The Alu domain homolog of the yeast signal recognition particle consists of an Srp14p homodimer and a yeast-specific RNA structure

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Abstract

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ABSTRACT
The mammalian Alu domain of the signal recognition particle (SRP) consists of a heterodimeric protein SRP9/14 and the Alu portion of 7SL RNA and comprises the elongation arrest function of the particle. To define the domain in Saccharomyces cerevisiae SRP that is homologous to the mammalian Alu domain [Alu domain homolog in yeast (Adhy)], we examined the assembly of a yeast protein homologous to mammalian SRP14 (Srp14p) and scR1 RNA. Srp14p binds as a homodimeric complex to the 5' sequences of scR1 RNA. Its minimal binding site consists of 99 nt (Adhy RNA), comprising a short hairpin structure followed by an extended stem. As in mammalian SRP9/14, the motif UGUAAU present in most SRP RNAs is part of the Srp14p binding sites as shown by footprint and mutagenesis studies. In addition, certain basic amino acid residues conserved between mammalian SRP14 and Srp14p are essential for RNA binding in both proteins. These findings confirm the common ancestry of the yeast and the mammalian components and indicate that Srp14p together with Adhy RNA represents the Alu domain homolog in yeast SRP that may comprise its elongation arrest function. Despite the similarities, Srp14p selectively recognizes only scR1 RNA, revealing substantial changes in RNA–protein recognition as well as in the overall structure of the complex. The alignment of the three yeast SRP RNAs known to date suggests a common structure for the putative elongation arrest domain of all three organisms.

Keywords: elongation arrest; homodimer; RNA–protein; scR1 RNA; secretion; SRP9/14

INTRODUCTION
The process of cotranslational translocation into the endoplasmic reticulum (ER) is mediated by a cytoplasmic targeting factor, the signal recognition particle (SRP). Components of SRP have been identified in organisms of all three kingdoms by sequence comparison. Functional evidence for the role of SRP in protein secretion has been provided in mammalian, yeast, and bacterial organisms. Although most proteins appear to require SRP for their translocation into mammalian microsomes, only a subset of proteins use SRP as a targeting factor in yeast and in bacteria (for reviews, see Walter & Johnson, 1994; Rapoport et al., 1996; De Gier et al., 1997).

The mammalian SRP has been used as a model for the extensive characterization of SRP functions. It comprises six proteins and one RNA molecule (SRP or 7SL RNA). The SRP 54 protein specifically recognizes signal sequences and together with stem VIII of 7SL RNA is required for the targeting of the nascent chain–ribosome complex to the ER membrane (for review, see Lütcke, 1995). SRP54 and stem VIII of 7SL RNA represent the evolutionarily most highly conserved SRP structures and have been recognized in many organisms of all three kingdoms (for references, see Samuelsson & Zwieb, 1999).

The 5' and 3' sequences of 7SL RNA that are homologous to the Alu family of repetitive sequences as well as two proteins, SRP9 and SRP14, constitute the Alu domain of mammalian SRP. The Alu domain mediates a transient arrest in the elongation of nascent chains that increases the efficiency of translocation in vitro (Siegel & Walter, 1985; Thomas et al., 1997; for a review, see Bui & Strub, 1999). SRP9 and SRP14 are structural homologs and members of the family of small α/β RNA-binding proteins (Birse et al., 1997). Binding of SRP9/14 to the Alu portion of 7SL RNA appears to
induce conformational changes in the protein as well as in the RNA moieties (Janiak et al., 1992; Bui et al., 1997; Weichenrieder et al., 1997). Such adaptive changes have been suggested to play a crucial role in the direct interaction between the Alu domain and the ribosome that affects elongation arrest (Thomas et al., 1997).

The most highly conserved feature in the Alu domain comprises a mostly single-stranded motif within the context of two hairpin structures at the 5’ end of 7SL RNA (CCUUAAYCY; Strub et al., 1991). It is part of the binding sites of SRP9/14 (Strub et al., 1991; Chang et al., 1997; Weichenrieder et al., 1997) and is found in SRP RNAs in organisms of Eukarya and Archaea as well as in two Bacillus species. Many bacterial SRP RNAs lack the Alu domain entirely. Homologous proteins of mammalian SRP9 and SRP14 have so far only been identified in eukaryotic organisms.

Although signal recognition and targeting functions of SRP have been studied in mammalian, yeast, and bacterial organisms, the elongation arrest domain in SRPs of distantly related organisms has not been characterized. We therefore decided to define a putative elongation arrest domain of Saccharomyces cerevisiae SRP based on the assumption that its components are ancestrally related to the components of the mammalian Alu domain. The S. cerevisiae SRP appeared to be most suitable for these studies for two reasons. First, compared to the mammalian species it is the most distantly related organism for which structural components characteristic for the Alu domain have been identified (Strub et al., 1991; Brown et al., 1994) and second, the results of these studies should facilitate the genetic analysis of this function.

Saccharomyces cerevisiae SRP (scSRP) has been shown to play a role in the translocation of a number of proteins, but interacts preferentially with proteins bearing strongly hydrophobic signal sequences (Hansen & Walter, 1988; Hann & Walter, 1991; Stirling & Hewitt, 1992; Ng et al., 1996). In contrast to bacteria and other yeast species, scSRP is not essential for the survival of this organism. Presumably, it can adapt to the loss of SRP by using alternative targeting factors (Ogg et al., 1992). The scSRP comprises at least six proteins and one RNA molecule (Hann & Walter, 1991; Stirling & Hewitt, 1992; Brown et al., 1994). Five of the identified SRP proteins in S. cerevisiae, Srp72p, Srp68p, Srp54p, Srp19p, and Srp14p, are homologous to mammalian SRP proteins. Another protein, Srp21p, has so far only been identified in yeast. Yeast SRP RNA, called scR1 RNA, is almost twice as large as other known SRP RNAs. Its secondary structure remains elusive because computer-generated structures failed to reveal a similarity to the canonical secondary structure of SRP RNAs and to identify the conserved stem VIII (Felici et al., 1989; Hann & Walter, 1991). In contrast, SRP RNAs from Schizosaccharomyces pombe and Yarrowia lipolytica could be folded to resemble the canonical structure of SRP RNAs in the S domain, but lacked the typical two-hairpin structure at their 5’ ends (Brenwald et al., 1988; Poritz et al., 1988; Ribes et al., 1988; He et al., 1989).

The components of the mammalian Alu domain that are also present in yeast include Srp14p, which shares 30% sequence identity with mammalian SRP14 (Brown et al., 1994), and a short sequence at the 5’ end of scR1 RNA that might represent a shorter version of the previously mentioned motif identified in the mammalian Alu domain (Strub et al., 1991). However, the conserved motif has also been proposed to represent the A box of the RNA polymerase III promoter of the scR1 gene (Felici et al., 1989). Furthermore, a homolog of the mammalian SRP9 protein has not been identified in S. cerevisiae.

Our studies revealed that Srp14p binds as a homodimeric complex to scR1 RNA. Its binding sites comprise 99 nt at the 5’ end of the RNA, which form a short hairpin structure followed by a double-helical stem. Unlike in the mammalian complex, the Srp14p binding sites do not include sequences from the 3’ end of the RNA and lack the typical two-hairpin structure. In addition, the RNA-binding specificity of the homodimer is clearly distinct from the mammalian heterodimer, as it failed to bind Alu RNA. Despite these differences, certain homologous amino acid residues and a short single-stranded RNA motif are required for the assembly of the yeast and the mammalian complexes, consistent with a common ancestry of the two domains. Based on the secondary structure alignment of the RNAs, it is likely that the three yeast SRPs known to date share the typical features of the Alu domain homolog in S. cerevisiae SRP.

RESULTS

Srp14p binds as a homodimeric complex to scR1 RNA

In mammalian SRP, the proteins SRP14 and SRP9 bind specifically and exclusively as a heterodimeric complex to the Alu portion of 7SL RNA (Strub & Walter, 1990). In S. cerevisiae, a homolog of the mammalian SRP14 protein, Srp14p, has been identified, whereas a homolog of the SRP9 protein has not (Brown et al., 1994) suggesting that Srp14p alone, possibly as a homodimer, may bind scR1 RNA. This hypothesis was further strengthened by the finding that the mammalian proteins SRP9 and SRP14 were, despite their primary sequence divergence, structurally homologous (Birse et al., 1997) suggesting that the heterodimer may have evolved from a homodimeric protein.

To test whether Srp14p can bind S. cerevisiae SRP RNA, scR1 RNA, we synthesized the protein and the RNA in vitro. Both genes, SRP14 and SCR1, were am-
plified from the yeast genome and inserted into plasmids to allow their transcription by SP6 and T7 RNA polymerase, respectively (see Materials and Methods). The synthetic transcripts comprising the SRP14 coding region were used to program wheat germ extract for the synthesis of [35S]-labeled Srp14p. The translation reaction was then incubated with in vitro-synthesized biotinylated scR1 RNA and the RNA-bound protein separated from free protein with immobilized streptavidin. The bound protein was displayed by SDS-PAGE and visualized by autoradiography.

Reproducibly, ~40% of [35S]-labeled Srp14p was bound to scR1 RNA, whereas <5% of the protein was bound to a control RNA (Fig. 1). The control RNA (cRNA) represents a portion of the antisense strand of the murine SRP14 mRNA and was previously used as a negative control in RNA-binding assays with the mammalian SRP9/14 heterodimer (Bovia et al., 1994). The observed binding efficiency of Srp14p is practically identical to that found for SRP9/14 binding to Alu RNA (Bui et al., 1997). Furthermore, increasing the scR1 RNA concentration by 10-fold and varying the salt concentrations between 150 mM and 350 mM did not change the binding efficiency, indicating that the binding conditions were optimal (results not shown). In addition, we tested an RNA that was derived from an scR1 RNA gene lacking the most 5' adenosine residue (results not shown). It bound Srp14p with the same efficiency as scR1 RNA, demonstrating that the adenosine is dispensable for protein binding.

In the same experiments, we also examined whether a 160-nt RNA representing the Alu portion of 7SL RNA (Strub et al., 1991) can bind Srp14p. Alu RNA, which specifically binds the mammalian heterodimer SRP9/14, failed to bind Srp14p. Thus, Srp14p very specifically recognizes only scR1 RNA, suggesting that the structural features of the RNA recognized by the yeast Srp14p protein are clearly distinct from the ones recognized by the mammalian heterodimeric SRP9/14 protein.

For further analysis of the scR1 RNA–Srp14p complex, we wanted to narrow down the region in scR1 RNA that was required for Srp14p binding. A likely candidate for Srp14p binding was the motif CUGUAUAU at the 5' end of scR1 RNA, which has been suggested to represent a shorter version of a conserved motif in the SRP9/14-binding sites of 7SL RNA (Strub et al., 1991). To examine whether the 5' portion of scR1 RNA was indeed required for Srp14p binding, we made two RNAs, Rydel45 and Rydel70, lacking 45 and 70 nt at their 5' ends, respectively. In addition, we designed two RNAs, Rycom188 and Rycom71, comprising sequences from the 5' and the 3' ends of scR1 RNA. Rycom188 comprises 160 and 26 nt from the 5' and 3' ends of scR1 RNA, respectively (see Fig. 3 for exact sequence). Rycom71 includes the first 43 and the last 19 nt linked by the sequence ACAUUUCUU. In the absence of a known secondary structure for scR1 RNA, the design of these truncated RNAs was based on a secondary structure model (not shown) generated with the computer (Zuker et al., 1991, and references therein). In this model, nucleotides near the 5' and at the 3' ends are paired, which is a common feature in SRP RNAs (Samuelsson & Zwieb, 1999). The RNAs were synthesized in vitro and assayed for protein binding as described above.

Srp14p bound with comparable efficiencies to Rycom188 and scR1 RNAs, whereas Rycom71 RNA did not bind Srp14p (Fig. 1B). Both RNAs with deletions at their 5' ends had strongly reduced binding capacities (Fig. 1C). Hence, Rycom188 RNA is sufficient for Srp14p binding and the sequences within the 5' portion may be critical for this function. The lack of activity of Rycom71 RNA also suggested a role for the nt 44–160 in protein binding.

Analogous to SRP9/14, it was likely that the Srp14p protein might exist and bind to the RNA as a homodimeric complex. To address this issue, we decided to use recombinant epitope-tagged Srp14p (PhySrp14p) in immunoprecipitation and cross-linking experiments. At the same time, this allowed us to verify that Srp14p

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**FIGURE 1.** In vitro synthesized Srp14p binds to the 5' portion of scR1 RNA. A,B,C: In vitro-synthesized, [35S]-labeled Srp14p protein was incubated with in vitro-synthesized biotinylated RNA as indicated on top of each lane. In the binding reactions, the RNA and the potassium acetate concentrations were 0.05 mM and 150 mM, respectively. The bound protein was displayed by 15% SDS-PAGE and visualized by autoradiography. Reproducibly, around 40% of the protein bound to scR1 RNA, whereas less than 5% bound to Alu RNA and to the control RNA. Rycom188 comprises 160 and 26 nt from the 5' and 3' ends of scR1 RNA, respectively (see Fig. 3 for exact sequence). Rycom71 includes the first 43 and the last 19 nt linked by the sequence ACAUUUCUU. Rydel45 and Rydel70 lack 45 and 70 nt at their 5' ends, respectively.
binding to scR1 RNA was not due to a fortuitous complementation by a component of the wheat germ extract such as plant SRP9, which is most likely present in wheat germ extract (Prehn et al., 1987). The Srp14p gene was cloned into a PET vector that drives protein expression from a T7 polymerase promoter (Studier et al., 1990). The recombinant protein, PhySrp14p, contains at its N-terminus 12 additional amino acid residues representing the T7 Tag epitope. The protein was purified to homogeneity by heparin and hydroxyapatite chromatography (see Materials and Methods). PhySrp14p had the expected size and was recognized by antibodies against the T7 Tag epitope (Fig. 2A, lane I; see below).

The RNA-binding activity of the recombinant protein PhySrp14p was confirmed with glycerol gradients (see Materials and Methods). The free protein was predominantly found in fraction 2 (Fig. 2A). In the presence of the RNA, the protein shifted into the fractions that also contained the RNA (Figs. 2B and 2C, fraction 5 and, to a lesser extent, fractions 4 and 6). These results confirmed that Srp14p binds alone to scR1 RNA. As negative controls, we also made gradients with the control RNA used in the RNA-binding experiments (cRNA) and with tRNA. As expected, PhySrp14p did not bind to either of these RNAs (results not shown).

We used glutaraldehyde to examine whether PhySrp14p exists as a homodimeric complex. The cross-linking conditions used in these experiments have been established for efficient cross-linking of murine SRP9 and SRP14 in the mammalian heterodimer (Bui et al., 1997). Briefly, glutaraldehyde was added at a final concentration of 0.08% to an aliquot of each fraction of the two gradients. The reaction was allowed to continue for 2 min on ice before the addition of Tris-HCl to quench the reaction. Addition of glutaraldehyde to the protein alone (Fig. 2A) and to the RNA–protein complex (Fig. 2B) resulted in the formation of cross-linked products with apparent sizes of 21 kDa and 42 kDa, respectively. Thus, the cross-linked product matched the size of a homodimeric Srp14p complex. Reproducibly, we observed a reduced cross-linking efficiency of the two subunits in the presence of the RNA (Fig. 2, compare A and B). Because addition of scR1 RNA had no effect on the coimmunoprecipitation efficiency as shown below, the difference in cross-linking efficiency is most likely explained by a reduced accessibility of the cross-linking site(s) in the RNA–protein complex.

For the immunoprecipitation experiments, we synthesized [35S]-labeled Srp14p in wheat germ lysate in the presence of recombinant PhySrp14p. After synthesis,

![FIGURE 2](image-url)
the translation reactions were preabsorbed on protein A-Sepharose beads before adding to immobilized T7 Tag-specific antibodies (see Materials and Methods). The antibodies specifically coprecipitated the [35 S]-labeled Srp14p in the presence (Fig. 2D, lanes 1 and 2) but not in the absence of PhySrp14p (Fig. 2D, lane 3). Addition of scR1 RNA before immunoprecipitation did not change the coprecipitation efficiency (Fig. 2D, lane 2). In contrast, adding PhySrp14p posttranslationally reduced the coprecipitation efficiency of the newly synthesized protein by a factor of two (Fig. 2D, lanes 4 and 5). These results demonstrate that Srp14p forms a stable complex comprising at least two Srp14p subunits in the absence of scR1 RNA. In addition, efficient subunit exchange between the recombinant and the newly synthesized protein appears to require a prolonged incubation at 26 °C or may be facilitated by factors associated with nascent chains.

In conclusion, Srp14p exists free and bound to scR1 RNA as a homodimeric complex. The 5′ end of scR1 RNA appears to be critical for protein binding. Our results do not formally exclude the possibility that more than one homodimer binds to scR1 RNA. Analogous to the mammalian complex, this possibility appears rather unlikely.

The most prominent footprint of Srp14p covers the conserved motif CUGUAAU in scR1 RNA

To identify Srp14p binding sites within Rycom188 RNA, we decided to perform a footprint analysis. As a cleaving reagent we used hydroxyl radicals, which have been shown to react with the ribose moiety of nucleotides independently of the secondary structure of the RNA (Latham & Cech, 1989). Regions in the RNA that are protected from hydroxyl radical attack in the presence of the protein reveal either contact sites with the protein or ternary structure changes induced by complex formation. In conjunction with other methods, hydroxyl radicals have been successfully used to determine regions in mammalian Alu RNA that are critical for binding of the SRP9/14 heterodimer (Strub et al., 1991; Weichenrieder et al., 1997).

Complexes of Rycom188 and recombinant Srp14p were formed at molar ratios of 1:2 and 1:6. The RNA was subsequently cut with hydroxyl radicals and the cleaved products analyzed by primer extension using reverse transcriptase (see Materials and Methods). The cleavage products of the Srp14p–Rycom188 RNA complex (Fig. 3, RP lanes) were compared to the negative control samples in which Srp14p was replaced with the same amount of bovine serum albumin (Fig. 3, RC lanes). As additional controls, the primer extension products of untreated RNA–protein complex and of RNA alone were also analyzed (Figs. 3A and 3B, lanes 1 and 6).

The analysis revealed two regions within the first 30 nt of the RNA that were specifically protected against cleavage by hydroxyl radicals in the presence of PhySrp14p. The strongest protections were seen in region I at the very 5′ end of Rycom188 over the highly conserved nucleotides CUGUAAU. Region II is in close proximity...
proximity to region I, spanning nt 20–28. It comprises two nucleotides (G24, A25) that represent strong stop sites for reverse transcriptase (Fig. 3A, lane 1). It remains therefore unclear whether the protein also protects these nucleotides.

Reproductibly, we also observed two weaker protections in regions III and IV (Fig. 3B, lane 4). To detect protected sites in the last 50 nt, we used 3′ end-labeled RNA in the cleavage assays. No specific protection was observed at a 1:2 ratio of RNA to protein within this region (results not shown). However, in the presence of a sixfold excess of Srp14p, we observed complete protection of the last 38 nt (results not shown) and of the region spanning nt 44–56 (Fig. 3A). Because these protections were only seen with an excess of protein, we assumed that they resulted from nonspecific binding of PhySrp14p (see also below). One position adjacent to region III (A88) and one position outside the footprint regions (A57) reproducibly showed an increased sensitivity to hydroxyl radical cleavage consistent with protein-induced conformational changes in the RNA.

The minimal RNA, which binds Srp14p, comprises the first 99 nt of scR1 RNA

The footprint studies were consistent with an important role for the first 30 nt in protein binding. In addition, the results of the RNA-binding experiments suggested a possible role for nt 44–160 in protein binding. To determine the 3′ boundary of a functional RNA, we produced RNAs with sequential deletions at their 3′ ends. To this end, different restriction enzymes were used to linearize the plasmid comprising RYCOM188, which resulted in the synthesis of RNAs of different length (Ry plus number of nucleotides). The quality and the quantity of each RNA was determined by comparative denaturing gel analysis (not shown) and protein-binding assays carried out as before. Only Ry124 bound Srp14p as efficiently as Rycom188 RNA (Fig. 4A). This result demonstrated that sequences from the 3′ end of scR1 RNA are dispensable for protein binding (see also Fig. 3C for the RNA sequence). The RNA-binding efficiency of Ry91 is reduced to 30% as compared to Rycom188 RNA, whereas the shorter RNAs had a binding efficiency similar to control RNA. The low binding efficiency of Ry42 is due to a decrease in nonspecific binding, which may be explained by its small size.

Computer-assisted folding of Ry91 RNA indicated that the 5′ conserved sequences CUGUAAA can base pair to sequences at the 3′ end of the RNA. Analogous to Alu RNA (see Fig. 6), these nucleotides might have to be single stranded to bind the protein. In addition, the presence of a strong stop site for reverse transcriptase at nucleotide U14 (Figs. 3A and 3B, lanes 1) is consistent with the existence of a stable hairpin structure at the 5′ end of scR1 RNA. A slightly bigger RNA comprising 99 nt of scR1 yielded a predicted secondary structure with an intact 5′ hairpin structure (Fig. 4D). To test its protein-binding capacity, we engineered a truncated SCR1 gene, RY99, comprising the 99 nt of SCR1 followed by three thymines, which are part of a restriction enzyme cleavage site. As a positive control, we produced the RY126 gene, which spans 126 nt of scR1 RNA followed by three thymines. In addition, we also produced a scR1 gene, RYCOM151, lacking the putative 5′ hairpin structure of Rycom188 RNA. It starts at nt 28 and ends at nt 176 of RYCOM188 (see Fig. 3C). The results of the protein-binding assays are shown in Figure 4B. Both Ry99 and Ry126 RNAs bound Srp14p equally well. As expected from the footprint analysis, Rycom151 RNA did not bind Srp14p.

We had previously noticed in the characterization of the Alu RNA–SRP9/14 complex that removing the complementary strand in the central stem of Alu RNA (Fig. 6D) diminished protein binding significantly at a 500-mM salt concentration as compared to binding at a 250-mM salt concentration (Strub et al., 1991). To test the salt sensitivity of the Ry126 RNA and of the Ry99 RNA–protein complexes, we repeated the binding experiments in the presence of 500 mM potassium acetate. Ry99 and Ry126 RNAs still bound equally well to Srp14p (Fig. 4C), demonstrating that Ry99 RNA includes all necessary elements for efficient Srp14p binding. In contrast, nonspecific binding of the control RNA to Srp14p was not detectable at a 500-mM salt concentration, confirming that the observed binding to the control RNA at a 150-mM salt concentration is nonspecific.

The secondary structure predicted for Ry99 RNA is shown in Figure 4D together with the regions that have been found protected from hydroxyl radical cleavage in the presence of protein (bold letters). The RNA is mostly double stranded; therefore we decided to use V1 nuclease to examine its secondary structure experimentally. V1 nuclease preferentially cleaves RNA with a double-helical conformation over at least 4–6 nt. Notably, cleavage by nuclease V1 does not require that all the bases be paired within such regions (Lowman & Draper, 1986). Ry99 RNA was end labeled with [32P]-pCp and incubated with V1 nuclease at the concentrations indicated for 10 min on ice, and the cleavage products were analyzed directly on a denaturing polyacrylamide gel (Fig. 4E). In parallel, we ran sequencing reactions to map the cleaved bases (not shown). To detect V1 cleavage sites within the 5′ portion of the RNA, we used primer extension analysis. Strong and weak cleavage sites are depicted with black squares and dots, respectively (Fig. 4D).

The observed cleavage pattern is in agreement with the secondary structure predicted for Ry99 RNA. Between nt 50 and 95, the RNA is mostly double helical. Noticeably, the different helical regions are clearly visualized by the observed cleavage pattern. V1 nucleo-
ase cleaves most efficiently in the middle of a double-helical region and less towards its boundaries (Lowman & Draper, 1986). In addition, the loop and bulge regions comprising nt 5–11, 36–42, and 56–62 are not cleaved by V1 nuclease. We observed few cleavages within the 30 nt at the 5' end of Ry99 RNA. Specifically, nt 20–30 are not cleaved by V1 nuclease despite their complementarity to region 84–95. Possibly, we failed to detect cleavages because there are two strong stop sites for reverse transcriptase in the middle of the duplex region where we would expect to find the strongest V1 cleavages. Alternatively, the access of V1 nuclease is sterically hindered. Similarly, the upper strand of the central stem in Alu RNA is also not cleaved by V1 nuclease (Weichenrieder et al., 1997). The adjacent region in Ry99 RNA comprising nt 16–20 might not actually be base paired, as formation of the stem is energetically not favored (see also Discussion).

In summary, the minimal RNA required for Srp14p binding is defined by a hairpin linked by a most likely single-stranded region to a double-helical stem. Footprint regions I and II are located on the 5' hairpin structure and on the upper strand of the adjacent double-stranded region. The strong protection of these regions

![FIGURE 4. The smallest portion of scR1 RNA that binds Srp14p is 99 nt long. A,B,C: In vitro-synthesized Srp14p was incubated with biotinylated RNAs that were progressively shorter at their 3' ends. The RNAs are labeled according to their length. Ry126 and Ry99 RNAs comprise the first 126 and 99 nt of scR1 RNA, respectively, followed by three U residues. Rycom151 RNA spans nt 28–176 of Rycom188 RNA (see Fig. 3C). The binding reactions contained 0.05 nM biotinylated RNA and either 150 mM (A,B) or 500 mM (C) potassium acetate. cRNA: Control RNA. Input: 1/3 of the [35S]-labeled Srp14p protein used in the binding experiments. % bound: The fraction of total protein bound to the different RNAs standardized to 100% binding efficiency of Rycom188 RNA. D: A secondary structure model of Ry99 RNA that was generated with the mfold program (Zuker et al., 1991). V1 nuclease cleavage sites are represented with black squares and dots indicating strong or moderate cleaving efficiencies, respectively. The regions labeled I, II, and III outline the regions protected from hydroxyl radical attack in the presence of recombinant PhySrp14p. E: [32P]-labeled Ry99 RNA was treated with two different V1 nuclease concentrations (U/μL) and the cleavage products were analyzed on a 15% sequencing gel. The positions are labeled according to a sequencing reaction that was run in parallel. F: The cleavage products in the 5' portion of Ry99 RNA were analyzed by primer extension reactions and the positions mapped by comparing to a sequencing reaction. Upper lane: Ry99 treated with 0.03 U/μL V1 nuclease; lower lane: untreated Ry99 RNA.]}
together with the finding that they are essential for protein binding and that region I comprises the evolutionarily conserved motif shown to bind SRP9/14 to Alu RNA argue that these regions represent bona fide protein-binding sites. Footprint region III is located on the opposite strand almost one helical turn apart in a hypothetical A-type structure of the RNA. Hence, it is feasible that the protein may contact the RNA on both faces of the helical stem. However, as region III is significantly less well protected than regions I and II, its role in protein binding remains to be confirmed. Region IV is not included in Ry99 RNA and its weak protection observed in Rycom188 is therefore most likely explained by protein-induced conformational changes in the RNA.

Evolutionarily conserved basic residues in Srp14p are important for RNA-binding

The mutational analysis and the atomic structure of murine SRP9/14 revealed regions or amino acid residues that are important in binding Alu RNA (Birse et al., 1997; Bui et al., 1997; for review, see Bui & Strub, 1999). These regions include the first α-helix and the adjacent loop in SRP9 and a flexible loop between β1 and β2 strands in SRP14. In the heterodimer, these two regions lie in close proximity. Furthermore, SRP9 and SRP14 form together a six-stranded β-sheet that is curved to fit duplex RNA and which is strongly positively charged, suggesting that the β-sheet may directly contact the RNA. Many of these basic residues are conserved in evolution and the biochemical analysis of mutagenized proteins is consistent with a role for a subset of these residues in RNA-binding: arginine 59 in SRP14 as well as arginine 32 and lysine 41 in SRP9. However, because of a simultaneous negative effect on dimerization, the role of these residues in RNA binding has not yet been established unequivocally (Bui et al., 1997).

Srp14p can be aligned with mammalian and plant SRP14 proteins based on sequence homology and on the conservation of structurally important residues (Birse et al., 1997; Fig. 5A). To examine whether the RNA-binding function has been conserved between the murine and the yeast SRP14 proteins, we produced mutagenized Srp14p proteins. The RNA-binding capacities of the mutated proteins were analyzed as described above using biotinylated RNAs. The dimerization functions of the altered proteins were analyzed by cross-linking and immunoprecipitation experiments. Cross-linking was done as described before except that the in vitro-synthesized mutated Srp14p proteins were first partially purified on heparin beads. We found that this procedure removes a large fraction of wheat germ components that give rise to nonspecific cross-linked products. In the immunoprecipitation experiments, PhySrp14p was added cotranslationally to the translation reactions of the different Srp14p proteins. Immobilized T7 Tag-specific antibodies were then used to assay formation of a homodimeric complex between the recombinant and the mutagenized protein.

We generated two mutated proteins in which two adjacent basic residues located before and in the middle of the putative loop region in Srp14p were changed into alanine and serine. These residues include lysine 35 and arginine 36 as well as lysines 61 and 62 (14p35A/36S, 14p61A/62S; asterisks in Fig. 5A). Both mutated proteins had a reduced RNA-binding capacity (Fig. 5B). Replacing lysine 35 and arginine 36 completely abrogated the RNA-binding function, whereas changing lysines 61 and 62 reduced it by 50% as compared to Srp14p. Neither protein bound to Alu RNA and the control RNA, confirming that the specificity in RNA recognition was unchanged. In addition, the mutations interfered exclusively with the RNA-binding functions of the proteins, leaving their dimerization functions intact as shown with cross-linking and the immunoprecipitation experiments (Figs. 5C and 5D). Cross-linked products of a comparable size were observed with the wild-type as well as with the mutated proteins, which migrated as expected of a homodimeric complex. In addition, we had established in an experiment shown in Figure 2 that the cross-linked product is not the result of a fortuitous interaction of Srp14p with a wheat germ component. The cross-linking efficiencies cannot be compared quantitatively in these experiments because the ratio of labeled to wheat germ proteins (which also get cross-linked) is not constant. The translation efficiencies as well as the retention efficiencies on the heparin beads are not the same for the different Srp14p proteins.

Notably, double mutations on homologous positions had a more prominent negative effect on the RNA-binding function of the yeast homodimeric complex than on the RNA-binding function of the heterodimer SRP9/14 (Bui et al., 1997). In murine SRP14, changing each set of residues individually did not interfere with RNA binding of the heterodimer. However, removing the first half of the loop including the four basic amino acid residues decreased RNA-binding activity by 50%. We favor a model in which the difference between the murine and the yeast protein is explained by assuming that certain residues in Srp14p may play a role in the RNA-binding function of both subunits of the homodimeric complex. This interpretation is consistent with previous results in which the combination of Srp14p and SRP9 proteins with partially defective RNA-binding functions resulted in the complete loss of the RNA-binding function of the heterodimer (Bui et al., 1997). However, in the absence of quantitative studies we cannot discount that the results are explained by a difference in the dissociation constants of the murine and the yeast complexes. A lower stability of the latter one could make small changes in RNA–protein interactions more easily detectable.
Next we examined whether the highly conserved arginine 81 in Srp14p, which is homologous to arginine 59 in SRP14, is important for RNA binding. Arginine 81 was replaced by alanine and the in vitro-synthesized mutagenized protein assayed for RNA binding as described above. The protein 14p81A had specifically lost its RNA-binding capacity, although it still formed homodimeric complexes with the same efficiency as did wild-

FIGURE 5. RNA-binding and dimerization capacities of mutated SRP14p proteins. A: Alignment of murine SRP14 (M) and S. cerevisiae Srp14p (C) sequences. Black: Identical amino acid residues; grey: amino acid residues with similar physicochemical properties. The black fields shown exclusively in the murine SRP14 sequence represent residues conserved between mammalian and plant SRP14 proteins. Asterisks highlight the sites where amino acid residues were replaced or where a stop codon was introduced for the synthesis of the truncated protein Srp14p-29C. The secondary structure of murine SRP14 is shown on top of the sequence (Birse et al., 1997). Notably, the first portion of the loop in murine SRP14 comprises nine amino acid residues (amino acids 33–42), whereas the corresponding region in Srp14p includes 24 residues (amino acids 37–61). B: Wild-type and mutated Srp14p proteins bound to biotinylated Ry99 and Alu RNAs. cRNA: Negative control RNA. Input: 1/3 of the protein used in the binding assay. % bound: fraction of the mutated protein bound to Ry99 RNA standardized to 100% binding efficiency of Srp14p. Dimerization capacities of wild-type and mutated proteins assayed by cross-linking with glutaraldehyde (C) and by immunoprecipitation (D). C: Lanes 1: mock reaction in which Triethanolamine/HCl buffer (quencher) was added before glutaraldehyde. Lanes 2: Cross-linked products 2 min after glutaraldehyde addition. The proteins were displayed on 15% SDS-PAGE and visualized by fluorography. D: Coimmunoprecipitation of the different [35S]-labeled Srp14p proteins with T7 Tag antibodies in the presence (CP) and, as negative controls, in the absence (NC) of recombinant PhySrp14p. IN: input lanes displaying 1/3 of the protein used in the experiment.
type protein (Figs. 5B, 5C and 5D). Thus arginine 81, which would be located within the β-sheet in a hypothetical SRP9/14-like structure of the homodimeric Srp14p complex, has a very critical role in RNA binding.

All SRP14 proteins identified so far share a very basic C-terminal region. In the murine protein, it is essential for the dimerization function but not required for Alu RNA binding (Bui et al., 1997). Indeed, residues 93–95 are part of the dimer interface in SRP9/14 (Birse et al., 1997). To determine the function of the C-terminal region in Srp14p, we made a construct with a stop codon at position 117 (Fig. 5A). In vitro-synthesized 14p-29 specifically bound Ry99 as well as Srp14p (Fig. 5B). In contrast, its dimerization capacity was strongly reduced as shown in immunoprecipitation experiments (Fig. 5D). In agreement with this experiment a band that migrated as expected for a cross-linked homodimeric complex of the truncated protein was only detected after prolonged exposure in the cross-linking experiments (Fig. 5C). Hence, as for murine SRP14, the C-terminal domain of Srp14p is important in homodimer formation but not in RNA binding.

These results demonstrate that the RNA-binding function of certain basic amino acid residues and the dimerization function of the C-terminal region have been conserved between the yeast and the mammalian proteins. Thus, they support the hypothesis that the homodimeric complex has a structure similar to the heterodimer SRP9/14 and provide evidence for the presence of an RNA-binding function in the loop and in the positively charged concave β-sheet of SRP14 proteins.

DISCUSSION

It has been difficult to assign functional domains to scR1 RNA, because its overall secondary structure has remained unknown. In addition, binding sites of scSRP proteins have not been mapped experimentally. To define the Alu domain homolog in yeast SRP (Adhy), we examined the assembly of Srp14p and scR1 RNA. Our results demonstrate that Srp14p binds as a homodimeric complex to an RNA comprising 99 nt from the 5′ end of scR1 RNA (Adhy RNA). Formation of the Srp14p–Adhy RNA complex is dependent on structural and functional elements that are also involved in the assembly of the mammalian SRP9/14–Alu RNA complex. These similarities confirm the common ancestry of the mammalian and yeast protein and RNA moieties and indicate that the 5′ end of scR1 RNA together with Srp14p constitutes the domain of scSRP that is homologous to the elongation arrest domain of mammalian SRP. Despite the similarities in the assembly of the two domains, the yeast complex has unique features. They are illustrated by the differences in the overall structure of Adhy and Alu RNAs as well as by the RNA-binding specificity of Srp14p, which did not recognize Alu RNA. Hence, the overall structure of the two domains may have diverged substantially. It remains to be examined whether these changes affect the putative elongation arrest function of scSRP. The structural alignment of the three yeast SRP RNAs known to date suggests that the elongation arrest domain of all three organisms may have the yeast-specific structure.

The secondary structure model of Adhy RNA shown in Figure 6A is consistent with the results of digestion experiments using the double-strand-specific V1 nuclease. In addition, a secondary structure comparison with S. pombe and Y. lipolytica SRP RNAs suggested that the proposed structure of Adhy RNA is common to all three of them. In contrast to scR1 RNA, S. pombe and Y. lipolytica SRP RNAs were found to be similar in size to 7SL RNA and they could be folded to resemble the canonical secondary structure of 7SL RNA within the S domain (see insets of Fig. 6). However, their 5′ portions apparently lacked all or a portion of the typical two-hairpin structure of the Alu domain (Brennwald et al., 1988; Poritz et al., 1988; Ribes et al., 1988). In fact, the previously suggested secondary structure of the 5′ portion of S. pombe RNA (Poritz et al., 1988; Fig. 6C) is very similar to the one of Adhy RNA described here. However, in contrast to the Adhy RNA, the homologous folding domain includes sequences from the 5′ and the 3′ ends of S. pombe SRP RNA that pair to form the central stem. Similarly, nucleotides from the 5′ and the 3′ ends of Y. lipolytica SRP RNA can be folded to resemble Adhy RNA (Fig. 6B). In a previously proposed secondary structure model of Y. lipolytica SRP RNA, an additional hairpin structure was placed at the very 5′ end of the RNA (Poritz et al., 1988; He et al., 1989). Our alignment argues against the presence of this structure at the 5′ end. We have placed it now along the central stem (Fig. 6, inset) in agreement with the secondary structure predicted by the mfold program (Zuker et al., 1991).

Besides a similar overall secondary structure and a conserved primary sequence in the loop, the three yeast-specific SRP RNAs share additional structural features. They include the two GC base pairs and the guanosine nucleotide flanking the UGUAAU sequence. An additional primary sequence conservation is also observed between region II of Adhy RNA and the putative same regions in the other yeast SRP RNAs (see Fig. 6, asterisks). The nucleotides highlighted with asterisks are at the same distance from the conserved two GC base pairs as the ones in region II of Adhy RNA. Furthermore, the footprint region III also shares sequence identity with the equivalent region in Y. lipolytica SRP RNA (Fig. 6B). Finally, in all yeast SRP RNAs, a U-rich region links the short stem loop and the adjacent stem. This region is likely to be single stranded, because base-pairing interactions are not conserved and are not favored energetically in all three SRP RNAs. In addition, the removal of the 5′ adenosine, which could base pair with one of the uridines, does not interfere with Srp14p binding. High-affinity binding of
SRP9/14 requires flexibility between the two-hairpin structure and the central stem in Alu RNA (Weichenhrieder et al., 1997). Similarly, the U-rich region in yeast SRP RNAs may represent a hinge region that allows bending of the short hairpin structure to bind Srp14p. In summary, the many common features between yeast SRP RNAs strongly indicate that all three organisms contain a yeast-specific Alu domain.

As alluded to before, a unique feature of scR1 RNA is the position of the helical region that binds the protein within complete RNA. The canonical SRP RNA structure contains a long central stem (stem V) in which the 3'-terminal portion is paired to nucleotides near the 5' end and that links the elongation arrest domain to the signal recognition domain of the particle. Presumably, the long central stem between the two domains may allow simultaneous contacts of both domains with distant sites on the ribosome (Andrews et al., 1987). In 7SL RNA, the footprints of SRP9/14 are located on this central stem, whereas in scR1 RNA the Srp14p-binding sites are located on an additional short stem that does not link the two domains (Fig. 6, insets). Whether the different location of the Srp14p-binding site is relevant for function remains to be seen. However, the remaining portion of scR1 RNA is still large enough to form the central stem V that links the two domains.

**FIGURE 6.** Secondary structure models of the Alu domain portion of different SRP RNAs. A: Adhy RNA represented by the 99 nt at the 5' end of scR1 RNA. B, C, D: The Alu and the Alu-like domains comprise sequences from the 5' and the 3' ends of the different SRP RNAs. Bold letters designate footprint regions of the proteins and the conserved nucleotides. Asterisks highlight regions of sequence comparison. The insets show a schematic representation of the secondary structure of the complete RNAs and the black arrows indicate the position of the conserved motif. The secondary structures of *Y. lipolytica* and *S. pombe* SRP RNAs were drawn based on their alignment with the canonical secondary structure model of SRP RNAs (Samuelsson & Zwieb, 1999). The secondary structure of complete scR1 RNA is as yet unknown.
The Srp14p-binding sites within Adhy RNA include the universal single-stranded motif CUGUAUU also present in the binding sites of the heterodimeric SRP9/14 in the Alu portion of SRP RNA (Fig. 6). In yeast, the conserved motif overlaps with a putative polymerase III promoter element (Felici et al., 1989). The results of a previous analysis of S. pombe strains carrying mutated SRP RNAs were consistent with a role for this region in protein binding rather than RNA synthesis (Liao et al., 1992). Although changing a nucleotide in a conserved position of the protein-binding motif resulted in a growth defect, changing a nucleotide in a conserved position of the promoter element had no phenotype.

Although our studies confirm the importance of the UGUAUU motif in protein binding, its high degree of conservation is apparently not explained by direct protein recognition and therefore remains a puzzle. Mutation experiments have shown that the universal G in 7SL RNA can be changed into uridine or adenosine without greatly interfering with protein binding, whereas a transversion into cytosine reduced the binding constant by 200-fold (Chang et al., 1997). Similarly, in S. pombe SRP RNA, changing the same guanosine into cytosine resulted in a growth defect, whereas changing it to adenosine had no phenotype (Liao et al., 1992). Possibly, the nucleotide sequence is conserved, because it defines a functionally important structure. This interpretation is consistent with the finding that the simultaneous substitutions of G4A and G9A in S. pombe RNA resulted in a conditional growth phenotype, whereas strains carrying SRP RNAs with only one of the two substitutions had no phenotype. Based on the primary sequence within the loop (Fig. 6), the double mutation was expected to perturb the structure more dramatically than each mutation alone (Liao et al., 1992).

The structural studies on SRP9/14 revealed that the two polypeptides are structural homologs sharing the same $\alpha_1-\beta_1-\beta_2-\beta_3-\alpha_2$ fold. Their structure is closely related but not identical to the dsRBD domain (Bycroft et al., 1995; Kharrat et al., 1995) and was termed $\text{Alu}$ binding module (Alu bm; Birse et al., 1997). Together SRP9 and SRP14 form a six-stranded $\beta$-sheet stacked against the four $\alpha$-helices. The structure-based alignment is consistent with a similar structure for the heterodimeric and the homodimeric complexes. This hypothesis is further supported by our findings that the RNA-binding and dimerization functions are, to a certain extent, conserved between the yeast and the mammalian proteins. The concomitant conservation of the conserved motif UGUAUU in the RNA and the RNA-binding function of the loop region in the mammalian and the yeast proteins suggests a role of the flexible loop in recognizing the conserved RNA motif. This supports a previously proposed model of the mammalian complex. In this model, the curved $\beta$-sheet surface of the protein may contact the central stem, whereas the flexible loop of SRP14 and the N-terminal portion of SRP9 may contact the two-hairpin structure, including the conserved motif in SRP RNA (Bui et al., 1997; for review, see Bui & Strub, 1999). Analogously, the putative $\beta$-sheet surface of Srp14p comprising arginine 81, which is required for RNA binding, may contact the double-helical region II in Adhy RNA and one of the putative flexible loops in Srp14p may contact the conserved motif. In contrast to SRP9/14, the homodimeric Srp14p complex has two loop regions. A likely candidate for contacts with the second loop is region III in Adhy RNA, which lies on the opposite face of region II in a hypothetical A-helix structure of the RNA. In Alu RNA, the two contact sites of the protein in the central stem lie on the same face of the double-helical RNA (Fig. 6D; Strub et al., 1991). In addition, mutations in the yeast protein had a much stronger effect on RNA binding than the equivalent mutations in the murine protein, consistent with a role for both loops of Srp14p in RNA binding.

The homologous structure suggests a common ancestor for the mammalian SRP9 and SRP14 proteins. Our studies indicate that in yeast, a homodimeric complex replaced the heterodimeric protein. The finding that the presence of a homodimeric complex coincides with a change in the specific RNA structure that binds the protein further supports this interpretation. Srp14p fails to bind Alu RNA whereas SRP9/14 does not bind scR1 RNA (N. Bui and K. Strub, unpubl. results). Similarly, a histone-like protein, HBsu, has recently been found to bind, most likely as a homodimer, to the Alu-like portion of SRP RNA in Bacillus subtilis (scRNA; Nakamura et al., 1999). The structure of a close relative of HBsu, the HU protein of Bacillus stearothermophilus, has an $\alpha_1\beta_2\beta_3\alpha_2$ fold and forms a homodimeric complex comprising a curved six-stranded $\beta$-sheet flanked by two large loops (Tanaka et al., 1984). Analogous to SRP9/14, the $\beta$-sheet surface and the two loops have been suggested to play a role in scRNA binding of HBsuSRP9/14 (Nakamura et al., 1999).

Our results therefore suggest that a functional homolog of the mammalian SRP9 protein is absent in S. cerevisiae. Previously, an approximately 7-kDa protein that copurified with other yeast SRP components was suggested to represent a putative SRP9 protein (Brown et al., 1994). The primary sequence of this protein remains unknown and a SRP9 homolog has not been identified in the yeast genome. The RNA moiety of yeast SRP is rather large and may bind additional proteins. One of them has already been identified, Srp21p (Brown et al., 1994), and the 7-kDa species may represent another one.

The elongation arrest function of scSRP has so far not been confirmed experimentally. However, its existence is supported indirectly by the observations that certain yeast proteins can only be translocated cotranslationally in vitro (Hansen & Walter, 1988) and that scSRP interacts intimately with the ribosome during
the elongation cycle (Ogg & Walter, 1995). Based on the high conservation of ribosomes and SRP functions in evolution, it is likely that the features that are relevant for the elongation arrest function are also highly conserved and present in both complexes. Mammalian SRP14 has been shown to be critical to confer elongation arrest activity to the particle (Thomas et al., 1997) and the region that is required for this function is highly conserved in primary sequence between Srp14p and SRP14 (Fig. 5, amino acids 90–100 in SRP14). This region is part of the dimer interface in the SRP9/14 protein and was therefore suggested not to interact directly with the ribosome. However, it is feasible that this region has a different function in the RNA–protein complex. The same studies also indicated that a putative pseudoknot structure present in Alu RNA may be critical for elongation arrest activity. This structure is absent in the Srp14p-binding sites. We cannot completely discount the possibility that such a structure might be located outside the protein-binding sites in scR1 RNA. Alternatively, a small tRNA-like RNA might be part of yeast SRP. Such an RNA has recently been found associated with 7SL RNA of *Thermus brucei* (Beja et al., 1993).

Another conserved feature between the two homologous domains is the universal single-stranded motif. As discussed before, the available results indicate that the primary sequence conservation may not be explained solely by its requirement for protein binding. However, a role in elongation arrest activity has so far not been confirmed, as mutation of the conserved guanosine residue (G14) in 7SL RNA had only marginal effects on elongation arrest activity of the particle (Chang et al., 1997). Possibly, a single mutation is not sufficient to interfere with elongation arrest activity. Indeed, a genetic analysis of SRP RNA function revealed that this molecule is very robust and conditional lethality was only observed for multiple mutations (Liao et al., 1992). Clearly, a role of this region in elongation arrest needs to be studied more thoroughly.

Alternatively, scSRP may represent a functional intermediate between mammalian SRP and the *Escherichia coli* particle, which lacks the Alu domain. The Adhy domain may have another function, which may be related to SRP assembly or SRP RNA transport. It has been shown that 7SL RNA accumulates in nucleoli before transport into the cytoplasm in mammalian cells (Jacobson & Pederson, 1998) and nuclear export of the RNA was found to be facilitated by the Alu portion of 7SL RNA (He et al., 1994). Similar functions would not be required in *E. coli*.

**MATERIALS AND METHODS**

**Expression of Srp14p and scR1 RNA**

The Srp14p and scR1 RNA genes were amplified from genomic DNA of the yeast strain RMY326 (kindly provided by Dr. Didier Picard) with the polymerase chain reaction (PCR) using Pfu DNA polymerase (Stratagene, La Jolla, California) and the appropriate primers. The primer sequences were derived from the published sequences of *SRP14* and *SCR1* (Felici et al., 1989; Brown et al., 1994). *SCR1* was amplified using the primers GGA ATT CTA ATA CGA CTC ACT ATA AGG CTG TAA TGG CTT T and GCT TAA AAA ATA TGG TTC AGG AC, which add the T7 RNA polymerase promoter at the 5′ end of the gene and a Dral restriction site at the 3′ end. The fragment was cloned into the pUC plasmid linearized with Hin cII (pURy). *SRP14* was amplified from genomic DNA with the primers GCT TTC TTA CCA CC TAA AAC and CAA ATT GAT CAC GGC GGT ACC. The amplified fragment of the expected size was cloned into the plasmid SP64 linearized with Hin cII (pSY2). Several clones were isolated from independent amplification reactions and the inserts sequenced. In all these clones, the genes contained the same few nucleotide changes when compared to the published sequences. In *SRP14*, nucleotide changes in the coding region altered amino acid residues S69 into G69 and G137 into A137. The scR1 gene we isolated contained an additional G at positions 50 and 99 as shown in Figure 3. Truncations and mutations in both genes were introduced using PCR technology and the changes were verified by sequence analysis. Rycom188 comprises nt 1–160 and 491–516 at the 5′ and 3′ ends, respectively, of scR1 RNA linked by two adenosine nucleotides (see Fig. 3 for the exact sequence). Rycom71 RNA comprises the first 43 and the last 16 nucleotides of scR1 RNA linked by the sequence ACAUUCUU.

Proteins were expressed using wheat-germ lysate prepared according to Erickson and Blobel (1983). Uncapped synthetic transcripts were produced from the linearized plasmids with SP6 RNA polymerase (Promega, Madison, Wisconsin). Proteins were labeled with [35S]-methionine (1,500 Ci/mmol; Amersham Pharmacia Biotech, Zürich, Switzerland) as described in Strub and Walter (1990). For expression in *E. coli*, the ϕ-epitope (12 amino acid residues recognized by T7 Tag antibodies; Novagen, Madison, Wisconsin) was added to the N-terminus of Srp14p using PCR, and the new gene was inserted into the pET9a vector (Studier et al., 1990; Novagen, Madison, Wisconsin). Lysis of the bacteria and purification of the recombinant protein on an 18-mL heparin column (BioRad Laboratories, Hercules, California) as described in Thomas et al. (1997). For further purification of the protein, we used hydroxyapatite gel chromatography (Bio-Gel HTP, Bio-Rad Laboratories) and the buffers described in Siegel and Walter (1985). The protein eluted with a 400-mM sodium phosphate buffer and was at least 95% pure based on Coomassie staining. It was quantified by comparing it to a Coomassie-stained lysozyme standard.

The plasmids comprising the scR1 and the modified Ry RNA genes were generally linearized with Dral and the RNA synthesized with T7 RNA polymerase as described in Bui et al. (1997). Biotinylated RNAs were synthesized in the presence of 1 mM each ribonucleotide and 0.1 mM of biotinylated uridine triphosphate (Roche Molecular Biochemicals, Mannheim, Germany). For the synthesis of Ry42 and Ry91, the plasmid pURycom188 was linearized with EcoRI and BglII (GIBCO BRL, Life Technologies, Zürich, Switzerland), respectively. For the production of Ry50, Ry73, and Ry124, pURycom188 was linearized with BsaJI, BflI, and BsrI1286I (New England Biolabs GmbH, Schwalbach, Germany), respec-
tively. The RNAs were purified on Nucleobond AX columns (Machery and Nagel GmbH, Düren, Germany) and quantified by measuring the optical density at 260 nm. To confirm their concentration and to examine their quality, the RNAs were analyzed on a denaturing 6% polyacrylamide gel and stained with the fluorescent dye Gelstar® (FMC BioProducts, Rockland, Maine). The control RNA (cRNA) was synthesized from the plasmid pG14-2 linearized with Pvu II and represents 368 nt of the antisense strand of the murine SRP14 cDNA. For secondary structure predictions we used the mfold program by Zuker et al. (1991) adapted to the Wisconsin package (Genetics Computer Group, Madison, Wisconsin).

RNA binding experiments

The RNA-binding experiments have previously been described in Bui et al. (1997). Instead of streptavidin-coated agarose beads, we used Streptavidin Dynabeads M-280 (Dynal A.S., Oslo, Norway) in the binding assays. The binding reactions were carried out in 20 μL containing 5 μL translation reactions and 1 μL of 1 μM biotinylated RNA (or higher concentration when indicated), 100 ng/μL competitor tRNA (50 ng/μL tRNA of E. coli and approximately 50 ng/μL calf liver tRNA from the translation reaction), 150 mM potassium acetate (or 350 mM), 1.5 mM magnesium acetate (or 3.5 mM), 50 mM N-2-hydroxyethyl piperazine-N’-2-ethanesulfonic acid/potassium hydroxide (HEPES/KOH), pH 7.5, 1 mM dithiothreitol (DTT), 0.01% Nikkol (Nikko Chemical, Tokyo, Japan). After incubations of 10 min on ice and 10 min at 26 °C, the reactions were added to 20 μL of the same buffer containing 0.1% Triton X-100 and 12 μL Streptavidin beads that were previously equilibrated in the same buffer. At higher RNA concentrations (0.15 or 0.5 mM), 20 μL of Streptavidin beads were used in the binding reactions. After 20 min incubation with the beads, they were washed three times for 5 min with 200 μL of the same buffer.

To prepare glycerol step gradients, 150 μL each of 25, 20, 15, and 10% glycerol solutions in 350 mM potassium acetate, 3.5 mM magnesium acetate, 50 mM HEPES/KOH, pH 7.5, 1 mM DTT, and 0.01% Nikkol were overlaid and left in the cold for 2 h. PhySr14p and Rycom188 RNA (30 pmol each) were combined in the same buffer and incubated on ice and at 26 °C for 10 min each before layering on top of the gradient. Gradients were run overnight at 4 °C, 40,000 rpm in a TST 55.5 ultracentrifuge rotor. The gradients were collected in six fractions, of which 20% was used for the analysis of the RNA and 40% each for the analysis of the protein and for the cross-linking experiments. The RNA was analyzed after phenol/dichloromethane extraction and ethanol precipitation in the presence of 1 μg carrier tRNA from E. coli on a 8% denaturing polyacrylamide gel and visualized with Gelstar®. Cross-linking was done as described previously (Bovia et al., 1994) with 0.08% glutaraldehyde. Proteins were precipitated with 10% final concentration of trichloroacetic acid and analyzed by 15% SDS-PAGE and visualized with silver staining or, in the case of [35S]-labeled proteins, with fluorography.

Immunoprecipitation experiments

Sr14p and the mutated proteins were synthesized in wheat germ lysate in the presence of recombinant PhySr14p (1 pmol/10 μL translation reaction) and [35S]-labeled methionine. In the negative control reactions, PhySr14p was omitted from the reaction. After translation, 10 μL of the reaction were diluted with 90 μL of IP buffer (20 mM HEPES/KOH, pH 7.5, 350 mM potassium acetate, 0.1% Triton X-100, 2 mM methionine, 0.01% Nikkol and a cocktail of 1× protease inhibitors (a 200× stock solution contains 20 μg/mL of pepstatin A, leupeptin, antipain, chymostatin, and 100 μg/mL aprotinin) and added to 30 μL protein A beads (Amersham Pharmacia Biotech, Zürich, Switzerland). Preabsorption was allowed to continue for 30 min at 4 °C by rotating the tubes end over end. After centrifugation, the supernatant was incubated with immobilized T7 Tag antibodies (1 μL antibodies bound to 20 μL beads/reaction) for 1 h and the beads washed three times with 200 μL IP buffer. The bound proteins were displayed by SDS-PAGE and visualized by fluorography. When indicated, 5 pmol of scr1 RNA was added to the preabsorbed supernatant and the samples incubated for 10 min at 37 °C to facilitate complex formation.

Hydroxyl radical reactions and V1 nuclease digestions

Hydroxyl radical reactions were carried out as described earlier (Strub et al., 1991). The cleavage reactions contained 1 pmol of Rycom188 RNA and 2 or 6 pmol of PhySr14p. In the control reactions, PhySr14p was replaced by 35 ng and 105 ng of bovine serum albumin. Rycom188 was labeled at its 3′ end with [32P]-pCp as described in Bruce and Uhlenbeck (1978).

Unlabeled or 3′ end-labeled Ry99 RNA in 100 mM potassium acetate, 1.5 mM magnesium acetate, 20 mM HEPES/KOH, pH 7.5 was digested with 0.03 and 0.01 U of V1 nuclease on ice for 15 min. The digestion products were analyzed directly on a 15% sequencing gel or by reverse transcription using a primer complementary to nt 80–100 in Ry RNA. In parallel, we run RNA sequencing reactions that were obtained with the RNA sequencing enzyme kit (Amersham Pharmacia Biotech, Zürich, Switzerland). In the primer extension analysis, the RNA was sequenced using deoxyxynucleotides as described in Strub et al. (1991) to map the cleavage positions.

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