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Small ribonucleoproteins in *Schizosaccharomyces pombe* and *Yarrowia lipolytica* homologous to signal recognition particle

(α-sarcin/RNA "footprinting"/polymerase III transcripts/secretion/yeast)

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ABSTRACT **We have partially purified ribonucleoproteins (RNPs) from *Schizosaccharomyces pombe* and *Yarrowia lipolytica* with properties resembling those of mammalian signal recognition particle (SRP). In both species of yeast we have identified a single major RNA species in the size range of SRP RNA (256 nucleotides in *S. pombe* and 270 nucleotides in *Y. lipolytica*) present in postribosomal salt extracts of the cytoplasm. The RNPs containing these RNAs sediment in sucrose gradients at 11 S and 10 S for *S. pombe* and *Y. lipolytica*, respectively. Analysis of genomic clones of these RNAs has revealed that (*i*) they are encoded by single copy genes; (*ii*) they share two short conserved sequences that match the A and B boxes defined for polymerase III promoters; (*iii*) they can be folded into secondary structures that closely match that defined by phylogenetic analysis of higher eukaryotic SRP RNAs; and (*iv*) they show primary sequence conservation in short regions predicted to be single stranded. Both of the yeast RNAs bind under stringent conditions to canine SRP proteins. Most importantly, RNase protection of the *S. pombe* RNA by the individual canine SRP proteins, p19 and p68/72, shows that the proteins recognize homologous elements of the mammalian and yeast RNA. Taken together these data suggest strongly that we have identified yeast SRP homologues.**

Fractionation of *in vitro* systems that mimic the translocation of nascent secretory proteins across the membrane of the mammalian endoplasmic reticulum (ER) has led to the identification of a soluble ribonucleoprotein (RNP), the signal recognition particle (SRP), which is essential for targeting of nascent secretory proteins to the ER membrane. SRP is thought to function as an adapter between the cytoplasmic translation and the ER membrane-bound protein translocation machineries (1).

SRP is a biochemically well-defined entity. It contains one RNA of 300 nucleotides (nt) (7SL RNA, here referred to as SRP RNA) and 6 polypeptides (2, 3). The canine SRP proteins can be separated from the RNA in native form and rebound to the RNA to reconstitute active SRP (4, 5). Studies on perturbed SRPs reconstituted either with modified SRP proteins or with only a subset of the proteins allowed us to dissect the functional contributions of individual SRP proteins (5, 6). Heterologous reconstitutions of the canine proteins with *Xenopus* or *Drosophila* SRP RNAs also yield active particles in the *in vitro* assay, thus demonstrating the evolutionary conservation of SRP RNA (4). Phylogenetic analysis of the sequences from the human, amphibian, and insect RNAs has defined the secondary structure (7, 8). The binding sites of two of the proteins (p19, a monomeric protein, and p68/72, a heterodimer) have been mapped (9) onto this structure.

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As a first step toward elucidating SRP function *in vivo* in genetically tractable organisms we have identified homologues of SRP in the yeasts *Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Our strategy involved fractionation of yeast extracts to enrich for RNPs of similar biochemical properties as mammalian SRP and then to characterize their RNA components with respect to the structural criteria described above.

METHODS

Materials. *S. pombe* strain sp972h$^-$ (10) and a genomic plasmid library constructed from this strain were a gift of M. Yanagida (Kyoto University). *Y. lipolytica* strain CX161-1B (11) and a genomic λ library constructed from this strain were a gift of R. Mortimer (University of California at Berkeley) and D. Ogrydziak (University of California at Davis), respectively. The plasmid pUC118 (12) was a gift of J. Vieira (Rutgers University). α-Sarcin was a gift of I. Wool (University of Chicago).

Preparation of Yeast Extracts. *S. pombe* sp972h$^-$ or *Y. lipolytica* CX161-1B was grown to 2–3 OD_{600} units/ml in YEPD or glycerol proteose/peptone medium (11), respectively. Cells were washed once in distilled water and suspended at a concentration of 400 OD_{600} units/ml in buffer A (1.2 M sorbitol/50 mM Tris·HCl, pH 7.5/1 mM EDTA/1 mM dithiothreitol) containing 0.4 mg of Zymolase 100T per ml (Miles). After 1 hr at 30°C the cells were pelleted and washed once in buffer A. All subsequent steps were carried out at 4°C.

The cells were lysed with five strokes in a tight-fitting Dounce homogenizer in buffer B (250 mM sucrose/50 mM triethanolamine·HOAc, pH 7.5/50 mM KOAc/6 mM $Mg(OAc)_2$/1 mM EDTA/1 mM dithiothreitol) at a concentration of 200 OD_{600} units/ml. The salt concentration was raised to 500 mM KOAc. After a 10-min incubation the cell lysate was centrifuged at 1000 × *g* for 10 min. The resulting postnuclear supernatant fraction was underlayered with one-third volume of buffer B containing 350 mM sucrose and 500 mM KOAc and centrifuged at 183,000 × *g* in a Beckman 50.2 Ti rotor for 3.5 hr. The postribosomal supernatant and the upper half of the cushion were collected and diluted with two-thirds volume of 50 mM triethanolamine·HOAc/1 mM dithiothreitol/0.025% Nikkol (octaethyleneglycol dodecyl ether, Nikko Chemicals, Tokyo, Japan) before loading onto a DEAE-Sepharose CL-6B column (Pharmacia) equilibrated in buffer C [50 mM triethanolamine·HOAc/300 mM KOAc/5 mM $Mg(OAc)_2$/1 mM dithiothreitol/0.01% Nikkol]. One milliliter of resin was used for each 8000 OD_{600} units of yeast cells.

The column was washed with 3 volumes of buffer C and eluted in three steps with buffer C, but containing 600 mM

Abbreviations: ER, endoplasmic reticulum; nt, nucleotide(s); RNP, ribonucleoprotein; SP256, *S. pombe* 256-nt RNA; SRP, signal recognition particle; YL270, *Y. lipolytica* 270-nt RNA.

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KOAc/6 mM $Mg(OAc)_2$, 800 mM KOAc/8 mM $Mg(OAc)_2$, and 1 M KOAc/10 mM $Mg(OAc)_2$. RNA was prepared from each fraction by proteinase K treatment that was followed by phenol/chloroform extraction; RNA was analyzed on 50% urea/6% polyacrylamide gels.

Recombinant DNA Analysis. *S. pombe* 256-nt RNA (SP256) and *Y. lipolytica* 270-nt RNA (YL270) used for random priming (13) or ^{32}P pCp labeling (9) were prepared from the 600 mM K^+ and 800 mM K^+ elutions, respectively, from the DEAE column (Fig. 1*A*, lane 5, and Fig. 1*B*, lane 6) after elution from preparative gels (13). Hybridizations of Southern blots, RNA transfer blots, and colony or plaque lifts were carried out as described (14), except that the hybridization was done in 0.9 M NaCl/50 mM sodium phosphate, pH 7.4/5 mM EDTA (13)/50% formamide/7% $NaDodSO_4$ at 42°C. Washing steps were three times, 10 min each, in 36 mM NaCl/1 mM sodium phosphate/0.2 mM EDTA/0.1% $NaDodSO_4$ at 50°C.

Genomic clones were sequenced as described (12, 15, 16). The 3′ ends of the RNAs were determined by sizing the products of an RNase T1 sequencing ladder of 3′ pCp-labeled RNA. The 5′ end of SP256 was determined by using the blocked T4 DNA polymerase assay (17). The 5′ end of YL270 was determined by primer extension of a ^{32}P end-labeled oligonucleotide complementary to bases 52–64.

RNase protection ("footprinting") of SP256 by the purified SRP proteins was carried out according to Siegel and Walter (9).

RESULTS

Partial Purification of Yeast RNPs. In homogenates from pancreatic cells, mammalian SRP is found to partition about equally among free, ribosome-bound, and membrane-bound forms (18). SRP can be extracted with high salt, rendering all of the particles soluble. Thus, as the first step in the purification scheme of putative yeast SRP homologues, we adjusted extracts of *S. pombe* and *Y. lipolytica* to 0.5 M salt and then prepared a high-speed supernatant. Encouragingly, in postribosomal supernatants from both yeasts we found a major RNA species in the 300-nt size range (Fig. 1 *A* and *B*, lanes 3)—i.e., of similar size as mammalian SRP RNA (Fig. 1*A*, lane 9). These RNAs are 256 and 270 nt long for *S. pombe* and *Y. lipolytica*, respectively (see below), and henceforth are referred to as SP256 and YL270.

Further purification of the RNPs containing these RNAs was achieved by chromatography on DEAE-Sepharose. Most protein flowed through the column at 300 mM salt, whereas the RNPs containing SP256 and YL270 were quantitatively retained (Fig. 1 *A* and *B*, compare lanes 3 and 4). The column was then eluted sequentially with steps of 600 mM, 800 mM, and 1.0 M salt. Under these conditions mammalian SRP elutes at 600 mM salt, whereas naked SRP RNA elutes only when the salt is raised above 1.0 M. SP256 and YL270 were recovered in all three elution steps with peaks in the 600 mM and 800 mM salt eluates, respectively, indicating that these RNPs have a somewhat higher negative charge density than mammalian SRP or suffered partial disassembly during the purification. On sucrose velocity gradients, the RNPs containing SP256 and YL270 sedimented as discrete peaks at 11 S and 10 S, respectively (not shown), compared to a value of 11 S for mammalian SRP (3).

Molecular Cloning of the SP256 and YL270 RNA Genes. To address the question of whether these RNPs indeed constitute yeast SRP homologues, we characterized their RNA components in more detail. For this purpose we isolated the genes encoding the SP256 and YL270 by using random-primed cDNA probes made from gel-purified RNAs to screen genomic libraries. Restriction and Southern hybridization analysis mapped the SP256 RNA gene to a 1.1-kilobase (kb)

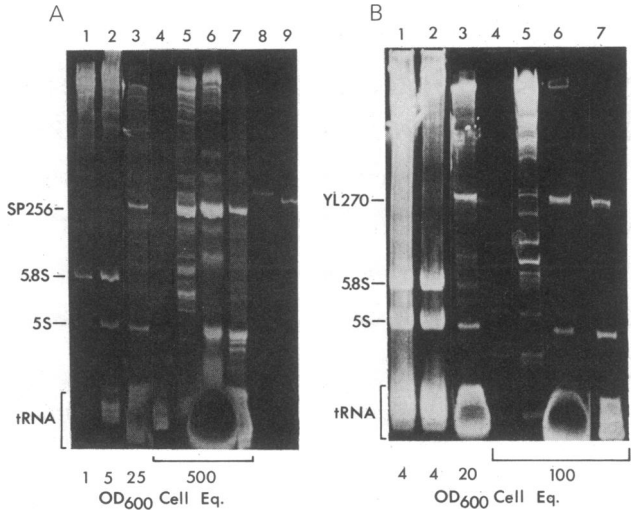

FIG. 1. Purification of the yeast RNPs monitored by denaturing PAGE. RNA samples from the fractionation of *S. pombe* (*A*) and *Y. lipolytica* (*B*) extracts were resolved by denaturing PAGE and stained with ethidium bromide. The amounts of sample loaded [OD_{600} of cells starting material (OD_{600} Cell Eq.)] are indicated. The positions of SP256, YL270, 5.8 S, 5 S, and tRNA are shown. Lanes: 1, cell lysate; 2, postnuclear supernatant; 3, postribosomal supernatant; 4, flow-through fraction of DEAE-Sepharose column; 5, 600 mM KOAc eluate; 6, 800 mM KOAc eluate; 7, 1 M KOAc eluate; 8, 100 ng of canine SRP RNA; 9, gel-purified YL270 (for size comparison).

*Hin*dIII–*Eco*RI fragment (Fig. 2*A*) and the YL270 RNA gene to a 560-base-pair (bp) *Nde* I–*Mlu* I fragment (Fig. 2*B*). The

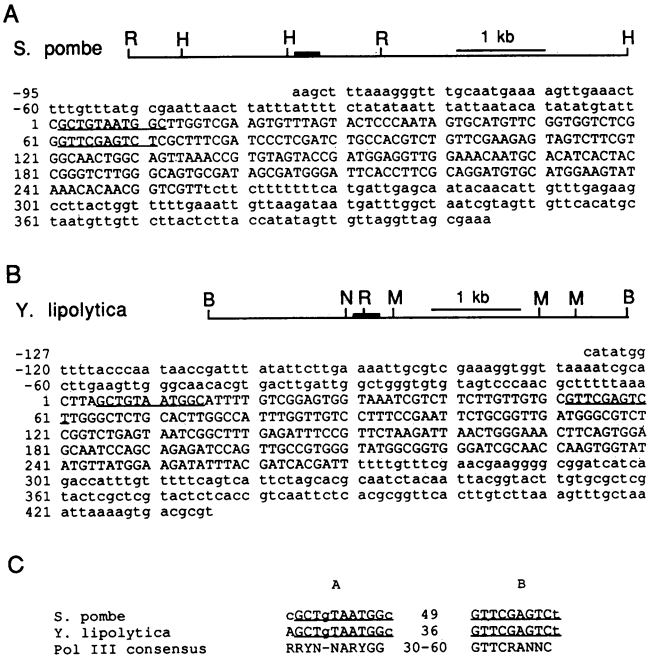

FIG. 2. Restriction map and sequence analysis of the genes encoding SP256 and YL270. The RNA coding sequences of SP256 (*A*) and YL270 (*B*) are indicated by thick lines and are oriented 5′ → 3′ as drawn. B, *Bam*HI; R, *Eco*RI; H, *Hin*dIII; N, *Nde* I; M, *Mlu* I. The coding sequences are in uppercase letters in the sense strand of the RNA. The underlined segments indicate the longest stretches of sequence identity between the two genes. (*C*) The sequences underlined in *A* and *B* are aligned with the consensus defined for the A and B box elements of RNA polymerase III promoters (19). R, purine; Y, pyrimidine; N, any base. Underlined bases indicate identity between the SP256 and the YL270 RNAs. Uppercase letters indicate matches to the A or B box consensus. The nucleotide spacing between the elements is indicated.

DNA sequences of the RNA genes (Fig. 2 *A* and *B*, uppercase letters) and flanking regions were determined. The ends of the RNAs were mapped as described in *Methods*. Genomic Southern analysis demonstrated that both genes were single copy in their respective genomes (not shown).

Primary and Secondary Structure of SP256 and YL270. Comparison of the two RNA sequences revealed two small regions of sequence identity of 11 and 10 bases (underlined in Fig. 2 *A* and *B*) that match closely the consensus sequences defined for the A and B boxes of RNA polymerase III genes

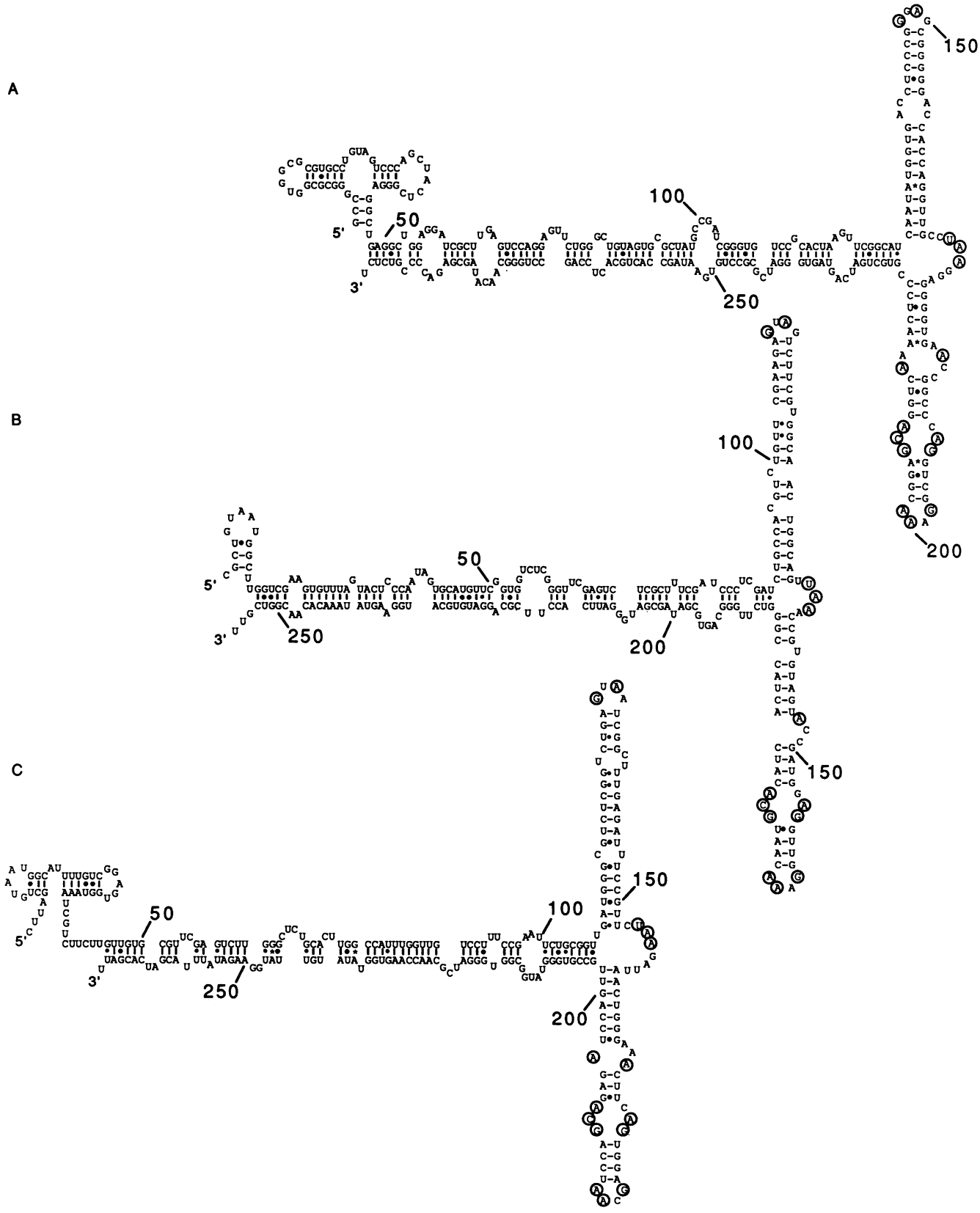

FIG. 3. Secondary structure of human SRP RNA (*A*), SP256 RNA (*B*), and YL270 RNA (*C*). Bases in single-stranded regions that are conserved among the human, *Xenopus*, and *Drosophila* (not shown) SRP RNAs and SP256 and YL270 are circled.

(ref. 19; Fig. 2*C*). Except for this, there are no significant stretches of primary sequence similarity between these RNAs and any other RNA sequence in the Genbank data base. Despite this lack of similarity it is possible to generate secondary structures for these RNAs that are strikingly similar to those proposed on phylogenetic grounds for the higher eukaryotic SRP RNAs (Fig. 3): a long interrupted stem containing the paired 5′ and 3′ ends (the *Alu* domain in mammalian SRP RNA) that ends in a bifurcation into two shorter stems (the S region of mammalian SRP RNA).

By using the secondary structure as a guide for alignment, small but significant regions of primary sequence conservation between these evolutionarily distant molecules become apparent (Fig. 3, circled nucleotides). Regions predicted as stems are divergent and show the expected compensatory base changes.

Binding of Mammalian SRP Proteins to SP256 RNA. The data presented so far suggest that SP256 and YL270 may indeed be yeast SRP RNA homologues. As an independent test of this assignment, we asked whether the structure of the RNAs would be sufficiently conserved so that mammalian SRP proteins would interact with the yeast RNAs. By using a nitrocellulose filter binding assay, we found that SP256 and YL270 bound specifically and under very stringent conditions (0.5 M salt) to canine SRP proteins (not shown). Unfortunately, we have not been able to demonstrate that these heterologous particles have reconstituted any of the known SRP activities in functional assays. Therefore, to determine whether the observed binding is indeed due to a meaningful recognition event, we identified the binding sites on SP256 of the two purified mammalian SRP proteins (p19 and p68/72) whose binding sites on the mammalian RNA are known.

α-Sarcin, a ribonuclease that cleaves preferentially after purines and does not discriminate between single- or double-stranded regions (20), was used as a reagent to footprint p19 (Fig. 4, compare lane 7 with lanes 5 and 6) and p68/72 (Fig. 4, compare lane 7 with lanes 8 and 9) on 3′ pCp-labeled SP256. Protections conferred to SP256 by the bound proteins are schematically summarized in Fig. 5 *B* and *D*. Previous experiments using this reagent on mammalian SRP RNA have shown that p19 protects a discrete set of sites at the terminal loops of the S region (9) (Fig. 5*A*). In contrast, p68/72 protects an extensive set of sites in the central S region of the mammalian SRP RNA (Fig. 5*C*).

The footprinting patterns of p19 and p68/72 on SP256 and mammalian SRP RNA are strikingly similar (compare Fig. 5 *A* with *B* and *C* with *D*). Most characteristically, p19 protects regions of SP256 at the terminal loops (regions b and c in Fig. 5*B*). We note the presence of several additional protections on the RNA (Fig. 4, lane 5 regions a and e). These sites may represent nonspecific binding site(s), since they become

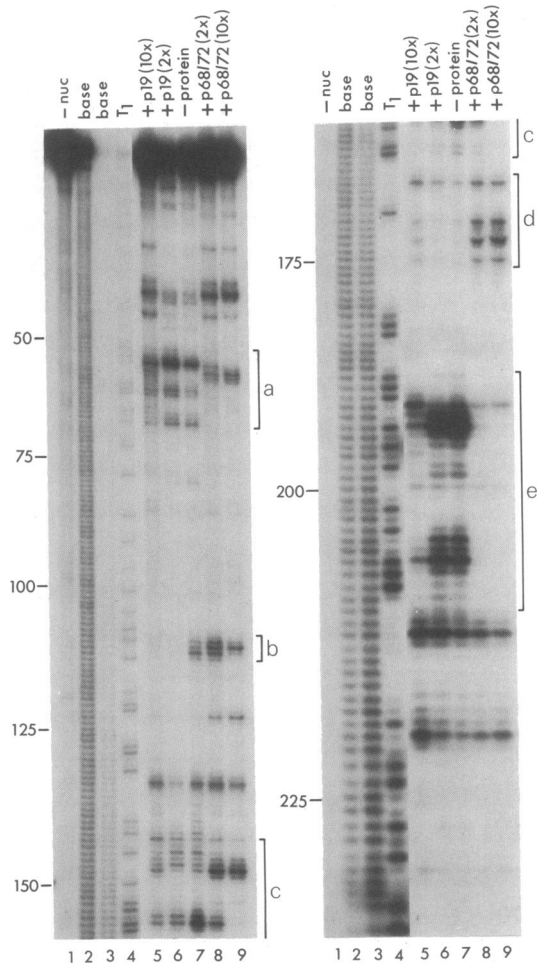

FIG. 4. Footprinting of p19 and p68/72 on SP256. 3′ pCp-labeled SP256 (75 nM) was reconstituted with either no protein (lane 7) or else a 2-fold (lanes 6 and 8) or a 10-fold (lanes 5 and 9) molar excess of p19 or p68/72 as indicated. The sample was digested with 0.5 μM α-sarcin as described (9) and the products were displayed on a 50% urea/5% acrylamide wedge gel. Lanes 1, uncut RNA; lanes 2 and 3, base-hydrolyzed RNA; lanes 4, ribonuclease T1-digested RNA. The regions labeled a–e correspond to those in Fig. 5. Parallel experiments using 0.05 μM α-sarcin produced a similar spectrum of digestion products, implying that the observed enhancements are not due to multiple α-sarcin cuts per molecule.

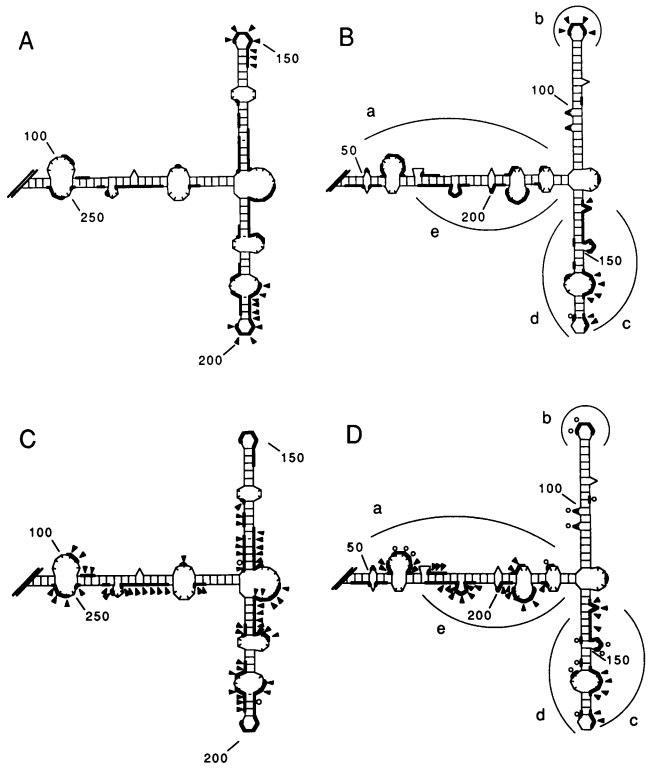

FIG. 5. Positioning of the p19 and p68/72 α-sarcin protection sites onto the secondary structures of mammalian SRP RNA and SP256. The secondary structures of the central portion of mammalian SRP RNA (*A* and *C*) and SP256 (*B* and *D*) are shown schematically. The cleavage sites of α-sarcin on naked RNA are indicated by thick lines. The protections (arrowheads) and enhancements (circles) of digestion due to the presence of p19 (*A* and *B*) or p68/72 (*C* and *D*) are indicated. Only the strong protections are indicated for the mammalian SRP RNA sequence (9). Data for SP256 are from Fig. 4 (lanes 6 and 8; 2-fold molar excess of protein over RNA). The regions labeled a–e correspond to those in Fig. 4.

apparent only at 10-fold molar protein excess—i.e., when the primary, cognate binding sites appear to be occupied.

As on mammalian SRP RNA, p68/72 protects a large number of sites on SP256. The same sites are seen at a 2- or a 10-fold excess of protein. Those at regions a, c, and e (Fig. 5*D*) correspond to similar protections on mammalian SRP RNA. Binding of p68/72 also causes a number of enhanced cleavages (region d). These enhancements occur at sites that are protected by p68/72 in mammalian SRP RNA. One possible explanation for these enhancements is that p68/72 sits more loosely on SP256 than on its cognate RNA and by electrostatic interactions attracts nuclease to sites that would otherwise not be cut.

DISCUSSION

We have identified small RNPs in the yeasts *S. pombe* and *Y. lipolytica* that by several criteria are homologues of SRP in higher eukaryotes. The RNPs were purified based on the similarity of their fractionation properties to those of mammalian SRP. The sequences of their RNA components (SP256 and YL270) reveal striking similarities of their secondary structures to that of mammalian SRP RNA as well as small regions of highly conserved primary sequence identity. Most importantly, we have been able to demonstrate that mammalian SRP proteins are capable of recognizing both RNA species under highly stringent conditions. These proteins bind to corresponding regions on the secondary structure projection of SP256 and mammalian SRP RNAs. In addition to providing strong support for the proposed secondary structure, these data demonstrate that the tertiary structures as seen by these specific RNA binding proteins must be very similar between the yeast and mammalian RNA species. Given these striking similarities on the RNA level, it should be straightforward (once the protein complement of the yeast RNPs is identified) to relate these proteins to their mammalian counterparts that have been well characterized and that define structural and functional domains on SRP (6).

Two other observations lend further indirect support to the notion that these RNPs are SRP homologues. From our sequence data we infer that SP256 and YL270 are likely to be polymerase III transcripts, which is also the case for mammalian SRP RNA. We estimate that the RNPs are present in the yeast cytoplasm at about the same relative stoichiometry with respect to the ribosomes as SRP is in mammalian cells [about 10,000 copies of SP256 per cell—i.e., about one RNP per 10 ribosomes (18)].

By comparing the sequences of the two yeast RNAs with the higher eukaryotic SRP RNAs, we have been able to refine the proposed secondary structure in the central portion of the molecule. This phylogenetic evidence supports a more tightly base-paired structure than that previously suggested when only human, amphibian, and insect RNA sequences were available (8). The increased level of base pairing highlights the presence of invariant nucleotides in single-stranded regions (Fig. 3, circled). A more extensive phylogenetic analysis will be published elsewhere; however, we note that the sequence of a 7S RNA identified in *Halobacterium* (21) is compatible with the modified structure and conserved nucleotides described here. An alternative base-pairing scheme, proposed to function as a "dynamic switch," has been described in the central portion of SRP RNAs (8, 22). We do not find phylogenetic support for a second structure in the yeast RNAs.

Zwieb (8) has also noted a region of sequence similarity between the human SRP RNA (bases 236–255) and 5S RNA (bases 84–110). This similarity is well conserved in *Xenopus* but only moderately conserved in *Drosophila*. However, there is no significant sequence similarity between the two yeast RNAs and their corresponding 5S RNAs, and therefore we find no phylogenetic evidence to support the functional significance of this similarity.

Two short stems present on the 5′ ends of higher eukaryotic SRP RNAs can be formed on YL270, but only one, relatively weak stem can be formed on SP256. This region may form a tRNA-like domain, possibly involved in the elongation arrest function of mammalian SRP (1, 8), which may thus be absent or be structurally distinct in the RNP from *S. pombe*.

Note Added in Proof. Recently Tollervey and colleagues (23) as well as Wise and colleagues (24) have independently cloned the gene for SP256 and shown by gene disruption experiments that SP256 is essential for viability.

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