A RAC Protein-binding Site in the Internal Transcribed Spacer 2 of Pre-rRNA Transcripts from Schizosaccharomyces pombe

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The interdependence of steps in the processing of the eukaryotic preribosomal rRNA transcripts indicate that rRNA processing, at least in part, acts as a quality control mechanism to help ensure that only functional rRNA is incorporated into mature ribosomes. In search of structural components that underlie this interdependence, we have isolated a large protein complex or RAC that contains an independent binding site for all four of the transcribed spacers in the nascent pre-rRNA. In this study the RAC-binding site in the internal transcribed spacer 2 sequence of Schizosaccharomyces pombe rRNA transcripts was identified, and the influence of this site on rRNA maturation was assessed. Modification exclusion analyses indicate that the protein complex interacts with a helical domain previously shown to contain features common to both the internal transcribed spacer 1 and the 3′-external transcribed spacer. Mutagenic analyses in vitro confirm an interaction with this sequence, and parallel analyses in vivo indicated a critical role in both the maturation of the rRNA components of the large subunit as well as the 18 S rRNA component of the small subunit. Hybridization analyses also indicated greatly elevated levels of unprocessed nascent RNA. These effects are contrasted with mutations in other regions of the secondary structure that resulted in some reduction of plasmid-derived mature rRNA but no elevated levels of the precursor molecules. The significance with respect to rRNA maturation and the interdependencies in rRNA processing are discussed.

The rRNAs of eukaryotic ribosomes are cleaved from a large 35–45 S pre-rRNA nucleolar precursor, which initially must be fully transcribed, modified through RNA methylation and base conversions, and assembled into an 80–90 S nucleolar ribonucleoprotein particle (1, 2). Electron micrographs of chromatin spreads from actively transcribed nucleoli (3) as well as structural analyses of the nucleolar RNA precursors (4) have demonstrated such transcripts in many organisms. The processing of the transcripts, however, has been pictured as a cleavage pathway (5) of many rapid and independent steps beginning with the two external transcribed spacers (5′-ETS and 3′-ETS) and followed with cleavages in the internal transcribed spacers (ITS1 and ITS2). Although some variations in the details have been reported, in general, this cleavage pathway is considered a conserved feature of eukaryotic cells.

In recent years, a number of genetic and biochemical analyses have begun to indicate that, at least in Schizosaccharomyces pombe, the cleavage steps in rRNA processing are not independent as previously believed. In this fission yeast, distant interactions between the external and internal transcribed spacers can dramatically influence the efficiency of rRNA processing and ribosomal integration. For example, deletion of a conserved 3′-ETS structure has been found to dramatically affect the processing of the ITS2 and the 5.8 S rRNA sequences, located more than 3000 bases upstream (6, 7). Equally, the deletion of the ITS2 spacer not only prevents the maturation of the large subunit but severely affects maturation of the small subunit rRNA (8). Mutations in the 5′-ETS also can dramatically affect the production of large subunit RNA constituents (9), and even changes to the position of termination appear to critically alter the maturation efficiency (10). All of these observations have been taken as evidence that interdependencies in rRNA maturation act as quality control mechanisms to help ensure that only functional rRNA is incorporated into ribosomes.

To identify structural components that underlie the observed interdependencies, searches have been made for proteins that interact specifically with the transcribed spacers (11, 12). With the application of affinity chromatography and the use of the transcribed spacers as ligands, a large protein complex of 20 or more polypeptides has been isolated (13) and putatively called RAC (ribosomes assembly chaperone). Many of the polypeptides appeared to contain RNA-binding sites, but no RNA cleavage activity could be demonstrated with this protein complex. Subsequently, the complex was shown to contain independent binding sites for each of the transcribed spacers in the pre-rRNA (14), and consistent with a role in rRNA processing, the disruption of an RAC-binding site in the ITS1 rRNA spacer was observed to severely affect rRNA processing (13). Although the RAC complex itself appears not to have any nuclease activity, more recent studies with the PacI RNase III-like endonuclease have shown that, in the presence of the RAC complex, this enzyme specifically cleaves the ribosomal RNA precursor at the 3′-end of the 25 S rRNA sequence (15). Taken together, these results provide evidence that the RAC complex does indeed function as a chaperone for ribosome maturation.

Because deletion of the ITS2 region is critical not only to the maturation of the large ribosomal subunit but also has been found to dramatically affect the efficiency of 18 S rRNA production, further study now has been made of essential structural features in the ITS2 sequence from S. pombe, particularly with respect to RAC binding. The results identify the protein-binding domain in the central extended hairpin structure and show that this site is critical to rRNA maturation in vivo.
**EXPERIMENTAL PROCEDURES**

**Preparation of Ribosomal RNA Precursor**—A DNA template for the ITS2 region in *S. pombe* rDNA was prepared by PCR amplification using primers specific for the 3' end of the 5.8 S rRNA (ATGCCTGGTTTGAGTGTC), beginning 20 nucleotides from the 3' end, and the 5' end of the 25 S rRNA (GCGAATAACTATACGAA), ending 55 nucleotides downstream of the 5' end. This 374-bp fragment was cloned in the pTZR19 plasmid for expression by bacteriophage T7 RNA polymerase after cleavage with EcoRI restriction endonuclease, as described previously (12). The precursor RNA transcript was purified on a 6% denaturing polyacrylamide gel and labeled at the 5' end using bacteriophage T4 polynucleotide kinase and [γ-32P]ATP, (16), after dephosphorylation with calf intestinal alkaline phosphatase (17). The phosphatase was inactivated by heating to 75 °C for 10 min in 5 mM EDTA (pH 8.0) before the RNA was labeled, and the labeled RNA again was purified on a 6% denaturing polyacrylamide gel. To prepare just the central extended stem RNA in ITS2, the same methodology was applied using primers specific for the 5' (GATGAGGTGTTG) and 3' (GTTAAGGTTCAA) ends of the 222-nucleotide sequence.

**Preparation of RAC Protein**—The RAC protein complex was purified by affinity chromatography using ITS1 RNA bound to a poly(C)-agarose support (Sigma-Aldrich) as recently described (13, 14). The RNA ligand was immobilized on the column matrix using a poly(G) sequence at the 3' end that 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

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**Fig. 1. Preparation of PCR-mediated mutations in the ITS2 region of the rDNA from *S. pombe*. a, a 1634-base pair *Sst*I-*Acc*I (A) restriction fragment containing a tagged intragenic region from the *S. pombe* rDNA was cloned in the pBluescript KS vector and used as a template for PCR-based mutagenesis (22, 23). The closed arrow (M) indicates ITS2-specific mutagenic primers, and the open arrows (arrows 1 and 2) indicate plasmid-specific universal primers used in the first and second steps, respectively. b, a unique *Hpa*I-*Bgl*II (B) restriction fragment of *S. pombe* rDNA cloned in the pTZ19R vector. PCR-amplified mutant sequences (a) were digested with *Sst*I and *Acc*I restriction enzymes to replace the original rDNA sequence. c, the pFL20/Sp18Pst5.8i4 yeast shuttle vector containing neutral tags (dark grey) in the 18 and 5.8 S rRNA sequences (8). Cloned mutant sequences (b) were digested with *Hpa*I-*Bgl*II restriction enzymes to replace the original rDNA sequence. The inset describes the normal ITS2 sequence including an estimate of its secondary structure as deduced by computer analyses and enzymatic probes (12). Darkly shaded nucleotides are those reported common to the ITS1, ITS2, and 3'-ETS regions in the *S. pombe* rDNA.
chromatography buffer (5 mM Mops, 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 RNA. The column was washed with chromatography buffer containing 0.15 M and then 1 M KCl, respectively. As previously reported, the RAC complex was present in the 1 M KCl fraction.

Electrophoretic Mobility Shift Assay—Ribonucleoprotein complex formation was assayed by gel retardation as described previously (11, 12). Aliquots of in vitro transcribed and labeled spacer RNA (0.3–2 ng/20,000–25,000 cpm) were incubated with 5 μg of RAC protein in binding buffer (100 mM KCl, 5 mM MgCl2, 0.5 mM dithiothreitol, and 12 mM Tris-HCl, pH 8.0) containing 8% glycerol and 0.25 mg/ml calf liver RNA to eliminate nonspecific interactions. For comparative 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 with normal RNA, allowing about 60–70% of the labeled RNA to be incorporated into the ribonucleoprotein complex. The complexes were fractionated from free RNAs on 2% (w/v) agarose gels at 4°C; following electrophoresis the gels were exposed to x-ray film to detect the bands by autoradiography.

Modification Exclusion Analyses—Critical residues in protein-binding sites within the RNA spacer were determined by modification exclusion as described previously (18, 19). After dephosphorylation with calf intestinal alkaline phosphatase, in vitro transcribed ITS2 precursor RNA or just the central extended stem region was labeled at the 5′-end using bacteriophage T7 polynucleotide kinase and [γ-32P]ATP and purified by electrophoresis on a nondenaturing 6% polyacrylamide gel at 4°C. The free and complexed labeled RNA bands were detected by autoradiography and eluted by gel homogenization. After SDS/phenol extraction, each RNA fraction again was purified by electrophoresis on a denaturing 6% polyacrylamide gel, dissolved in 20 μl of freshly prepared aniline solution (1 M aniline in 2.5% acetic acid), and incubated at 60 °C for 20 min in the dark. The mixture was cooled, the reaction was stopped with 200 μl of 0.3 M NaAc, and the RNA was precipitated with 0.5 ml of ethanol. The RNA fragments were dissolved in loading buffer (50% formamide, 0.001% xylene cyanol, and 0.001% bromphenol blue) and fractionated on a denaturing 8% polyacrylamide sequencing gel; following electrophoresis the gels were exposed to x-ray film to detect the bands by autoradiography.

Quantitative analyses of the cleaved bands were performed using a scanner (Umax Technologies) to capture the images and Molecular Analyst PC software, version 1.5 (Bio-Rad). The level of modification for each nucleotide in protein-associated RNA was determined as a percentage of that observed with the free RNA fraction.

Construction and Expression of Mutant rRNA Genes—Site-specific mutations were incorporated into the ITS2 sequence of a S. pombe rRNA transcriptional unit that had previously been subcloned (20) into the pFL20 yeast shuttle vector and “tagged” with a PstI restriction site in the 18 S rRNA sequence (8) and a four-base insert in the 5.8 S rRNA (21). The base substitutions and some deletions were introduced by two-step PCR amplification (22) using a pBluescript KS plasmid template containing the entire intragenic region with adjacent 5′- and 3′-ends 25 S rRNA sequences. In some instances when deletions were not successful with this strategy, mutations were introduced by plasmid-amplified PCR-based mutagenesis (23) using the same template. In either case, the resulting mutated and amplified DNA was used to replace the normal sequence in the shuttle vector containing the tagged S. pombe rRNA transcriptional unit. The recombinants subsequently were amplified in Escherichia coli strain C490, and used to transform S. pombe strain h- leu 1-32 ura 4-D18 using the method described by Prentice (24). Each mutation initially was confirmed by DNA sequencing (25), and subsequently, where required, the presence of mutant rRNA in transformed cells was confirmed again by PCR amplification of the ITS2 rDNA region followed once more by DNA sequencing.

Characterization of the Expressed Mutant Ribosomal RNAs—Transfected S. pombe cells expressing normal or mutant rRNAs were grown with constant aeration at 30 °C in minimal medium broth (26). For all analyses, the cells were rapidly cooled with ice, harvested by centrifugation, and extracted with SDS/phenol after disruption by vortexing with an equal volume of glass beads (27). For 5.8 S rRNA analyses, the RNA was fractionated on an 8% (w/v) polyacrylamide gel and stained with methylene blue to detect the separated RNA components (21). For 18 S rRNA or precursor analyses, the RNA was fractionated on a 0.8% agarose, 0.2 M formaldehyde gel and transferred to nylon by capillary blotting (8). The membrane was stained with methylene blue to confirm that equivalent amounts of RNA were transferred and then hybridized with a 32P-labeled oligomer probe specific for the tagged 18 S rRNA transcribed from the plasmid-associated rDNA (8).

RESULTS

Gel retardation studies with the ITS2 region in the S. pombe rRNA precursor indicate that the central extended hairpin structure in this rRNA sequence forms a specific and stable ribonucleoprotein complex (12). As indicated in Fig. 1 (inset), based on sequence comparisons with the ITS1 and 3′-ETS regions in the same rRNA precursor molecule, a protein-binding site was putatively identified at that terminal end of this hairpin structure. In the present study direct evidence for this site initially was sought using modification exclusion (19). Whole ITS2 rRNA or just the central extended hairpin was prepared by transcription using T7 rRNA polymerase, and the RNA was labeled at the 5′-end using [γ-32P]ATP. To maintain protein limited conditions, sufficient affinity-purified RAC complex was incubated with labeled RNA (ca. 2 × 106 cpm) in about 90% of the RNA to ribonucleoprotein complex (Fig. 2, lane a). To probe the protein-binding site, the labeled RNA was first modified using an RNA chemical sequencing reagent containing diethylypyrocarbonate (28) and then incubated with RAC protein. As also shown in Fig. 2 (lane c), when the resulting ribonucleoprotein complex was fractionated by gel electrophoresis, a significantly reduced amount of ribonucleoprotein was evident, with only about half of the RNA being able to form RNP. The RNA in both the free and the protein-bound RNA fractions was eluted, purified by SDS/phenol extraction, and treated with aniline to cleave the chemically modified...
FIG. 3. Probing of the protein-binding site by modification exclusion. Labeled ITS2 RNA was prepared, modified with diethylpyrocarbonate, and used to form ribonucleoprotein as shown in Fig. 2. The gel purified protein-associated (RNP) or free RNA (RNA) bands were eluted and extracted with SDS/phenol, and the modified bases were cleaved with aniline. The resulting fragments were fractionated on 8% polyacrylamide sequencing gels for comparison and image analyses. Both ribonucleoprotein formed with the original cellular protein extract (A) and affinity-purified protein (B) were examined. The gel images were captured and used to determine the level of modification in protein-associated RNA as compared with the free RNA fraction. The average for three replicate experiments with affinity-purified protein is presented as a histogram (C). The two regions with reduced modification (regions I and II) in the secondary structure are indicated with light shading (D); nucleotides common to the ITS1, ITS2 and 3′-ETS structures are indicated by dark shading.
residues (19, 28). The fragments were fractionated on polyacrylamide sequencing gels as shown in Fig. 3 (A and B), and after exposure to x-ray film, the images were analyzed to determine the relative reactivity at each base in the protein-associated fraction as compared with the free rRNA. Although the diethylpyrocarbonate-induced cleavage was primarily directed at the purine residues (28), as noted previously (13), sufficient cleavage occurred at pyrimidine sites to permit the analysis of all of the nucleotides. As also shown in Fig. 3C, when images of replicated experiments were analyzed, the relative reactivity at each base in the protein-associated fraction was reduced in two areas, U\textsuperscript{114}G\textsuperscript{137} and U\textsuperscript{196}A\textsuperscript{192}. More important, when the sites are examined with respect to the secondary structure (Fig. 3D), they appear on opposite sides of the helix near the end of the extended central stem. As indicated in Fig. 3D, this is the same region that contained nucleotides that were previously noted to be conserved in the extended hairpin structures from the ITS1, ITS2, and 3′-ETS regions in the \textsuperscript{32}S pre-rRNA of \textit{S. pombe} (12).

Although the correspondence between conserved nucleotides and the protein-binding site was striking, the biological significance of these observations remained unclear. To further establish the protein-binding site and any role in rRNA maturation, mutations were introduced in this region to assess their effects on rRNA processing in vivo and protein binding in vitro. Initially, the entire end of the extended hairpin (Fig. 4A) was deleted using a plasmid-enhanced PCR-based mutagenesis strategy (23). As shown in Fig. 4B, when rRNA containing this mutation was expressed in \textit{vitro} and the amount of plasmid-derived 5.8 S rRNA was determined after fractionation by polyacrylamide gel electrophoresis, the observed effect was dramatic. As described previously, in control cells that were transformed with rDNA containing the normal ITS1 sequence, about 50% of the total 5.8 S rRNA was plasmid-derived. In cells that were not transformed with plasmid, no mutant RNA was evident. In all of the mutational analyses, to ensure a reproducible conclusion, at least three separate transformants were examined. As shown in Fig. 4B, in all three examples for this deletion (\textit{lunes a–c}), the substantial amount of plasmid-derived RNA clearly was absent when the mutation was present. As also shown in Fig. 4C, when hybridization analyses were conducted using an oligonucleotide probe that was specific for the plasmid-derived 18 S rRNA sequence, precursor rRNA was clearly detectable, although, again, no mature rRNA was present. Untransformed cells contained no RNA that was homologous with the oligonucleotide probe, and in normal transformed cells, only a very large amount of mature rRNA was evident under the exposure conditions that were used. In strong contrast, only greatly elevated levels of 37 S RNA transcript or transcript trimmed of ETS sequence was evident in any of the three mutant colonies (\textit{lunes a–c}). To ensure a uniform transfer, all of the membranes were stained with methylene blue prior to hybridization analyses. As shown in Fig. 4D, comparable amounts of RNA were evident in each lane.

Because sequence comparisons and the RAC ITS2 RNA footprint indicated that the binding site was centered in the top of the second base-paired section in the central extended hairpin, specific base substitutions also were introduced into this region of the hairpin structure to further evaluate the critical features. As shown in Fig. 5, in some cases the effects again were very dramatic. For example, when the hairpin was disrupted with a substitution of three nucleotides at G\textsuperscript{187}, U\textsuperscript{189}, and G\textsuperscript{191} (mutant II) or a smaller disruption with a substitution of two adenyl acid residues at U\textsuperscript{188} and U\textsuperscript{190} (mutant III), essentially no 5.8 S RNA was detected (Fig. 5, \textit{left panels}) and levels of the mature plasmid-derived 18 S rRNA were severely im-

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contrast, when this hairpin region was not disrupted but a substitution of three nucleotides at U186, U190, and G191 (mutant IV) simply further stabilized the hairpin structure, little or no effect was observed with respect to either 5.8 or 18S rRNA maturation. Furthermore, when significant changes were made in the terminal loop (mutant I), modest effects were evident, but as indicated by the hybridization analyses, increased levels of precursor intermediates were not observed, suggesting an effect on precursor stability rather than an inhibition in a defined step in the maturation pathway. Taken together these mutational analyses indicated that the protein-binding site, identified by sequence comparisons and modification exclusion, was uniquely critical to rRNA maturation.

To further confirm the RAC protein-binding site, the mutant rRNA also was examined with respect to RAC protein binding in vitro. As shown in Fig. 6, the simple substitution of two adenylic acid residues at U188 and U190 that strongly impacted rRNA maturation also dramatically affected protein binding. This relatively modest disruption in the hairpin structure completely disrupted protein binding, and no complex was formed with RNA that contained these changes (Fig. 6, lane d).

Based on comparative analyses the core structure of the ITS2 region has been postulated to comprise of a single extended hairpin that includes the interacting termini of the mature 5.8 and 25S rRNAs (12). In S. pombe and all of the other examples that have been examined, various smaller hairpin branches were also always present. Such features appear not to be conserved in any consistent fashion and tend to be characteristic of individual ITS2 sequences (12). In addition, in the present study, modification exclusion did not indicate contacts between these structures and the RAC protein complex, further suggesting that they may not be important with respect to rRNA maturation. Nevertheless, to detect any other influences on rRNA maturation, the three branch structures in the S. pombe ITS2 were each deleted separately, and the effects of these changes also were examined in vivo. As indicated in Fig. 7, some effects were evident, but they were far less dramatic than observed with the RAC-binding site. In replicated experiments with all three of the new mutations, the amount of plasmid-derived 5.8S rRNA was reduced to about 60% of the control value (Fig. 7, left panels), and the amount of 18S rRNA also was similarly depressed (Fig. 7, right panels). More important, however, when these mutants were examined with respect to changes in specific precursor intermediates, no elevated precursor levels were evident as was observed with the RAC-binding site mutations. These observations again suggest that this group of mutant RNAs were simply less stable with no specific failure in rRNA processing.
As shown in Fig. 1, based on computer-aided modeling and experimental probing, the ITS2 region has been predicted extensively paired into a relatively simple secondary structure (12). Nevertheless, the hairpin structure proximal to the mature termini appears to be more complex with some evidence of a higher order structure, making this region less susceptible to enzyme digestion (12). In view of these observations and the potential importance of these features with respect to the removal of the ITS2 sequence, a number of substitutions also were introduced into the large loop in this region (Fig. 7, mutant IV). When *S. pombe* cells were transformed with this sequence, and the effects were examined in vivo, as observed with that terminal helix, these changes proved critical, and little or no mature rRNA was observed. As shown in Fig. 7, the large amount of plasmid-derived 5.8 S rRNA was clearly absent, and only small amounts of mature 18 S rRNA could be detected by hybridization analyses. More important was the fact that the amount of initial nascent rRNA transcript was greatly elevated, consistent with a direct inhibition in rRNA processing.

**DISCUSSION**

Although the sequencing of rDNAs from many diverse eukaryotes has revealed highly conserved core structures in the mature rRNAs, the spacer regions have been shown to vary greatly, both in size and sequence, with little conservation even...
between relatively close examples. This has caused some to speculate that the processing mechanisms may vary with phylogeny (29, 30). An alternative explanation could be a role in which the spacers act as “biological springs” to maintain processed sites in close proximity (31). In more recent analyses, computer modeling and probes for nuclease protection were applied in searches of conserved core features (11, 12). Taken together, these analyses have suggested that all of the spacer regions may act in a similar fashion not only to organize the maturing terminal sequences but also to organize transacting factors in a manner that may be analogous with that of many small nuclear RNAs (12). The mutation analyses in the present study are consistent with these two basic functions. On one hand the ITS2 spacers interact with the RAC complex, an interaction that has been shown to be critical to rRNA maturation. Equally critical is the sequence surrounding the mature termini, where changes also can eliminate rRNA maturation. The influence of the remaining secondary structure clearly was less important and appeared primarily to result in precursor instability.

Early studies on the removal of internal transcribed spacers from preribosomal RNP particles in S. cerevisiae suggested a split maturation for two portions of the 50–90 S nuclear precursor particle. Removal of large portions from the 5’-ETS or almost half of ITS1 appeared to only affect the formation of tagged 17 S rRNA (32, 33) and complete or partial deletions of ITS2 prevented the production of mature 26 S rRNA (33) but did not interfere with the synthesis of the 17S rRNA (34). These conclusions differ from more recent studies in S. pombe (e.g. 6, 8), including the present analysis, primarily because of differences in the tagged experimental systems that were used. As noted previously, in the initial studies a low copy plasmid vector resulted in very small amounts of mutant RNA that had to be detected by very sensitive hybridization analyses. In the more recent studies, at least half of the cellular RNA normally is plasmid-derived, and the tagged 5.8 S rRNA can be detected by a simple staining procedure (21). This ensures that large amounts of mutant RNA precursors are forced to compete with normal RNA during quantitative analyses and provides a more accurate measurement of interdependences in the process.

Although alternate conclusions were reached regarding interdependence in rRNA processing, the earlier studies in S. cerevisiae first demonstrated that although the spacers vary widely in size and sequence (35), they do contain structural features that are essential for their removal. In their study of the role of ITS2 in processing, Raue and co-workers (34, 36) concluded that this sequence is more sensitive to structural alteration than ITS1. They also concluded that elements of ITS2 that play a major role in processing were confined largely to structures that were conserved in other yeast species. The deletion of individual nonconserved segments appeared to cause little or no disturbance in processing.

With respect to a generalized estimate of the secondary structure for ITS2 regions (12, 37), the regions of sequence conservation and deleterious mutations in S. cerevisiae primarily correspond with the terminal helix in the extended central hairpin and the region surrounding or adjacent to the interacting mature termini of the 5.8 and 25 S rRNAs. Changes in the branch helices or in the middle portion of the central stem were those that, individually, caused little or no disturbance. Similarly, in the present studies, mutations in the branched regions again were not critical. With large amounts of plasmid-derived RNA and more accurate quantitative analyses, there was a clear reduction in the mature RNA yield, but, as indicated by the precursor profiles, no specific steps were inhibited, and the precursors appeared simply to be less stable.

Also in agreement with the studies in S. cerevisiae, in S. pombe the end of the extended hairpin and the sequence adjoining the mature termini proved to be critical to rRNA maturation. In both regions, the mutations also induced large changes in the precursor profile as characterized by hybridization analyses, and substantial build-up in the initial precursors were evident. With changes near the maturing termini, some 18 S rRNA was clearly evident; this was essentially eliminated entirely with changes in the RAC-binding site. Taken together, the previous studies in S. cerevisiae and the present studies in S. pombe are consistent with two critical requirements in the ITS2 spacer: a site that interacts with the RAC complex, possibly to contribute to a common processing domain in the nuclear precursor particle (14), and a defined structure at the maturing termini that is recognized by the processing enzyme(s). As previously suggested (31), the remaining ITS2 structure simply may act to organize the critical domains in a stable fashion. This would be consistent with the wide range of size and composition that is observed among ITS2 sequences.

Although the present study documents the critical nature of the ITS2-RAC interaction, the reason why RNA processing is inhibited remains unclear. In a recent study of the 3’-ETS (15), the RAC complex, which, independently, exhibits no nuclease activity, was observed to dramatically alter the efficiency and specificity of the RNase III-like PAC nuclease, a primary processing enzyme in the maturation of the 3’-ETS. This observation was consistent with and provided new direct evidence of a “chaperone” role for this protein complex. The effects of the RAC-binding site mutations in the present study are completely consistent with this function and raise the possibility that the RAC complex can affect the efficiency and/or specificity of enzyme activities in ITS1 sequence removal, including RNase MRP (38), the nuclear RNase P (39), or the Rrp4p protein (40). Studies with these enzymes will be required to examine this possibility. In the interim, the RAC-binding site analyses provide an explanation for the critical nature of mutations at the end of the central extended hairpin in the ITS2 region and further underlines the structural equivalence (12) in the transcribed spacers of pre-rRNA transcripts.

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