of autoclaved water and used for primer extension. When [^{32}P]pCp-labeled RNA was used in the cleavage reaction, the samples were dissolved in sample buffer and directly loaded onto an 8 M urea–15% polyacrylamide gel.

Primer extension reaction. Canine SRP RNA (0.1 pmol) treated with hydroxyl radical or, as a control and for the sequencing reaction, uncut SRP RNA (0.1 or 0.5 pmol, respectively) was annealed to 1.5 pmol of ^{32}P-labeled primer (24) in a buffer containing 50 mM Tris [pH 8.3], 60 mM sodium chloride, and 10 mM dithiothreitol by heating to 65°C for 3 min and incubation at 42°C for 45 min. For the primer extension reactions, the following reagents were combined: 2 μl of primer template mix, 1 μl of 2 mM dNTPs in annealing buffer, 1 μl of ddNTP or water (either 0.4 mM ddATP, 0.4 mM ddGTP, 0.2 mM ddCTP, or 0.125 mM ddTTP for the sequencing reactions), and 1 μl of reverse transcriptase (1.36 U) in 50 mM Tris (pH 8.3)–60 mM sodium chloride–10 mM dithiothreitol–30 mM magnesium acetate. The samples were incubated for 3 min at 37°C and then for 30 min at 50°C, and the reactions were stopped by addition of 4 μl of sample buffer. The reaction products were displayed by autoradiography following separation on a 6% sequencing gel.

RNase protection experiment. SRP RNA or the *Alu* portion of it was homogeneously labeled by in vitro transcription in the presence of [α-^{32}P]ATP or [α-^{32}P]GTP (specific activity of SRP RNA, 112 μCi/pmol of RNA; specific activity of *Alu* RNA, 56 μCi/pmol of RNA). After gel elution, 2 pmol of each RNA sample was reconstituted with a twofold molar excess of SRP9/14 in SRP buffer. As a negative control, SRP9/14 was substituted in parallel samples with the same amount of bovine serum albumin. The RNA-protein complex (0.5 pmol) was digested at 26°C for 30 min with RNase T_1 at different concentrations and in SRP buffer containing 250 mM potassium acetate. After digestion, the samples were filtered through nitrocellulose (see below). The filters were incubated in 200 μl of 50 mM Tris (pH 7.5)–0.3 M sodium acetate–1 mM EDTA–0.5% sodium dodecyl sulfate (SDS)–100 μg of proteinase K per ml at 55°C for 15 min to remove the RNA from the filters. The samples were then extracted with phenol-chloroform, and the RNA was precipitated with 2.5 volumes of ethanol. The dried samples were dissolved in 4 μl of sample buffer, and the RNA was fractionated on an 8 M urea–15% polyacrylamide gel. RNA fragments A, B, and C were eluted from the gel and identified by the T4 DNA polymerase-dependent mapping technique as described previously (55).

Filter binding experiments. The [^{32}P]pCp-labeled RNA species (0.1 pmol) were incubated in SRP buffer containing either 500 or 250 mM potassium acetate and in the presence of 100 ng of calf liver tRNA with 2 pmol of SRP9/14 or, as a negative control, with the same amount of bovine serum albumin. The protein-RNA complexes (5 μl) were added to 50 μl of wash buffer (SRP buffer without Nikkol) containing either 500 or 250 mM potassium acetate, immediately filtered through nitrocellulose, and subjected to three washes of 200 μl each of the same buffer and a final wash with 100 μl of wash buffer containing 100 mM potassium acetate. The amount of ^{32}P-labeled RNA retained on the filter was determined by liquid scintillation counting. A control experiment indicated that the dilution of the samples before filtration did not affect the results.

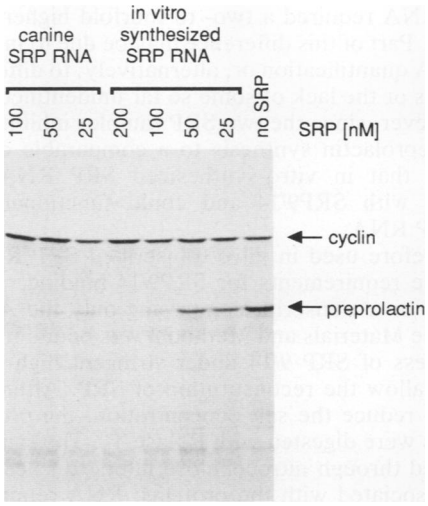


FIG. 2. Elongation arrest activity of SRP samples reconstituted from canine SRP RNA or in vitro-synthesized SRP RNA. Reconstituted SRPs, containing either RNA isolated from purified canine pancreatic SRP or in vitro-synthesized SRP RNA, were added at the concentrations indicated above the lanes to 10-μl wheat germ translation reactions programmed with synthetic bovine preprolactin and sea urchin cyclin mRNAs. After incubation for 25 min at 26°C, the proteins were trichloroacetic acid precipitated and analyzed by SDS-PAGE followed by autoradiography. The concentrations of the reconstituted SRPs were based on the respective concentrations of the SRP RNAs (see Material and Methods).

RESULTS

SRP RNA fragments protected from RNase T_1 digestion in the RNA-SRP9/14 complex. To determine the binding site(s) of the heterodimeric protein SRP9/14 on SRP RNA, we analyzed SRP RNA fragments that were protected from T_1 nuclease digestion in the RNA-SRP9/14 complex. A ^{32}P-labeled, in vitro-transcribed SRP RNA was used to reconstitute the RNA-protein complex. The SRP RNA gene was synthesized from overlapping oligonucleotides and cloned behind the T7 RNA polymerase promoter (see Materials and Methods).

We first confirmed that a functional SRP could be reconstituted from the in vitro-synthesized RNA and isolated SRP proteins. SRP RNA was either transcribed from the synthetic gene by T7 RNA polymerase or extracted from SRP and reconstituted with the full complement of SRP proteins. Reconstituted SRPs, containing either RNA, were assayed for their ability to arrest the elongation of secretory proteins, since this activity of SRP requires SRP9/14 to be bound to the particle (40).

As shown in Fig. 2, increasing concentrations of both reconstituted SRP preparations specifically inhibited the synthesis of the secretory protein preprolactin in comparison with the control nonsecretory protein cyclin. Both SRP preparations reached a similar efficiency in elongation arrest, although the reconstituted particle containing in vitro-syn-

thesized RNA required a two- to fourfold higher SRP concentration. Part of this difference may be due to inaccuracies of the RNA quantification or, alternatively, to different RNA conformers or the lack of some so far unidentified modification. However, since the two SRP samples inhibited elongation of preprolactin synthesis to a comparable degree, we concluded that in vitro-synthesized SRP RNA properly associated with SRP9/14 and could functionally replace native SRP RNA.

We therefore used in vitro-transcribed SRP RNA to determine the requirements for SRP9/14 binding. ^{32}P-labeled SRP RNA or a transcript comprising only the *Alu* portion thereof (see Materials and Methods) was bound to a twofold molar excess of SRP 9/14 under stringent high-salt conditions that allow the reconstitution of SRP. After a twofold dilution to reduce the salt concentration, the protein-RNA complexes were digested with RNase T_1. The samples were then passed through nitrocellulose filters to select for RNA species associated with the proteins. RNA retained on the filters was extracted and analyzed by polyacrylamide gel electrophoresis (PAGE). In several experiments, we found that the digestion patterns of the complete SRP RNA and those of the *Alu* portion of SRP RNA were identical (Fig. 3; compare the three lanes labeled A, GTP with the three lanes labeled C, GTP). Also, the same fragments were generated whether SRP RNA was labeled with [α-^{32}P]ATP or with [α-^{32}P]GTP during transcription.

Three distinct RNA fragments were reproducibly obtained (Fig. 3, bands A, B, and C) with apparent lengths of 71, 27, and 26 bases, respectively, in comparison with DNA size markers. Variable amounts of two smaller RNA fragments (19 and 20 nucleotides long) were also present. When higher ratios of RNase T_1 to SRP RNA were used, fragment A could not be quantitatively converted into the smaller fragments. Rather, the amounts of fragments B and C increased only slightly when most of fragment A was degraded (data not shown). This finding suggested that the fragment A-SRP9/14 complex was most stable, whereas the smaller fragments, albeit still associated with SRP9/14, became increasingly RNase sensitive.

To identify the protected RNA species, fragments were mapped by primer extension with T4 DNA polymerase (55). The RNA fragments were eluted from the gel and annealed together with a 5′-labeled oligonucleotide primer to a single-stranded antisense DNA which included SRP RNA and flanking vector sequences. During extension of the labeled primer with T4 DNA polymerase, the annealed RNA fragments are not displaced. As a result, the polymerase stalls when it encounters the 5′ end of an RNA fragment, giving rise to a discretely sized extention product. The results of this experiment are shown in Fig. 4A and schematically represented in Fig. 4B. According to this assay, the 5′ end of fragment A mapped to the first nucleotide of the SRP RNA (Fig. 4A, lane 3, labeled a). Since the fragments were generated by RNase T_1 which cuts specifically after G and since fragment A had an apparent length of 71 nucleotides in comparison with the DNA size marker (Fig. 3), fragment A encompasses the complete 5′ end of SRP RNA up to the G residue located at position 68 (or possibly 73) in the SRP RNA (Fig. 4B). Additional signals were observed in this lane (Fig. 4A, labeled a*). Most likely, these fragments resulted from degradation of fragment A during elution from the gel, because (i) two of the three dominant extension products do not map to a nucleotide following a G residue as would be expected from fragments resulting from a RNase T_1 digest (the strongest band maps to A-26 in SRP RNA) and (ii) nucleotides in this single-stranded region of SRP RNA were previously noted to be sensitive to nuclease cleavage (11).

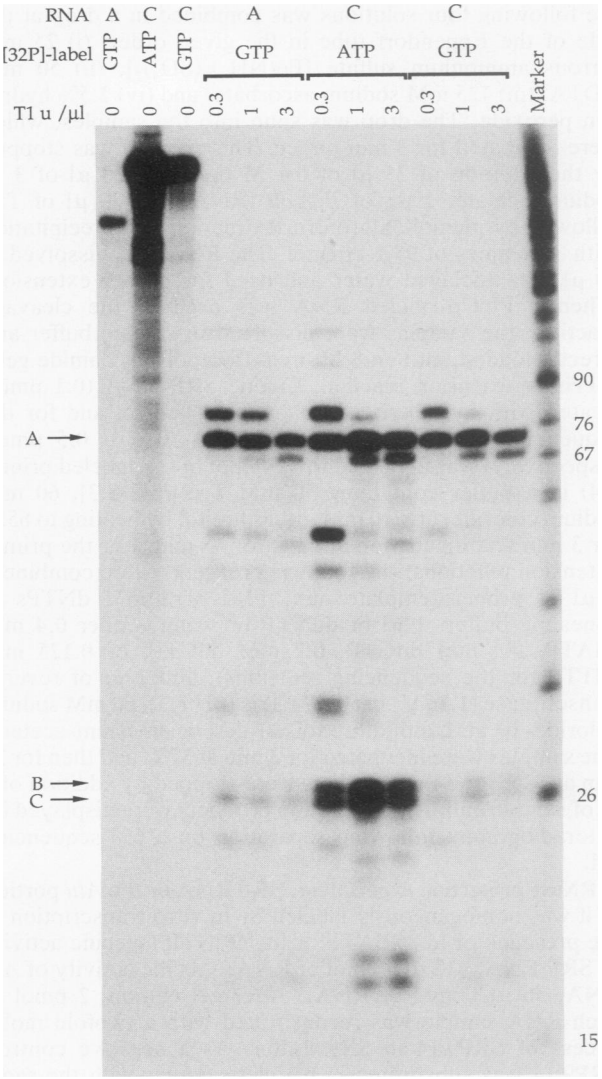
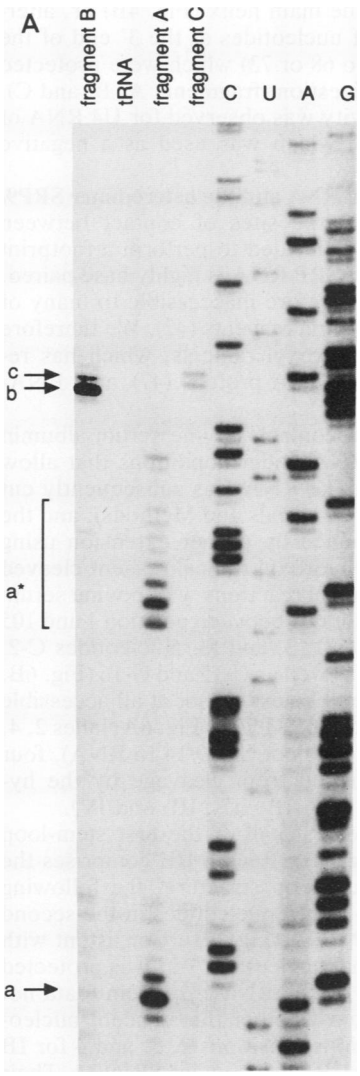


FIG. 3. SRP RNA fragments protected from RNase T_1 digestion by SRP9/14. Complete SRP RNA (RNA labeled C above the lanes) and the *Alu* portion of it (RNA labeled A) were prepared by in vitro transcription using T7 RNA polymerase in the presence of [α-^{32}P]ATP or [α-^{32}P]GTP as indicated. Aliquots of one-fifth of the total amount of RNA used in the experiment are shown for each RNA sample in the leftmost lanes. The RNAs were incubated with SRP9/14 under conditions that allow reconstitution of SRP, and the complexes were treated with RNase T_1 at the concentrations indicated above the lanes. Protein-associated RNA fragments were isolated on nitrocellulose filters, fractionated on an 8 M urea–15% polyacrylamide gel, and visualized by autoradiography. No protected fragments were observed when SRP9/14 was replaced by bovine serum albumin in the protection assay (data not shown).

The 5′ ends of fragments B and C mapped to nucleotides G-42 and A-43, respectively (Fig. 4A, labeled b and c). Given their apparent lengths of 26 and 27 nucleotides (Fig. 3), they extend up to position 68 in SRP RNA (Fig. 4B). For all three fragments (A, B, and C), the extension products were slightly heterogeneous (note the shadow bands in Fig. 4A spaced by one nucleotide), most likely because of a contam-



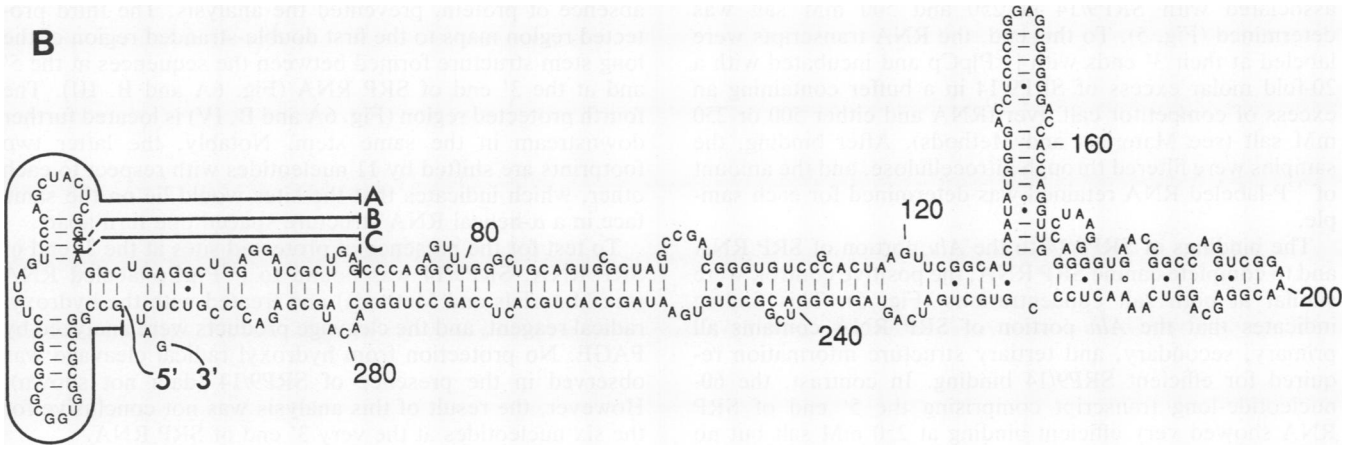


FIG. 4. (A) Mapping of the 5′ ends of the RNase T_1-protected fragments. The 5′ ends of the protected fragments were mapped onto the primary structure of SRP RNA by primer extension using T4 DNA polymerase. In this assay, the ^{32}P-labeled extension products terminate when the polymerase encounters the 5′ ends of an annealed RNA fragment. A sequencing reaction was run in parallel to determine the precise location of the 3′ ends of the extension products. The protected fragments analyzed (A, B, and C from Fig. 3) are indicated above the lanes. *E. coli* tRNA was used as a negative control. The 3′ ends of the extension products are indicated (a, b, and c). Extension products resulting from possible RNA degradation (a*) are discussed in the text. (B) Secondary structure of SRP RNA. Bars indicate the locations of the protected fragments as deduced from the positions of their 5′ ends (panel A) and their total lengths (Fig. 3).

inating exonuclease activity. Mapping experiments using the smaller fragments of RNase T_1 digestion (Fig. 3) gave inconclusive results and were not pursued further.

Binding of SRP9/14 to SRP RNA deletion mutants. These results suggested that the *Alu* portion of SRP RNA and possibly only the first 68 (or 73) nucleotides might be sufficient for binding of SRP9/14. We tested this notion directly in a filter binding assay using canine SRP RNA, the in vitro-synthesized *Alu* portion of SRP RNA, or a transcript consisting of only 60 nucleotides from the 5′ end of SRP

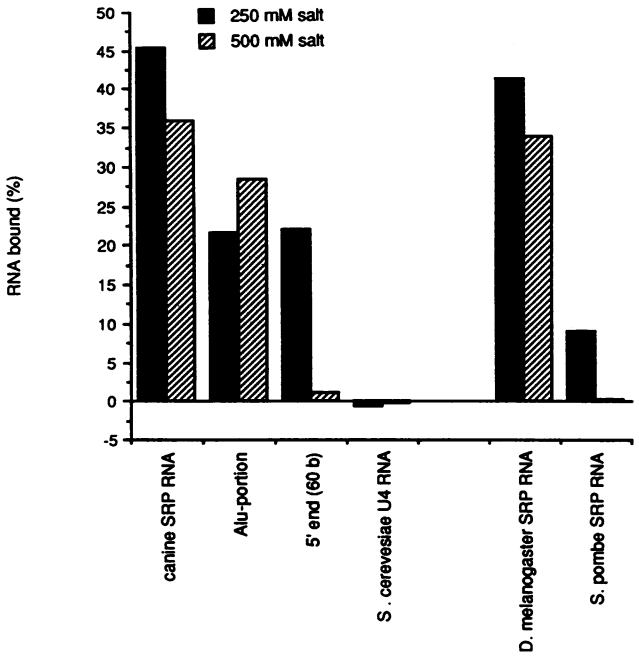


FIG. 5. Binding of SRP9/14 to SRP RNAs and SRP RNA fragments at different salt concentrations. [^{32}P]pCp-labeled RNAs were incubated with a 20-fold molar excess of SRP9/14 in SRP buffer containing 250 or 500 mM potassium acetate (for preparation of the different RNA samples, see Materials and Methods). The relative amount of RNA associated with SRP9/14 or with bovine serum albumin (background binding) was assessed by filtration through nitrocellulose. The values obtained for background binding were subtracted from those obtained with SRP9/14. Binding of *S. cerevisiae* U4 RNA to SRP9/14 served as a control for nonspecific binding. Since the 3′ labeling as well as the recovery from the gel of the various RNA samples occurred with different efficiencies, the concentrations of the different RNAs were only approximate, and the relative binding efficiencies to SRP9/14 between different RNA species may not be directly comparable.

RNA. The relative amount of each RNA sample specifically associated with SRP9/14 at 250 and 500 mM salt was determined (Fig. 5). To this end, the RNA transcripts were labeled at their 3′ ends with [^{32}P]pCp and incubated with a 20-fold molar excess of SRP9/14 in a buffer containing an excess of competitor calf liver tRNA and either 500 or 250 mM salt (see Materials and Methods). After binding, the samples were filtered through nitrocellulose, and the amount of ^{32}P-labeled RNA retained was determined for each sample.

The bindings of SRP9/14 to the *Alu* portion of SRP RNA and to complete canine SRP RNA, the positive control, were similar at both salt concentrations (Fig. 5). This finding indicates that the *Alu* portion of SRP RNA contains all primary, secondary, and tertiary structure information required for efficient SRP9/14 binding. In contrast, the 60-nucleotide-long transcript comprising the 5′ end of SRP RNA showed very efficient binding at 250 mM salt but no binding at 500 mM salt (Fig. 5). Thus, this truncated transcript contains most sequence elements needed to bind SRP9/14, as expected from the analysis of the RNase T_1-protected fragments described above. However, changes in the overall structure resulted in a decrease of the affinity of the protein to the RNA, and binding at 500 mM salt was no longer sustained. This change could be due to the lack of the complementary strand of the main helix (Fig. 4B) or, alternatively, to the absence of nucleotides at the 3′ end of the transcript (nucleotides 61 to 68 or 73) which were protected by SRP9/14 from RNase digestion (fragments A, B, and C). No significant binding activity was observed for U4 RNA of *Saccharomyces cerevisiae*, which was used as a negative control.

Contact sites between SRP RNA and the heterodimer SRP9/14. To determine precisely the sites of contact between SRP9/14 and SRP RNA, we decided to perform a footprint analysis. The *Alu* portion of SRP RNA is highly base paired, and most nucleotides are therefore inaccessible to many of the RNA-modifying or -cleaving reagents (42). We therefore chose RNA cleavage by hydroxyl radicals, which has recently been used to study DNA-protein (47) and RNA-protein (54) interactions.

Purified SRP9/14 or, as a control, bovine serum albumin was incubated with SRP RNA under conditions that allow the reconstitution of SRP. The RNA was subsequently cut with hydroxyl radicals (see Materials and Methods), and the cleavage sites were determined by primer extension using reverse transcriptase. The hydroxyl radical reagent cleaved SRP RNA, incubated in control reactions with bovine serum albumin, at almost all nucleotides between position 1 and 105 in SRP RNA (Fig. 6A, lanes 1, 3, and 5). Nucleotides C-2, C-3, and G-4 (Fig. 6B, IA), as well as C-15 and G-16 (Fig. 6B, IIA), seemed to be considerably less or not at all accessible for cleavage. In the presence of SRP9/14 (Fig. 6A, lanes 2, 4, and 6, at increasing molar ratios of SRP9/14 to RNA), four distinct regions were protected from cleavage by the hydroxyl radical reagent (Fig. 6A, IB, IIB, III, and IV).

Region IB comprises the first half of the first stem-loop structure of SRP RNA (Fig. 6B). Region IIB comprises the second half of the first stem-loop structure, the following bulge, and half of the base-paired nucleotides in the second stem-loop structure in SRP RNA (Fig. 6B). Consistent with this finding, we observed that nucleotide G-27 was protected by SRP9/14 from cleavage by the RNase α-sarcin (data not shown). It remains uncertain whether the adjacent nucleotides of both protected regions (positions 2, 3, and 4 for IB and positions 15 and 16 for IIB) also contact SRP9/14. Their intrinsic resistance to hydroxyl radical cleavage, even in the absence of protein, prevented the analysis. The third protected region maps to the first double-stranded region of the long stem structure formed between the sequences at the 5′ and at the 3′ end of SRP RNA (Fig. 6A and B, III). The fourth protected region (Fig. 6A and B, IV) is located further downstream in the same stem. Notably, the latter two footprints are shifted by 11 nucleotides with respect to each other, which indicates that the sites would lie on the same face in a α-helical RNA structure spaced one turn apart.

To test for the presence of protected sites at the 3′ end of SRP RNA, SRP9/14 was bound to [^{32}P]pCp-labeled RNA (see Materials and Methods) and treated with the hydroxyl radical reagent, and the cleavage products were analyzed by PAGE. No protection from hydroxyl radical cleavage was observed in the presence of SRP9/14 (data not shown). However, the result of this analysis was not conclusive for the six nucleotides at the very 3′ end of SRP RNA.

The results obtained in the footprint analysis (Fig. 6) are in good agreement with the results of the RNase T_1 protection experiments (Fig. 4B). All regions protected from the attack by hydroxyl radical are contained within the fragments which were found to be protected from RNase T_1 cleavage in the SRP RNA-SRP9/14 complex. Taken together, the results corroborate the interpretation that the sites protected by

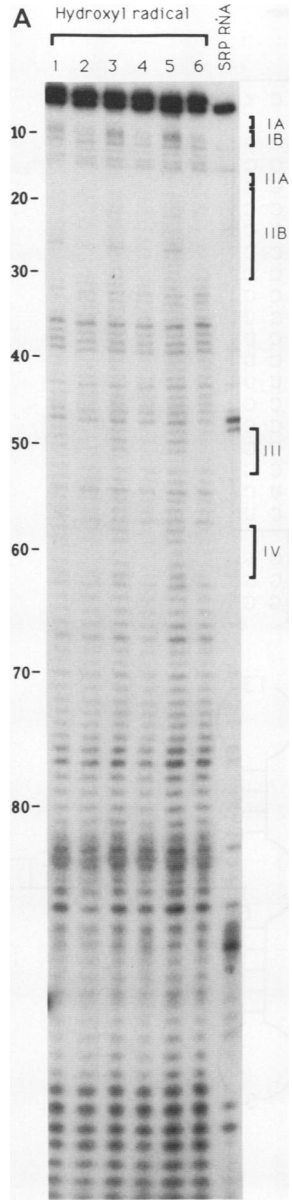
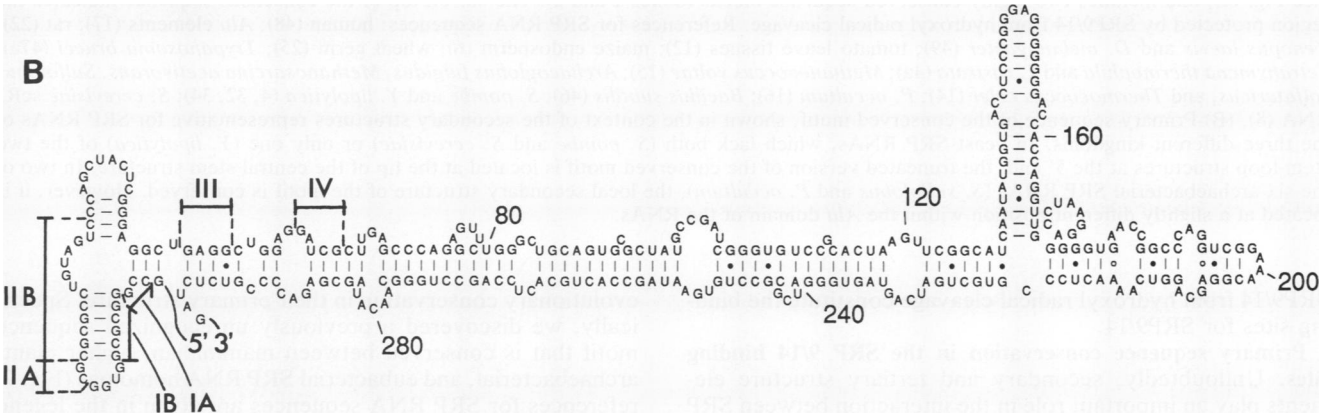


FIG. 6. (A) Alteration of the hydroxyl radical cleavage pattern of SRP RNA in the presence of SRP 9/14. SRP RNA was incubated with SRP9/14 or, as a negative control, with bovine serum albumin under conditions that allow reconstitution of SRP and subsequently cleaved with hydroxyl radical. The cleavage pattern of the RNA in each sample was determined by primer extension using the ^{32}P-labeled primer complementary to nucleotides 112 to 122 in SRP RNA. The primer extension products were fractionated on an 8 M urea–6% polyacrylamide gel and displayed by autoradiography. A sequencing reaction was run in parallel to map the extension products and to localize the footprints (data not shown). The regions protected from cleavage by the hydroxyl radical reagent in the presence of SRP9/14 are labeled IB, IIB, III, and IV. Regions IA and IIA are not cleaved by the reagent in naked RNA. The full-length primer extension product of SRP RNA was always seen as a doublet at the top of the gel. Lanes: 2, 4, and 6, cleavage patterns of SRP RNA in the presence of SRP9/14 at molar ratios of 1:1, 1:3, and 1:6, respectively; 1, 3, and 5, cleavage patterns of SRP RNA when SRP9/14 was replaced with the same amount of bovine serum albumin (variations in the band intensity may reflect tertiary RNA structure [5, 21]); SRP RNA, primer extension products obtained with uncleaved canine SRP RNA. (B) Secondary structure of SRP RNA. Bars outline the regions that are protected from hydroxyl radical cleavage in the presence of SRP 9/14.



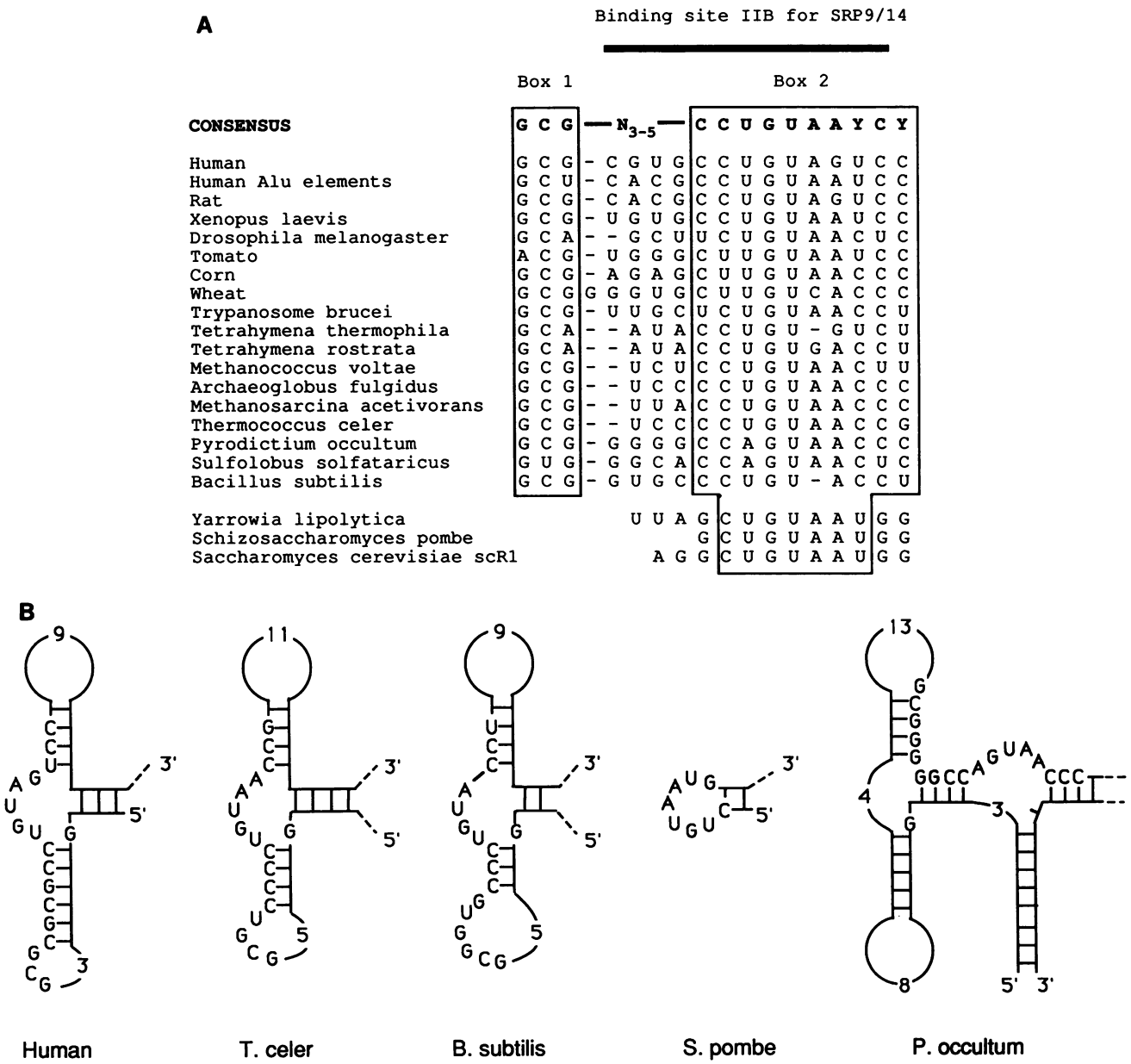


FIG. 7. (A) Sequence conservation in the SRP9/14 binding site IIB. The complete motif spans nucleotides 14 to 30 in canine SRP RNA (see Fig. 6B) and includes two highly conserved sequence blocks (boxes 1 and 2). The bar on top of the consensus sequence indicates the region protected by SRP9/14 from hydroxyl radical cleavage. References for SRP RNA sequences: human (48); *Alu* elements (17); rat (22); *Xenopus laevis* and *D. melanogaster* (49); tomato leave tissues (12); maize endosperm (6); wheat germ (25); *Trypanosoma brucei* (47a); *Tetrahymena thermophila* and *T. rostrata* (4a); *Methanococcus voltae* (15); *Archaeoglobus fulgidus*, *Methanosarcina acetivorans*, *Sulfolobus solfataricus*, and *Thermococcus celer* (14); *P. occultum* (16); *Bacillus subtilis* (46); *S. pombe* and *Y. lipolytica* (4, 32, 34); *S. cerevisiae* scR1 RNA (8). (B) Primary sequence of the conserved motif, shown in the context of the secondary structures representative for SRP RNAs of the three different kingdoms. In yeast SRP RNAs, which lack both (*S. pombe* and *S. cerevisiae*) or only one (*Y. lipolytica*) of the two stem-loop structures at the 5′ end, the truncated version of the conserved motif is located at the tip of the central stem structure. In two of the six archaebacterial SRP RNAs (*S. sulfolobus* and *P. occultum*), the local secondary structure of the motif is conserved. However, it is located at a slightly different position within the *Alu* domain of the RNAs.

SRP9/14 from hydroxyl radical cleavage constitute the binding sites for SRP9/14.

Primary sequence conservation in the SRP 9/14 binding sites. Undoubtedly, secondary and tertiary structure elements play an important role in the interaction between SRP RNA and SRP 9/14. However, we found that the sequences contained in binding site IIB (Fig. 6B) showed significant evolutionary conservation in their primary structure. Specifically, we discovered a previously unrecognized sequence motif that is conserved between mammalian, higher plant, archaebacterial, and eubacterial SRP RNA homologs (Fig. 7; references for SRP RNA sequences are given in the legend to Fig. 7). The core of the consensus sequence is also found in SRP RNA homologs from the yeasts *Yarrowia lipolytica*

and *S. pombe* as well as in scR1 RNA from *S. cerevisiae* (8). scR1 RNA is an abundant cytoplasmic RNA which does not contain an otherwise highly conserved stem structure (stem IV in references 33 and 45) indicative of SRP RNAs. However, recent experimental evidence shows that scR1 RNA indeed constitutes the SRP RNA homolog in *S. cerevisiae* (13a).

The conserved motif (Fig. 7) extends from positions 14 to 30 in canine SRP RNA (compare with Fig. 6B). It consists of two highly conserved regions interupted by a stretch of nonconserved sequences. The first conserved sequence block, box 1 (GCG), is located in the loop of the first stem-loop structure of human SRP RNA. It is not clear whether SRP9/14 is actually in contact with the nucleotides of box 1 because the last two nucleotides (C-15 and G-16) are inaccessible for hydroxyl radical cleavage, and the exact border of the protection is therefore not known (Fig. 6B, IIA). Interestingly, it was previously found that nucleotides C-15 and G-16 constitute an essential promoter element for SRP RNA gene transcription (3; for a discussion, see below). The second conserved sequence block, box 2 (CYUG UAAYCY), spans mainly the single-stranded region between the two stem-loop structures at the 5′ end of SRP RNA but also extends on both sides into the adjacent stems (Fig. 6B). The last nucleotide of box 2 is not protected by SRP9/14 from hydroxyl radical cleavage and thus may not be part of the SRP9/14 binding site. The sequences between the two boxes are part of a region predicted to be double stranded, which may explain why they are not conserved in sequence. In a sequence comparison of archaebacterial SRP RNAs, it was previously noticed that the motif GUAA, which is part of box 2, is highly conserved (14).

Considering this sequence conservation, we tested whether canine SRP9/14 could bind to SRP RNAs from evolutionarily distant species. The result of a binding experiment is shown in Fig. 5 (right columns). In the presence of a 20-fold molar excess of SRP9/14, SRP RNA of *D. melanogaster* was bound to a similar extent at high (500 mM) and at low (250 mM) salt concentrations as mammalian SRP RNA. Interestingly, the SRP RNA homolog from *S. pombe* also showed significant binding of mammalian SRP9/14 at the lower salt concentration (Fig. 5; compare with the negative U4 RNA control).

DISCUSSION

Recently, several components of SRP were found to be conserved throughout evolution, indicating that an SRP-mediated signal recognition and protein-targeting mechanism may have evolved much earlier than previously appreciated. Homologs of SRP54, the protein that interacts with the signal sequence of the secreted protein as it emerges from the ribosome, were identified in yeasts and eubacteria by virtue of primary sequence similarity (1, 2, 13, 36). Overall secondary structure conservation has served as a criterion for the identification of SRP RNAs from a wide variety of organisms. However, although adhering to a characteristic secondary structure, these RNAs show a very rapid evolutionary drift in their primary structure, with hardly any sequence similarities apparent even between the yeast and mammalian homologs. During an extended phylogenetic analysis of SRP RNA sequences, it was noted that a structural element in the central domain of SRP RNA (domain IV; 33, 45) is particularly well conserved. Recent evidence suggests that this domain is involved in linking SRP54 to the rest of the particle and hence is involved intimately in the signal sequence recognition function of SRP (37, 57).

In contrast, the *Alu* domain of mammalian SRP, albeit required for the elongation arrest function of SRP, was shown to be completely dispensable for the signal recognition and protein-targeting activity of SRP (40). No significant primary and secondary structure conservation was observed between the 5′ and 3′ regions of SRP RNAs. Through a combination of RNase protection, filter binding, and protection from hydroxyl radical cleavage, we have mapped the binding site of the heterodimeric protein SRP9/14 to four discrete regions within the *Alu* portion of SRP RNA. Surprisingly, the nucleotide sequence in the mostly single-stranded contact site IIB (Fig. 6B) was found to be evolutionarily conserved in SRP RNAs of organisms of all three kingdoms (Fig. 7). The finding of a conserved sequence motif in the SRP9/14 binding sites suggests the existence of SRP9/14 homologs in other species. Potentially this structural conservation reflects a functional conservation and indicates that the elongation arrest function is also very ancient and important for a vast variety of organisms.

In the *Alu* domain of SRP RNAs of the three kingdoms, some general features of the secondary structure are conserved, such as the presence of two stem-loop structures at the 5′ end followed by an extended yet frequently interrupted double-stranded region. However, the exact secondary structure (e.g., length of stems and loops) is quite variable between the RNAs of different species. In addition, yeast SRP RNAs lack one (*Y. lipolytica*) or both (*S. cerevisiae* and *S. pombe*; Fig. 7B) of the stem-loop structures at the 5′ end (20a, 32). Notably, in the context of the general overall structure, the location of the identified consensus motif of the SRP9/14 contact site is very conserved (Fig. 7B). It spans the single-stranded region between the first and second stem-and-loop structures, extending into both flanking stems. At present, the precise end of the first stem remains uncertain. Results from experiments in which the secondary structure of SRP RNA was analyzed by using the double-stranded specific RNase from *N. oxiana* suggest that G-4 is base paired with U-24. However, the extreme conservation of this nucleotide may indicate that it is not base paired.

In the yeasts, a truncated version of the consensus motif (missing box 1) is found positioned at the tip of the central stem structure (Fig. 7B). The homology between the yeast RNAs in this region has been observed before, and it was suggested that this sequence would be part of the A box required for transcription by RNA polymerase III (8, 32). However, the transcription of human SRP RNA was shown in vivo and in vitro to depend solely on extragenic sequences located 5′ of the transcription initiation site, on the internal nucleotides C-15 and G-16 (region IIA, Fig. 6B), and on other intragenic sequence elements (proposed to constitute the A box) located further 5′ to box 1 (3, 18, 50). Most important, mutations in sequences in the box 2 of the SRP9/14 binding site consensus did not affect in vitro transcription of SRP RNA (3). The sequence elements essential for transcription of SRP RNA in yeasts have not been analyzed to date; therefore, the possibility remains that in yeasts these sequences play a role in binding of a putative SRP9/14 homolog(s) and in transcription of the SRP RNA gene. However, the results obtained in the mammalian system indicate that the high sequence conservation in the second block of the consensus does not reflect a requirement for transcription. In addition, the primary sequence conservation between yeast RNAs extends to nucleotides which

are defined as N within the polymerase III A-box consensus (9, 32).

In two of the six known archaebacterial SRP RNA homologs (*Sulfolobus solfataricus* and *Pyrodictium occultum*), the local secondary structure of the consensus sequence is conserved; i.e., the sequence AGUAA in box 2 is single stranded, whereas the flanking sequences are double stranded. However, the sequence motif is found shifted to a different location within the overall secondary structure. In these two organisms, the motif starts at the end of the loop of the second stem-loop structure and extends through a single-stranded region linking the stem-loop structures to the main helical stem of the SRP RNAs (Fig. 7B; see also references in legend to Fig. 7). In addition, the highly conserved U residue at position 3 in box 2 is replaced by an A residue in these two archaebacterial SRP RNAs. As the conserved motif is somewhat altered, it will be interesting to determine whether SRP9/14 homologs are also present in these organisms and to determine their binding sites on the RNA.

In the *Halobacterium halobium* SRP RNA homolog (30), we could not detect a sequence that was convincingly homologous to the consensus motif defined in Fig. 7. This RNA contains the domain IV consensus structure in the central domain and therefore is very likely to be an SRP RNA homolog. Furthermore, *E. coli* 4.5S RNA is homologous to SRP RNA in domain IV but lacks the *Alu* domain altogether. These exceptions indicate that the consensus motif, while being extremely highly conserved, is not universal in SRP-like RNAs.

The consensus motif is also conserved in the highly repeated *Alu* sequences (Fig. 7). While most of the about half a million *Alu* repeats are thought to be pseudogenes, not considered to give rise to any RNA products, a small subset of *Alu* genes has been identified to be transcriptionally active (26). It is possible that RNA transcripts from these genes assemble with SRP9/14 and that, in analogy to the function of the *Alu* domain as part of SRP, the resulting ribonucleoprotein complexes play some role in translational control.

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