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A Chaperone for Ribosome Maturation*

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The nascent pre-rRNA of eukaryotic ribosomes is fully transcribed and assembled into an 80–90 S nucleolar particle before being cleaved into mature ribosomal RNA. The interdependence of steps in the processing of this precursor RNA indicates that RNA processing, at least in part, acts as a quality control mechanism that helps ensure that only functional RNA is incorporated into mature ribosomes. In search of structural components that underlie this interdependence using the *Schizosaccharomyces pombe* internal transcribed spacer 1 (ITS) as a ligand for affinity chromatography of ITS1-specific proteins, we have isolated a large spliceosome-like protein complex, a ribosome assembly chaperone (RAC) of 20 or more polypeptides (Lalev, A. I., Abeyrathne, P. D., and Nazar, R. N. (2000) *J. Mol. Biol.* 302, 65–77). When the ITS2 spacer was used in the present study to isolate ITS2-specific proteins, the same proteins were identified consistent with a complex containing multiple specific binding sites. Subsequent competition binding studies indicated that the protein complex actually contains independent binding sites for all four of the transcribed spacers in the pre-rRNA. Because disruption of protein-binding sites in these spacer RNAs is known to severely affect rRNA processing, taken together these results suggest that the RAC complex is a chaperone for ribosome maturation acting as a "rack" on which critical structure is organized.

The introns of eukaryotic mRNA precursors interact with snRNAs[1] and many proteins to form a large "spliceosome" complex (1) that catalyzes both the excision of the intron and the splicing of the remaining RNA to produce the mature coding sequence (2). Ribosomal RNA transcripts also represent large precursor molecules from which transcribed spacers are excised to form the RNA components of the mature ribosomal subunits. Although RNA splicing is not necessary in this process, several snoRNAs also have been implicated in the cleavage of the pre-rRNA (3) and a U_3 snoRNA·5′-ETS complex has been postulated to be essential for the initiation of rRNA processing (4).

Initially, studies of rRNA processing reported a split processing scheme for the independent maturation of the large and small subunit RNAs (5). Indeed, some mature rRNA is observed when the sequences are not linked genetically (6). More recent studies, however, have begun to demonstrate interdependences that dramatically affect the efficiency of maturation and/or the stability of the products. For example, an extended hairpin structure in the 3′-ETS has been shown to be critical not only to the processing of the 3′-end in the large subunit rRNA but also to the maturation of the 5.8 S RNA some 3000 nucleotides upstream of it (7). In addition, the deletion of the ITS2 sequence has been shown to be critical to the maturation of the large subunit but also to have severe effects on the level of 18 S rRNA production (8), and as already indicated, a U_3 snoRNA·5′-ETS complex appears to be essential for the initiation of pre-rRNA processing overall. Because the pre-rRNA is fully transcribed and assembled into a large 80–90 S nucleolar precursor particle before rRNA processing is initiated (9), these observations pose intriguing questions about the nature and role of the nucleolar precursor ribonucleoprotein particle. Apparently, at least in part, these processes contribute to a quality control mechanism that helps ensure that only functional RNA is incorporated into mature ribosomal particles (8).

In search of structural features that underlie the interdependences as well as possible roles of the transcribed spacers in rRNA maturation, we initially looked for interactions between the spacer regions and soluble cellular constituents (10, 11). Using gel retardation studies we were able to demonstrate specific interactions between individual transcribed spacer sequences and cellular proteins (10), and using ITS1 RNA we were able to isolate a complex of proteins that interacted specifically with this spacer sequence (12). This complex, a ribosome assembly chaperone (RAC), contained at least 20 proteins ranging in size from 20 to 200 kDa. Mass spectroscopy and computer analyses suggested that many of the proteins contained RNA binding motifs or were nuclear in localization. Additional RNA components were not evident. In the present study, proteins interacting with the ITS2 region also were isolated for further characterization. Subsequent comparative analyses now indicate that a single protein complex has independent binding sites for all the transcribed spacers in the pre-rRNA.

EXPERIMENTAL PROCEDURES

Preparation of ITS RNA—Schizosaccharomyces pombe ITS1 and ITS2 RNA for ribonucleoprotein formation and affinity chromatography were prepared by transcription using T_7RNA polymerase (13) as previously described (11, 12). For labeled molecules, the RNAs were labeled at the 5′-end using bacteriophage T_4 polynucleotide kinase and [γ-^{32}P]ATP after dephosphorylation with calf intestinal alkaline phosphatase. All RNAs were purified on 6% denaturing polyacrylamide gels.

Affinity Chromatography—RNA binding proteins were purified by affinity chromatography using ITS RNA bound to a poly(C)-agarose support (Sigma) as recently described (12, 14). The RNA ligand was immobilized on the column matrix using a poly(G) sequence at the 3′-end which was initially inserted into the DNA template sequence. Protein was extracted from logarithmically growing *S. pombe*, strain h$^-$ leu 1–32 ura 4-D18 and applied at a concentration of 10 mg/ml in chromatography buffer (5 mM $MgCl_2$, 0.3 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol and 10 mM Tris-HCl, pH 7.5) containing 5% glycerol, 0.01% Triton X-100 and 5–10 mg of unrelated calf liver rRNA.

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[1] The abbreviations used are: snRNA, small nuclear RnA; snoRNA, small nucleolar RNA; ETS, external transcribed spacer; ITS, internal transcribed spacer; RAC, ribosome assembly chaperone.

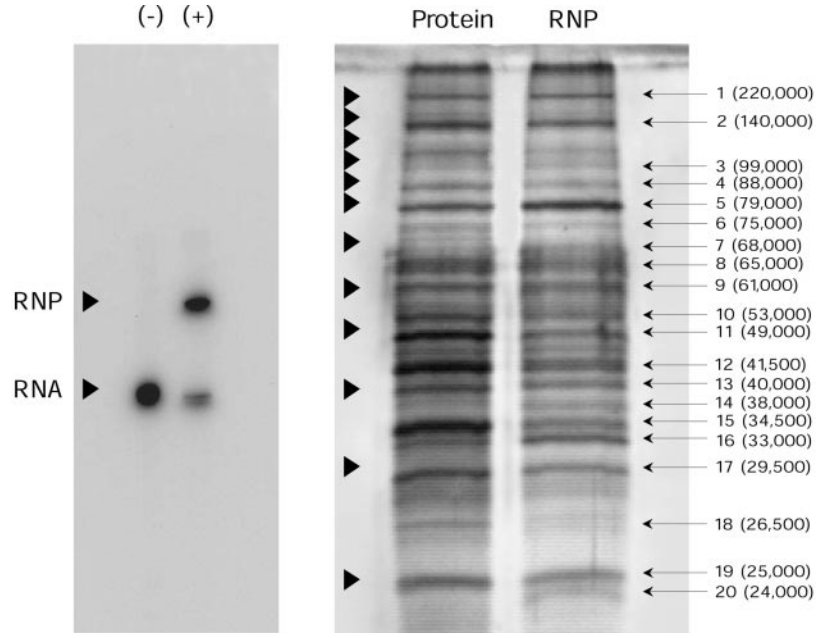

FIG. 1. **Protein constituents of gel-purified ribonucleoprotein.** *A*, *S. pombe* ITS2 RNA, transcribed *in vitro* and labeled at the 5′-end (−), was incubated with affinity-purified protein (0.3 M fraction) and unrelated carrier RNA (+) before fractionation on a 6% nondenaturing polyacrylamide gel (*left*). The ribonucleoprotein (*RNP*) band (indicated on the *left*) was excised and applied to a 10% SDS/polyacrylamide gel (*right*) together with the initial cellular protein extract (*Protein*) for further electrophoretic fractionation. The proteins were visualized by silver stain. The positions of molecular weight standards, as separately fractionated with the initial cellular protein extract, are indicated at the *left* and were used to estimate the molecular weight of each band as indicated in *parentheses*.

The column was washed with chromatography buffer containing 0.133 M KCl and then eluted in two steps using 1 ml of buffer containing either 0.3 M or 1 M KCl.

Electrophoretic Mobility Shift Assay—Ribonucleoprotein complexes were assayed by gel retardation as previously described (10, 11). Aliquots of *in vitro* transcribed and labeled spacer RNA (0.3–2 ng/20,000-25,000 cpm were incubated with 5 μl of protein extract in binding buffer (100 mM KCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 12 mM Tris-HCl, pH 8.0) containing 8% glycerol and 0.25 mg/ml calf liver rRNA to eliminate nonspecific interactions. For competition analyses, ~0.3 ng of RNA was incubated with RAC protein on ice for 10 min in 20 μl of buffer; the protein concentration was adjusted to be limiting, allowing about 75–80% of the labeled RNA to be incorporated into a ribonucleoprotein complex. For competition experiments with unlabeled RNA, a 1–300-fold excess (0.03–0.1 μg) was added to the incubation mixture. Complexes were fractionated from free RNAs on 2% (w/v) agarose gels at 4 °C.

Electrophoretic Analysis of Protein Constituents—For analyses of column eluates, the proteins were fractionated directly on 10% SDS-polyacrylamide gels using a 5% stacking gel as described by Laemmli (23). The bands were visualized using silver stain as described by Merrill and co-workers (24). For ribonucleoprotein constituents, sufficient protein was added to convert essentially all the RNA (1–2 μg) into ribonucleoprotein. The complex was purified on a 5% polyacrylamide gel using labeled RNA to identify its position, and the excised band was polymerized in place of the normal 5% stacking layer of the 10% SDS-polyacrylamide protein analysis gel, described above. In some analyses, markers also were applied to gels to estimate the molecular weights.

RESULTS AND DISCUSSION

In a previous study (11), gel retardation analyses indicated that the extended hairpin in the *S. pombe* ITS2 sequence interacted specifically with one or more soluble proteins. In the present study, the ITS2 region of the *S. pombe* pre-rRNA was synthesized using T_7 RNA polymerase (13) and used as a ligand in affinity chromatography (12) to isolate and further characterize the ITS2-specific spacer binding proteins from a cellular extract. The ITS2 sequence first was fused to a polyguanylic acid cluster that permitted the preparation of RNA with a poly(G) tail, which subsequently was used to immobilize the RNA on a poly(C)-agarose matrix (12). Protein was applied in low salt buffer (14) with calf liver RNA to eliminate nonspecific binding. After an extensive wash with 0.133 M KCl, the bound protein was eluted sequentially with buffer containing 0.3 and 1.0 M KCl. As shown in Fig. 1 (*left*), the 0.3 M fraction contained the majority of protein that effectively formed a stable ribonucleoprotein complex with ITS2 RNA (+). When this band was excised and polymerized into the stacking layer of a standard SDS-acrylamide protein analysis gel and the protein components were electrophoretically fractionated, a diverse group of proteins was evident (Fig. 1, *right*). Despite the surprisingly large number of proteins, most bands were present in similar amounts, consistent with a homogeneous protein complex (RAC).

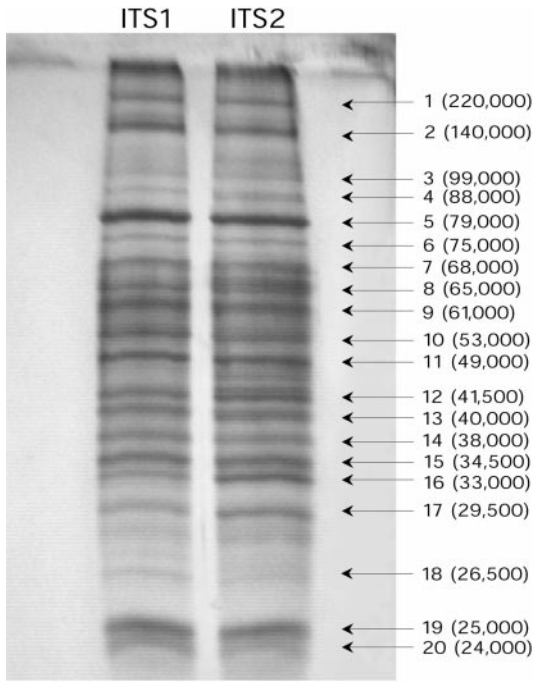

FIG. 2. **Comparison of RAC complex proteins purified with alternate ITS sequence ligands.** Ribonucleoprotein was prepared with labeled *S. pombe* ITS1 and ITS2 RNA, and the protein constituents were fractionated on an SDS-polyacrylamide gel as described in Fig. 1.

Because an equally large number of proteins also was isolated in the earlier study using ITS1 RNA (12), the ITS1- and ITS2-associated proteins were compared directly. In each case the fraction containing the majority of the spacer binding protein was used, the 1.0 M fraction for ITS1 and the 0.3 M fraction for ITS2. As shown in Fig. 2, despite differences in the RNA ligands and in the elution conditions, the protein components that formed a ribonucleoprotein complex were found to be

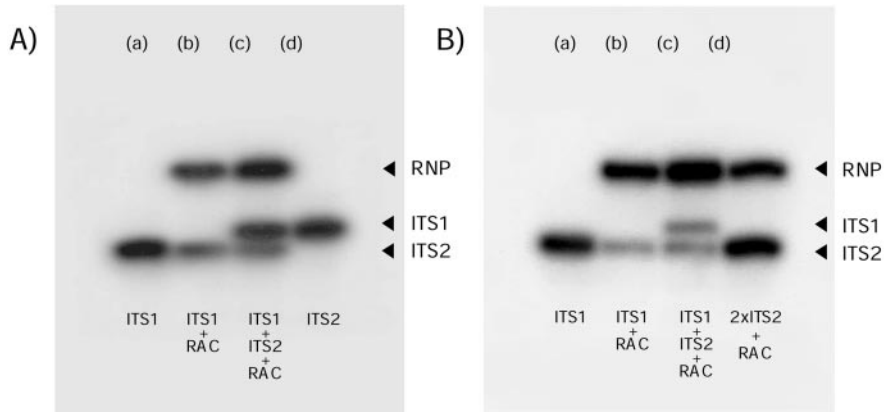

FIG. 3. **Distinct ITS binding sites in the *S. pombe* RAC complex.** *A*, *in vitro* synthesized and labeled ITS2 RNA (*a*) was incubated with affinity-purified protein (RAC) in the absence (*b*) or presence (*c*) of an equal amount of labeled ITS1 RNA (*d*). *B*, *in vitro* synthesized and labeled ITS2 RNA (*a*) was incubated with affinity-purified protein (RAC) in the absence (*b*) or presence (*c*) of an equal amount of labeled ITS1 RNA or an equal amount (total = 2 ×) of labeled ITS2 RNA (*d*). Ribonucleoprotein (*RNP*) formation was assayed by agarose gel electrophoresis. Equal amounts of ITS1 and ITS2 RNA are bound in two distinct sites (*c*); extra ITS1 is not bound in the ITS2 site (*B*, *lane d*).

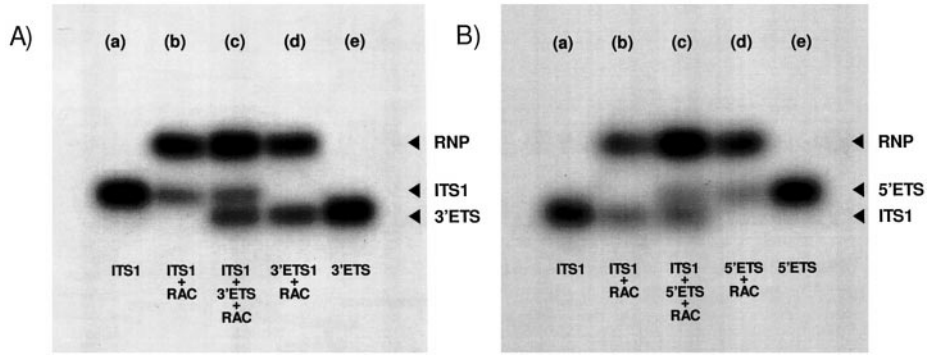

FIG. 4. **Distinct ETS binding sites in the *S. pombe* RAC complex.** *A*, *in vitro* synthesized and labeled ITS1 (*a*) or 3′-ETS (*e*) RNA was incubated with affinity-purified protein (RAC) individually (*b* and *d*, respectively) or together (*c*). *B*, *in vitro* synthesized and labeled ITS1 (*a*) or 5′-ETS (*e*) RNA was incubated with affinity-purified protein (RAC) individually (*b* and *d*, respectively) or together (*c*). Ribonucleoprotein (*RNP*) formation was assayed by agarose gel electrophoresis. The protein binds an equal amount of each spacer in distinct sites.

largely or completely identical. The fact that the same protein complex was eluted at different salt concentrations (1.0 *versus* 0.3 M KCl) raised the possibility that each ITS region interacted with a separate binding site in the protein complex.

Earlier studies already had suggested limited sequence equivalence in the ITS sequences, but some competition for protein was evident when large excesses of competing RNA were present (10, 11). This raised the alternate possibility that the two internal spacers competed for a common binding site, a possibility that also is consistent with the similarities in the protein components (Fig. 2). To evaluate these possibilities further, a complex formation was examined under more competitive conditions. As illustrated in Fig. 3*A* (*lane b*) with ITS2 RNA, in these studies the amount of affinity-purified protein was limited to allow ~80% of the labeled RNA to be incorporated into a ribonucleoprotein complex. As also shown in Fig. 3*A*, when an equal amount of ITS1 RNA was included (*lane c*), the amount of ITS2 RNA in the complex was not reduced and ~80% of the ITS1 molecules were incorporated into the ribonucleoprotein complex as well (*lane c*). This observation indicated that the two sequences were not directly competing for the same site; rather, they were equally bound in two separate sites. Such a model was further supported when an equal amount of ITS2 sequence was added instead of ITS1. As shown in Fig. 3*B*, under these conditions ITS1 was efficiently incorporated into the complex (*lane c*), but further ITS2 RNA was not incorporated (*lane d*) and ~60% of the total or all of the extra ITS2 RNA remained unbound.

Previous studies on the 3′-ETS region in the *S. pombe* pre-rRNA based on gel retardation analyses have indicated that a conserved, extended hairpin structure in this transcribed spacer also forms a stable ribonucleoprotein complex with cellular protein, a complex that has been experimentally linked with rRNA maturation (15). Studies on the 5′-ETS (16) again have identified an extended hairpin structure that is critical to rRNA maturation. Competition studies between the 3′-ETS and either unlabeled internal transcribed spacer did not show a relationship in protein-binding sites, although a limited sequence homology appeared present and raised the possibility of a structural equivalence (11). To explore the possibility that the RAC complex also contained binding sites for the external spacers, ribonucleoprotein formation again was examined using the affinity-purified protein. As shown in Fig. 4*A*, the results with the 3′-ETS were strikingly similar to those observed with the two internal spacers (Fig. 2). The 3′-ETS-derived RNA (*lane e*) was able to form a stable complex (*lane d*). More importantly, in the presence of ITS1-derived RNA (*lane c*) both RNAs were incorporated equally into the complex whereas the amount of complex-bound 3′-ETS remained constant. This observation again supported comparable but distinct binding sites for both spacer regions. As shown in Fig. 4*B*, the 5′-ETS-derived RNA also formed a stable ribonucleoprotein complex (*lane d*) with ITS-purified protein. Again, in the presence of ITS1-derived RNA (*lane c*) both RNAs were equally incorporated and no 5′-ETS was displaced.

The specific nature of this interaction was further demonstrated when complex formation was reexamined in the presence of excess unlabeled spacer RNA (Fig. 5). When ITS1 binding was compared with the 5′-ETS (Fig. 5*A*) in the presence of unlabeled ITS1-derived RNA (*lane d*), only labeled ITS1-derived RNA was displaced and in the presence of unlabeled 5′-ETS-derived RNA (*lane e*), only labeled 5′-ETS-derived RNA was displaced. These observations again were entirely consistent with separate and specific spacer binding sites. Similarly, when ITS1 binding was compared with ITS2 (Fig. 5*B*), equivalent results were obtained. In the presence of unlabeled ITS1-derived RNA (*lane c*), only labeled ITS1 RNA was displaced and in the presence of unlabeled ITS2-derived RNA (*lane d*), only labeled ITS2 RNA was displaced. Once more these results strongly supported separate and specific binding sites.

Taken together, the results of this study provided experimental evidence for a model of rRNA maturation (Fig. 6) that

we first suggested to explain distant effects in rRNA processing (17). The model proposes that as ribosomal proteins assemble on the mature ribosomal RNA sequences, nucleolar constituents simultaneously assemble on the spacer regions basically forming a nucleolar pre-rRNA particle consisting of three domains; two of those domains correspond with the ribosomal subunits and one domain is composed of spacers and nucleolar proteins or RNAs, comprising a common processing domain. The RAC complex described in this study would represent the core of this domain with spacer sequences and transacting factors appropriately organized on this core. Normally this particle fully forms before rRNA cleavages are initiated (9) and appears to be essential for an efficient maturation of the ribosomal RNA (7, 9, 15, 16). Our past studies on the RNA spacer sequences are entirely consistent with such a model. For example, mutations in the known protein-binding site of the 3′-ETS (15) or ITS1 RNA (12) also have been shown to dramatically affect RNA maturation with comparable effects on protein binding. Furthermore, mutations in the protein-binding regions in the ITS2 (11) and 5′-ETS (16) sequences have been

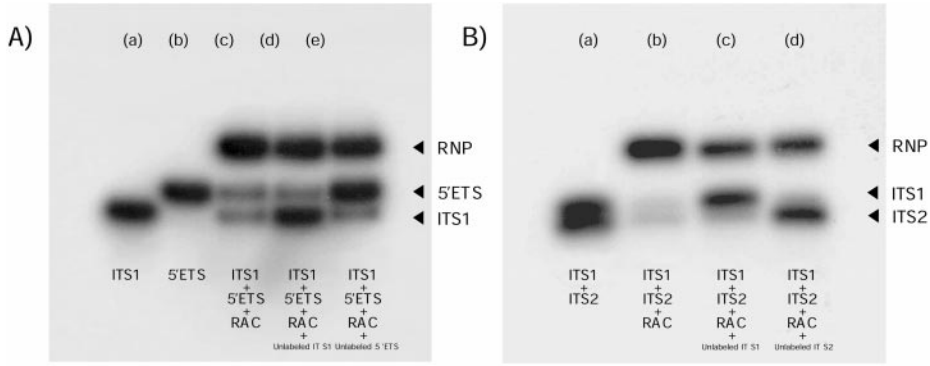

FIG. 5. **Spacer-specific binding sites in the *S. pombe* RAC complex.** *A*, *in vitro* synthesized and labeled ITS1 (*a*) and 5′-ETS (*b*) RNA were incubated together with affinity-purified protein (RAC) individually (*b* and *d*, respectively) or together (*c–e*) in the presence of a 100-fold excess of unlabeled ITS1 (*d*) or 5′-ETS (*e*) RNA. *B*, *in vitro* synthesized and labeled ITS1 and ITS2 RNAs (*a*) were incubated together with affinity-purified protein (RAC) (*c* and *d*) in the presence of a 100-fold excess of unlabeled ITS1 (*c*) or ITS2 (*d*) RNA. Excess unlabeled RNA displaces only equivalent labeled RNA. *RNP*, ribonucleoprotein.

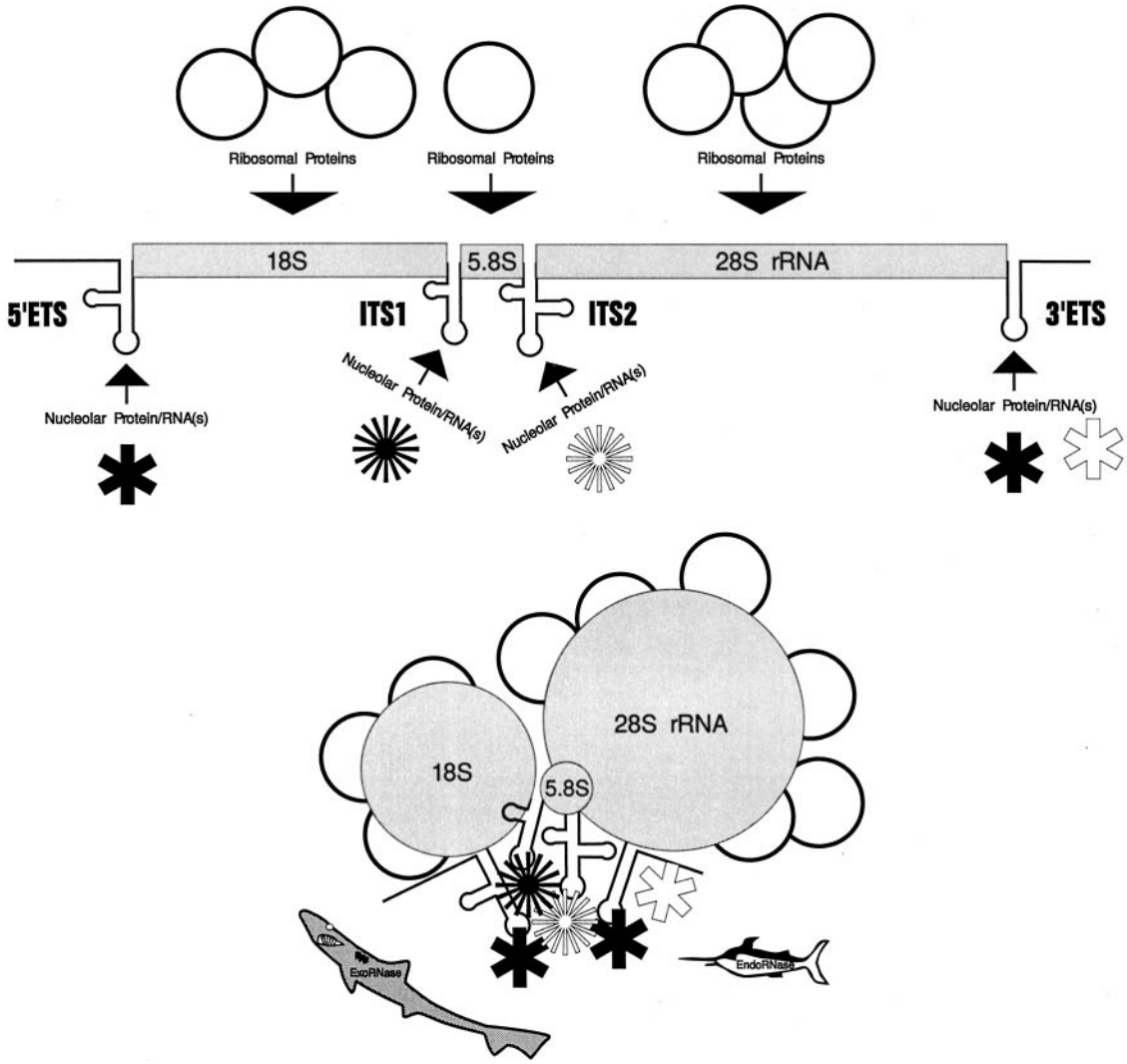

FIG. 6. **A model for the assembly of a nucleolar 80–90 S preribosomal particle.** Ribosomal proteins assemble on the rRNA sequences to form the ribosomal subunits whereas the nucleolar RAC proteins assemble on the spacer sequences to form a common processing domain that is acted on by nucleases and other nucleolar factors or RNAs.

shown to critically affect RNA maturation, and equivalent regions in the internal spacers of the preribosomal RNA of *Saccharomyces cerevisiae* also have been shown to be essential for rRNA processing (18).

As noted earlier, in many ways the particle described in this study resembles the spliceosomal complexes associated with mRNA processing. As in the case of spliceosomes, the roles of individual proteins in the present complex remain unclear. Our attempts to demonstrate specific cleavages with the affinity-purified RAC protein have failed (results not shown), but various alternate enzymatic activities associated with rRNA maturation previously have been reported (19). These observations suggest that the affinity-purified protein acts not to cleave the RNA but to organize it in a way that allows its precise and efficient cleavage by other peptides such as the RNase III-like enzymes (20, 21), the Rrp4p protein, and the related "exosome" (22). In this respect the new complex would act as a ribosome assembly chaperone or a kind of "rack" on which the maturing structures are organized. The protein complex may even act by sterically hindering the processing enzymes, allowing complexes such as the "exosome" to act only in a limited and defined fashion. Whatever the case, the new particle (RAC) raises a very different picture of ribosome maturation and provides an explanation for the previously described distant interdependences in rRNA maturation that, at least in part, serve as a "quality control" mechanism for ribosome biogenesis (8, 17).

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