Parallels in rRNA Processing: Conserved Features in the Processing of the Internal Transcribed Spacer 1 in the Pre-rRNA from *Schizosaccharomyces* pombe†

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ABSTRACT: Despite the large differences in their length and nucleotide composition, comparative analyses of the internal transcribed spacer 1 (ITS1) of widely divergent eukaryotes have suggested a simple core structure consisting of a central extended hairpin and lesser hairpin structures at the maturing junctions [Lalev, A. I., and Nazar, R. N. (1998) *J. Mol. Biol. 284*, 1341−1351]. In this study, the ITS1 in the pre-rRNA transcripts of *Schizosaccharomyces pombe* cells was examined with respect to structural features that underlie rRNA maturation. When plasmid-associated rRNA genes were expressed in vivo, a deletion of any major hairpin structure significantly reduced or eliminated both small and large subunit RNAs. Only changes in the central extended hairpin or junction regions, however, entirely eliminated plasmid-derived RNAs or resulted in elevated precursor levels. Structure-disrupting base substitutions within the RAC protein complex binding site in the extended hairpin indicated that the secondary structure was critical for rRNA maturation; composition or other changes with respect to the binding site had only modest effects. A similar disruption at the junction with the 18S rRNA also had striking effects on rRNA maturation, including a highly elevated level of unprocessed precursor and a surprisingly critical effect on 5.8S rRNA production. As previously observed with the 3′ external transcribed spacer, the results are consistent with a maturation mechanism in which an initial cleavage in the 5′ junction region may be directed by the RAC protein complex. Although not critical to rRNA processing, analyses of termini based on S1 nuclease protection as well as cleavage studies, in vitro, with Pac1 ribonuclease raise the possibility that in eukaryotes, as previously observed in bacteria, the RNase III homologues normally initiate the separation of the subunit RNAs.

In eukaryotes, three of the four RNA constituents (17−18S, the 5.8S, and 25−28S rRNAs) of the cytoplasmic ribosomes are encoded by tandemly repeated nucleolar transcriptional units. The large 35−45S pre-rRNA precursors are fully transcribed, modified through rRNA methylation and base conversion, and assembled with both ribosome and nucleolar proteins into 80−90S ribonucleoprotein particles before being processed into mature ribosome subunits (see refs *1* and *2*). Processing involves a complex cleavage pathway which begins with the removal of two external transcribed spacers (5′ETS and 3′ETS),[1] followed by cleavages in the internal transcribed spacers (ITS1 and ITS2) and RNA trimming to form the mature ribosomal RNA components. Studies over many years have shown that this complex maturation process is dependent on a large number of transacting factors including endo- and exonucleases and a wide assortment of nucleolar proteins, many of unknown function (see refs *3* and *4*). Specific nuclease activities have been linked with some of the processing steps (see ref *5*), but others remain unclear including that which initially separates the subunits. In addition, a number of snoRNAs, including snR30, U3, and U14, have been shown to be essential for early processing cleavages in yeast (*6*−*8*). Homologues of U3 and U14 and other snoRNAs also are required in vertebrates, and a large 5′ETS processing complex has been shown to form on a processing signal as an initiating step in rRNA processing (*9*, *10*).

Studies on important structural features or cis-acting elements in the spacer regions have been largely restricted to yeasts. While such studies in *S. cerevisiae* (e.g., *11*−*13*) initially suggested that the subunits matured independently (*14*), a number of genetic and biochemical analyses in *Schizosaccharomyces pombe* now indicate that the efficiency of rRNA processing is dramatically influenced by distant interactions between the transcribed spacers. For example, deletion of a conserved hairpin structure in the 3′ETS was found to completely block the processing of the ITS2 and 5.8S rRNA sequences, located more than 3000 bases upstream (*15*, *16*), and a subsequent study in *Saccharomyces cerevisiae* also has demonstrated this critical effect (*17*). Similarly, the deletion of the ITS2 spacer not only prevented the maturation of the large subunit but also severely affected the maturation of the small subunit rRNA (*18*). Equally, mutations in the 5′ETS can severely limit the production of large subunits (*19*), and even changes in the position of termination were observed to severely limit the production of ribosomal RNA (*20*). Taken together, these observations

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[1] Abbreviations: 5′ETS, 5′ external transcribed spacer; 3′ETS, 3′ external transcribed spacer; ITS1, internal transcribed spacer 1; ITS2, internal transcribed spacer 2; RAC, ribosome assembly chaperone.

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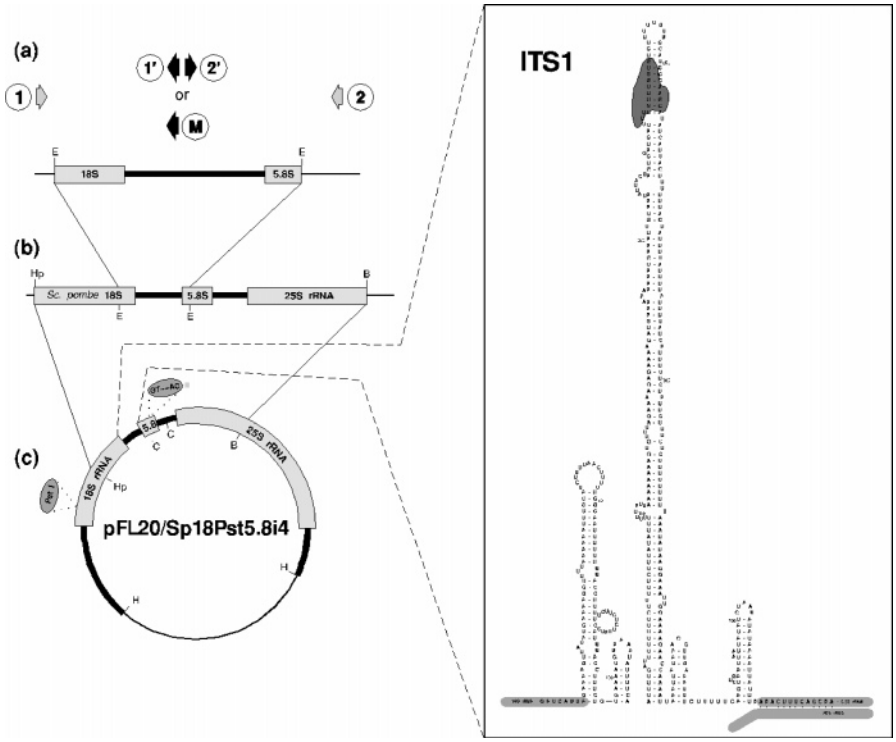

FIGURE 1: Preparation of PCR-mediated mutations in the ITS1 region of the rDNA from *S. pombe*. (a) A 725 base pair *Eco*RI-*Eco*RI (E) restriction fragment containing the ITS1 intragenic region from the *S. pombe* rDNA was cloned in the pTZ19R vector and used as a template for PCR-based mutagenesis using a "PEP" (*30*) or "Megaprimer" (*29*) strategy. The closed arrow (M) indicates ITS1-specific primers with nucleotide substitutions and closed arrows (1′, 2′) indicate ITS1-specific primers which border a deletion. The opened arrows (1, 2) indicate plasmid-specific universal primers used in both the first and/or second steps, as required. (b) A unique *Hpa*I (Hp)−*Bgl*II (B) restriction fragment of *S. pombe* rDNA cloned in the pTZ19R vector. PCR-amplified mutant sequences (a) were digested with *Eco*RI restriction enzyme to replace the original rDNA sequence. (c) The pFL20/Sp18Pst5.8i4 yeast shuttle vector containing neutral tags (dark gray) in the 18S and 5.8S rRNA sequences (*22*). Cloned mutant sequences (b) were digested with *Hpa*I−*Bgl*II restriction enzymes to replace the original rDNA sequence. The insert describes the normal ITS1 sequence including a prediction of its secondary structure as deduced by computer analyses and enzymatic probes (*24*). Lightly shaded nucleotides indicate the maturing rRNA sequences; the dark shading indicates a region previously shown to interact with RAC protein complex (*14*).

have suggested that the complex maturation pathway for eukaryotic ribosomes, at least in part, serves as a quality control mechanism which helps ensure that only functional ribosomes are released to the cytoplasm (*18*, *21*).

In more recent studies on interactions between the spacer regions, conserved structural features in these regions have been examined, including sites which interact with protein factors. Despite a wide divergence in length and nucleotide composition, analyses of the internal transcribed spacers from widely divergent eukaryotes have suggested simple core structures that include a central extended hairpin (*22*, *23*). The extended hairpin structures also have been shown to interact with cellular proteins, and affinity chromatography, with the extended hairpins used as RNA ligands, has been used to identify a large protein complex of 20 or more polypeptides which interacts with the spacer sequences (*24*). Putatively called RAC (ribosome assembly chaperone), the complex appears to function as a chaperone for ribosome maturation, perhaps acting as a "rack" on which the critical structure is organized. Indeed, although the RAC complex itself does not have a nuclease activity, recent studies with the Pac1 RNase III-like endoribonuclease have shown that, in the presence of the RAC complex, the enzyme specifically cleaves the ribosomal RNA precursor at the 3′-end of the 25S rRNA sequence (*25*, *26*).

To further explore the role of the spacer regions in rRNA processing, including their interactions with RAC proteins, in this study, mutations have been systematically introduced into the ITS1 structure of the pre-rRNA transcripts in *S. pombe* and the effects of these changes on rRNA processing have been examined in vivo. The results raise interesting parallels with observations in the other spacer regions and also the possibility that, as observed in bacteria, the eukaryotic RNase III enzyme also may be involved in separating the large and small subunits in the course of ribosome maturation.

MATERIALS AND METHODS

Construction and Expression of Mutant rRNA Genes. As outlined in Figure 1, site-specific mutations were introduced into the 1TS1 sequence of a *S. pombe* rRNA transcriptional unit that had previously been subcloned (*27*) into the pFL20 yeast shuttle vector and "tagged" with a *Pst*I restriction site in the 18S rRNA sequence (*18*) and a four-base insert in the 5.8S rRNA (*28*). The base substitutions and some deletions were introduced by a two-step PCR amplification strategy (*29*) using a pTZ19R plasmid template containing the entire ITS1 intragenic region with adjacent 3′-end 18S and 5′-end 5.8S rRNA sequences. In some instances when deletions were not successful with this approach, mutations were introduced by "PEP" mutagensis (*30*) 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* rDNA 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 (*31*). Each mutation initially was confirmed by DNA sequencing (*32*), and subsequently, when required, the presence of mutant rDNA in transformed cells was confirmed again by PCR amplification of the plasmid-associated ITS1 rDNA region followed once more by DNA sequencing. Evidence of gene conversion was not observed.

Characterization of the Expressed Mutant Ribosomal RNAs. Transformed *S. pombe* cells expressing normal or mutant rRNAs were grown with constant aeration at 30 °C in minimal medium broth (*33*). For 5.8S rRNA analyses, whole cell low molecular weight RNA was prepared as described by Steele et al. (*34*, *35*). Cells were grown to an OD_{550nm} of 0.6 and harvested by centrifugation, and 0.5 g of cells was resuspended in 3 mL of SDS buffer (0.3% w/v SDS, 1.4 M NaCl, and 0.05 M sodium acetate, pH 5.1) and an equal volume of phenol solution (phenol containing 16% w/v *m*-cresol, 10% w/v water, and 0.1% w/v 8-hydroxyquinoline). The mixture was incubated at 65 °C for 20 min with intermittent shaking, the phases were separated by centrifugation, and the aqueous phase was extracted further with chloroform. The nucleic acids were precipitated overnight at −20 °C with 2.5 vol of ethanol, dissolved in water, fractionated on a 10% (v/w) polyacrylamide gel, and stained with methylene blue to detect the separated RNA components (*28*). To capture the images, gels were dried and scanned using an Astra 600P scanner (UMAX Technologies, Inc., Dallas, TX). The 5S rRNA and tRNAs also were examined to ensure equal gel loading. For 18S rRNA or precursor analyses, whole cell RNA was prepared using the protocol of Rose and co-workers (*36*). One hundred milliliters cultures, grown to an OD_{550nm} of 0.6, were chilled rapidly with crushed ice, harvested by centrifugation, and resuspended in 0.65 mL of ice-cold LETS buffer (0.1 M LiCl, 10 mM EDTA, 0.2% w/v SDS, and 10 mM Tris-HCl, pH 7.4). The cell slurry was transferred to a tube containing 2.75 g of 0.5 mm diameter acid-washed glass beads (Biospec Products, Inc., Bartlesville, OK) in 0.75 mL of LETS buffer-saturated phenol, and the cells were disrupted by vortexing (*36*) with alternate cycles of agitation at top speed for 30 s followed by incubation on ice for 30 s to a total of 6 min, or until the broken cells appeared as nonrefractile "ghosts". After the addition of a further 1.25 mL of ice-cold LETS buffer, the phases were separated by centrifugation, and the aqueous phase was extracted twice with 3 mL of phenol/chloroform/isoamyl alcohol (25:24:1). After precipitation with ethanol, the RNA was fractionated on a 0.8% agarose/0.2 M formaldehyde gel (*18*, *37*) and transferred to a nylon membrane (Microseparations, Inc., Westborough, MA) by overnight downward blotting (*37*), using 10× SSC (1.5 M NaCl and 0.15 M sodium citrate, pH 7.0). The membranes were baked at 80 °C for 1.5 h, rinsed with water, and stained with methylene blue to confirm that equivalent amounts of RNA were transferred. They were then hybridized for 16 h at 37 °C (50% formamide, 6× SSPE, 0.5% SDS, 5× Denhardt's solution, and 100 μg/mL salmon sperm DNA) with a ^{32}P-labeled oligomer probe specific for the tagged 18S rRNA transcribed from the plasmid-associated rDNA (*18*), washed twice for 10 min at room temperature in 2× SSPE and 0.1% SDS, and then washed again in 0.2× SSPE and 0.1% SDS before exposure to film. Images of the membranes or films again were captured using a scanner.

S1 Nuclease Protection Assay. For S1 protection mapping of termini, a single-strand RNA probe was prepared by in vitro transcription using T7 RNA polymerase (*38*, *39*). A DNA template for the ITS1 region was prepared by PCR amplification using the pTZ19R recombinant described in Figure 1 with a primer specific for the 3′-end region of the 18S rRNA sequence (CAAGGTTTCCGTAGGTG) and a universal primer upstream of the T7 promoter element (CAGGAAACAGCTATGAC). The 645 bp runoff transcript was purified using a 6% polyacrylamide sequencing gel and labeled at the 3′-end using [^{32}P]pCp and RNA ligase (*40*). Whole cell RNA was extracted as descibed above, and 10 μg was mixed with 100 000 cpm of labeled RNA probe in 20 μL of hybridization buffer (4 M NaCl, 1 mM EDTA, 40 mM PIPES, pH 6.4, and 80% formamide), heated to 90 °C for 10 min, and incubated for 16 h at 30 °C (*41*). After incubation, the solution was diluted with 200 μL of S1 digestion buffer (4.5 mM $ZnSO_4$, 280 mM NaCl, 50 mM NaAcO, pH 4.5, and 20 μg/mL single-stranded DNA) and digested for 1 h at 30 °C with 200−400 units of S1 nuclease. The digestion was terminated on ice with 80 μL of 4 M NH_4-AcO and 50 mM EDTA, pH 8.0, together with 50 μg/mL carrier RNA, before extraction with phenol/chloroform. Products of digestion were recovered by ethanol precipitation and analyzed on a 6% polyacrylamide sequencing gel, together with standard dideoxy sequencing reaction products (*32*) as fragment length markers.

Pac1 Ribonuclease Digestion. The *S. pombe* ITS1 RNA for Pac1 nuclease digestion analyses was prepared by in vitro transcription using T7 RNA polymerase (*38*, *39*). A DNA template for the ITS1 region was prepared by PCR amplification using primers specific for the 3′-end of the 18S rRNA (CAAGGTTTCCGTAGGTG) and the 5′-end of the 5.8S rRNA (TTTCGCTGGGTTCTTCATC) sequences (*22*). The 506 bp fragment was cloned in the pTZ19R plasmid, and the resulting plasmid was digested with *Eco*RI endonuclease for runoff transcription as previously described (*16*). The transcription reaction was performed with 0.4−2 μg of a DNA template in the presence of 2 mM ATP, CTP, GTP, and UTP and 100 units of T7 RNA polymerase using N4 buffer (1 mM spermidine, 0.01% Triton X-100, 20 mM $MgCl_2$, and 40 mM Tris-HCl, pH 8.1) in a final volume of 100 μL. After incubation at 37 °C for 4 h, the RNA was precipitated with 2.5 vol of cold-salted ethanol. The transcript was then purified on a 6% denaturing polyacrylamide gel and labeled at the 5′-end using bacteriophage T4 polynucleotide kinase and [γ-^{32}P]ATP (*42*), after dephosphorylation with calf intestinal phosphatase (*40*). The phosphatase was heat-inactivated for 10 min at 75 °C in the presence of 5 mM EDTA (pH 8.0) before the RNA was labeled, and the labeled RNA was again purified on a 6% denaturing polyacrylamide gel.

Pac1 RNase was prepared, and cleavage reactions were performed essentially as described by Rotondo and Frendewey (*43*). The in vitro synthesized and labeled RNA was digested with Pac1 RNase for 60 min at 30 °C in 30 μL of buffer containing 5 mM $MgCl_2$, 1 mM DTT, and 30 mM Tris-HCl, pH 8.1. Reactions were stopped with the addition of an equal volume of loading buffer (formamide containing 0.05% xylene cyanol and 0.05% bromophenol blue); the solution

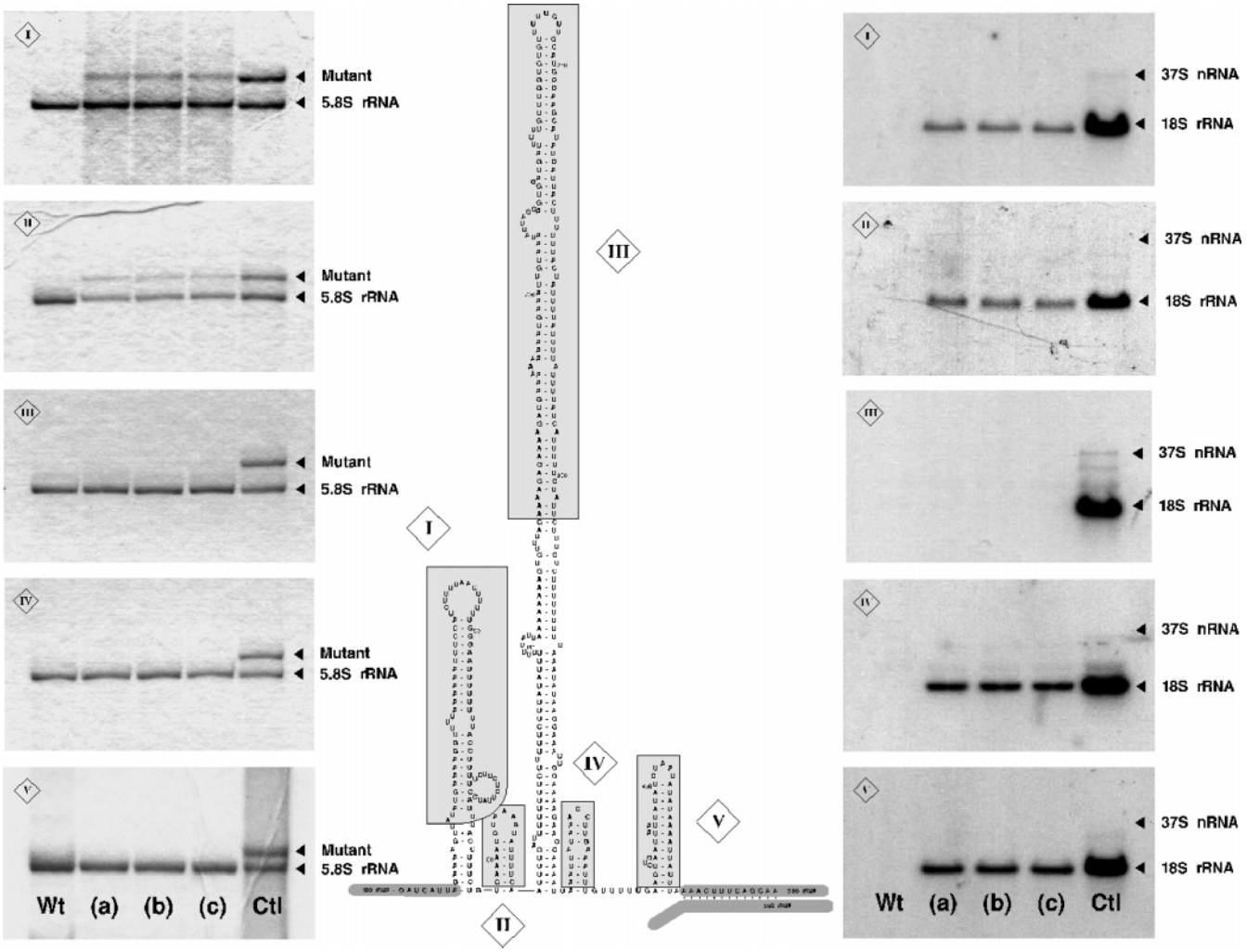

FIGURE 2: Effect of ITS1 structural deletions on the processing and stability of the *S. pombe* pre-RNA. Whole cell RNA was extracted from untransformed *S. pombe* cells (Wt), transformed cells expressing normal "tagged" rDNA (Ctl), or three transformants (a−c) expressing tagged rDNA containing structural deletions in the ITS1 sequence (I−V) as indicated with light shading. Aliquots of RNA were fractionated on a 10% polyacrylamide sequencing gel for direct 5.8S rRNA analyses (left panels) or on a 0.8% (w/v) agarose gel and transferred to nylon for hybridization analyses (right panels). The 5.8S rRNA was detected directly with methylene blue stain; the positions of the normal (5.8S rRNA) and plasmid-derived RNAs (Mutant) are indicated on the right. A ^{32}P-labeled oligomer probe, specific for the plasmid-derived tagged 18S rRNA, as described previously by Good et al. (*18*), was used for precursor analyses; the position of the mature 18S, as well as the 37S nucleolar transcript, is indicated on the right.

was heated for 2 min at 90 °C, and 5 μL aliquots were applied directly to a 6% (19:1; acrylamide/bisacrylamide) polyacrylamide gel containing 8.3 M urea. RNA partially digested with T1 ribonuclease or hydrolyzed with base (*22*) was also applied as length markers; after fractionation, the fragments were detected by autoradiography.

RESULTS

Although they contain large differences in their lengths and nucleotide composition, comparative studies on the ITS1 regions in rRNA precursors of divergent origins have identified core structural features which are present in all known examples (*22*). These features include a central extended hairpin structure (III in Figure 2) and smaller hairpins immediately adjacent to the maturing termini (I and V in Figure 2). Other structures may be present (e.g., hairpins II and IV in Figure 2), but these are not conserved and contribute to the large differences in the size and composition of the ITS1 regions. Similarly, central extended hairpin structures appear also to be common features in ITS2 regions (*23*) and even the 3′ETS (*15*). As noted earlier, at least in *S. pombe*, past studies have indicated that all of these extended hairpins interact with a common protein complex, RAC (*16*, *24*, *44*). To further evaluate the relative importance of the common structural elements in the ITS1 region of *S. pombe* pre-rRNAs, initially in this study, each of the hairpin structures in the ITS1 region was deleted systematically. As indicated in Figure 1, a "PEP" mutagenesis strategy (*30*) was used to avoid complications commonly observed with PCR-based mutagenesis in highly repeated sequence elements as are found in the ITS1 structures. Each mutated ITS1 sequence was substituted for the normal sequence in a tagged rDNA transcriptional unit which subsequently was used to transform *S. pombe* cells using a high copy number yeast shuttle vector (*27*, *28*). Under these conditions, the plasmid-derived transcripts must compete with the genomic transcripts for proteins and other factors, a condition which emphasizes any effect on rRNA processing. As observed with spacer mutations in past studies (e.g., *10*, *12*, *13*) and consistent with a surplus capacity for rRNA production, the growth rates of

cells expressing mutant genes were essentially unaffected with the doubling times ranging from 4.2 to 4.3 h.

When rDNAs containing the mutations were expressed in vivo and the amount of plasmid-derived 5.8S rRNA was determined after fractionation by polyacrylamide gel electrophoresis (Figure 2, left panels), the effects of specific mutations were found to vary greatly. In control cells that were transformed with rDNA containing the normal ITS1 sequence, about 50% of the total 5.8S rRNA was found to be plasmid-derived (Ctl). In cells which were not transformed with plasmid (Wt), no mutant RNA was evident and only one band corresponding to the untagged RNA was present (Wt). To ensure a reproducible conclusion, at least three separate transformants were examined for each mutation (lanes a−c). As observed previously (*24*) with a complete deletion of the central extended hairpin (III), with only a deletion of the upper half (mutant III), again no plasmid-derived 5.8S rRNA was evident. This also was true when hairpin IV or V was removed. In contrast, some plasmid-derived 5.8S rRNA was evident when most of hairpin I or all of hairpin II was removed. In these instances, however, levels of plasmid-derived RNA where reduced to about 20% of normal.

The results also differed significantly when levels of a plasmid-derived 18S rRNA were assessed by hybridization analyses. As shown in Figure 2 (right panels), when an oligonucleotide probe that was specific for the neutral tag in the plasmid-derived 18S rRNA (*18*) was used with RNA from cells transformed with the normal ITS1 sequence (Ctl), a strong signal corresponded to the 18S rRNA, with additional minor signals corresponding to the precursor molecules. As expected, untransformed cells contained no RNAs, which were homologous with the oligonucleotide probe (Wt). With four of the deletion mutations (I, II, IV, and V) the effects on 18S rRNA production were similar. In each case, the level of the mature 18S rRNA was reduced to about 20% of normal, and correspondingly, elevated levels of precursor molecules were not evident. With a deleted central stem sequence (mutant III), however, no plasmid-derived 18S rRNA precursor was evident; the central stem clearly had an equally critical effect on all rRNA production.

With the exception of mutant III, the presence of some plasmid-derived 18S rRNA indicated that none of the other structures were critical to subunit RNA separation, although, clearly, the mutations did affect the yield of the mature RNAs. Since the 18S rRNA-specific probe also would detect all precursors to this ribosomal RNA, intensified precursor bands might be anticipated. The lack of any elevated precursor levels strongly suggested that the reduced levels of plasmid-derived mature ribosomal RNAs were not the result of inefficient or delayed processing. Rather, they were likely the result of precursor degradation. With reduced levels of the 5.8 S rRNA, however, some elevated intermediate may not have been visible because the hybridization probe was specific for the plasmid-derived 18S rRNA sequence and would not be expected to detect intermediates such as the 27S pre-rRNA containing only the 5.8S and 25S rRNA sequences. To exclude the possibility that such precursors were overlooked with mutations IV and V, these deletions were reexamined using an ITS2-specific probe. As shown in Figure 3, the conclusions remained unchanged; no elevated

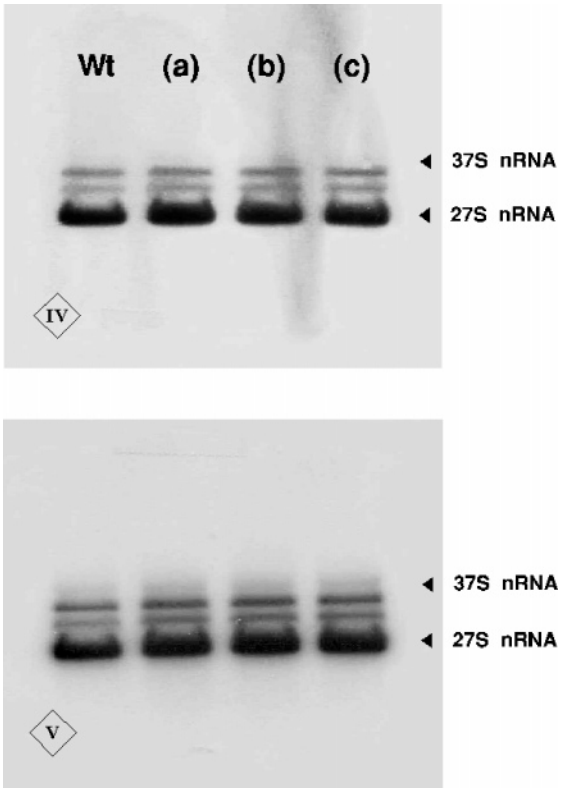

FIGURE 3: Effect of ITS1 structural deletions on the processing of the ITS2 region in the pre-rRNA of *S. pombe*. Whole cell RNA was extracted from untransformed *S. pombe* cells (Wt) or three transformants (a−c) expressing rDNA containing structural deletions in the ITS1 sequence (IV and V) as described in Figure 2. Aliquots of RNA were fractionated on a 0.8% (w/v) agarose gel and transferred to nylon for hybridization analyses. A ^{32}P-labeled ITS2 DNA fragment was used as a probe for precursor analyses; the positions of the 37S nucleolar transcript and 27S nucleolar intermediates are indicated on the right.

levels of the shorter intermediates were evident. Again, it was clear that substantial amounts of plasmid-derived precursors were rapidly degraded and were not present as elevated levels of intermediate precursors. The absence of mature plasmid-derived 5.8S rRNA (Figure 2) with mutants IV and V again appeared to result more from precursor instability than a block in a specific cleavage step. However, since unlike mutants I and II, no mature 5.8S rRNA was observed, blockage in 5.8S rRNA maturation in addition to rapid degradation could not be excluded.

Previous, more limited studies on RAC protein binding sites, based on modification exclusion, indicated that at least the primary site was localized in the upper region of the central extended hairpin (see Figure 1). To more precisely identify the essential structural features, substitutions were introduced into the binding region as well as above and below it. As shown in Figure 4, when expressed in vivo, the effects of these mutations on rRNA production again varied widely depending on their position or the structural change they induced. Changes which disrupted the helix (mutants II and IV), fully inhibited rRNA maturation as observed with a deleted stem (see mutant III in Figure 2). Plasmid-derived 5.8S or 18Ss rRNA could not be detected, and there was no elevation in the precursor levels. In strong contrast, when the base substitutions altered the sequence without disrupting the secondary structure (see mutant III in Figure 4), normal amounts of plasmid-derived 5.8S and 18S rRNAs clearly

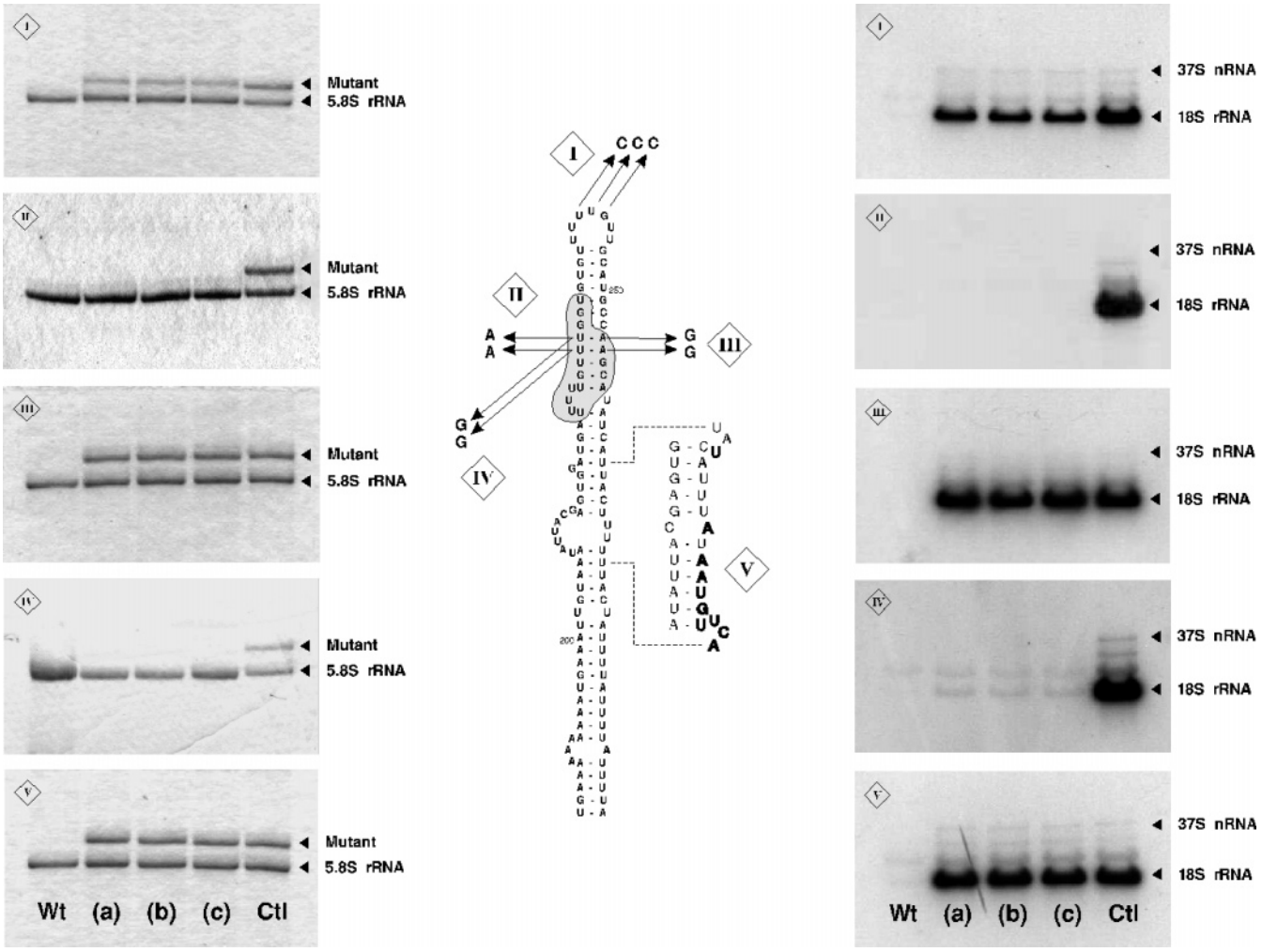

FIGURE 4: Effect of RAC binding site mutations on the processing and stability of the *S. pombe* pre-RNA. Whole cell RNA was extracted from untransformed *S. pombe* cells (Wt), transformed cells expressing normal "tagged" rDNA (Ctl), or three transformants (a−c) expressing tagged rDNA containing nucleotide substitutions in the ITS1 sequence (I−V) as indicated in the predicted structure. Aliquots of RNA were fractionated on a 10% polyacrylamide sequencing gel for direct 5.8S rRNA analyses (left panels) or on a 0.8% (w/v) agarose gel and transferred to nylon for hybridization analyses (right panels) as described in Figure 2. The 5.8S rRNA was detected directly with methylene blue stain; the positions of the normal (5.8S rRNA) and plasmid-derived RNAs (Mutant) are indicated on the right. A ^{32}P-labeled oligomer probe, specific for the plasmid-derived tagged 18S rRNA, was used for precursor analyses; the position of the mature 18S rRNA, as well as the 37S nucleolar transcript, is indicated at the right. The RAC protein complex binding site, as previously determined (*24*), is indicated with light shading.

were evident. Base substitutions in the terminal loop (see mutant I in Figure 4) had similar but much more modest effects on both 5.8S and 18Ss rRNA production. Base substitutions below the RAC protein binding site, even when they resulted in an elongated central extended hairpin and moved the binding site relative to the maturing termini, had essentially no effect. As also shown in Figure 4 (mutant V), in this case, levels of plasmid-derived 5.8S and 18S rRNA remained unchanged when compared with a normal plasmid sequence (Ctl).

In vitro studies on rRNA processing in the 3′ETS of *S. pombe* have indicated that the RNase III-like Pac1 nuclease cleaves the extended hairpin structure at two known intermediate processing sites (*43*, *45*). More recent studies also indicate that, in the presence of the RAC protein complex, the same enzyme completely removes the 3′ETS by recognizing and cleaving a helical structure at the 3′-end of the mature 25S rRNA (*25*). In bacteria, helices formed between the termini of the mature RNAs also are cleaved by RNase III as an initial step in rRNA maturation (*46*, *47*). Because hairpin structure appears to be conserved at the 3′-end of the 18S rRNA and deletion I (shown in Figure 2) did not remove the actual junction area, base substitutions were also introduced into this region to disrupt the terminal helical structure. As shown in Figure 5A, when *S. pombe* cells were transformed with such a mutant sequence, subsequent RNA analyses revealed surprisingly dramatic effects on rRNA maturation. Low molecular weight RNA analyses Figure 5B indicated very little or no plasmid-derived 5.8S RNA was formed, and hybridization analyses Figure 5C indicated only trace amounts of plasmid-derived 18S rRNA were present. Equally important, an elevated level of precursor was present corresponding not to nascent transcripts but to an intermediate precursor lacking the 5′- and 3′ETS regions. Such elevations were also previously observed with mutations in ITS2 and the 3′ETS (*15*, *44*). To ensure equal RNA loading and to eliminate any possibility that differences in the RNA profiles were artifacts of RNA transfer, in all hybridization analyses, the membrane was stained with methylene blue (D) prior to incubation with oligonucleotide probe. As shown

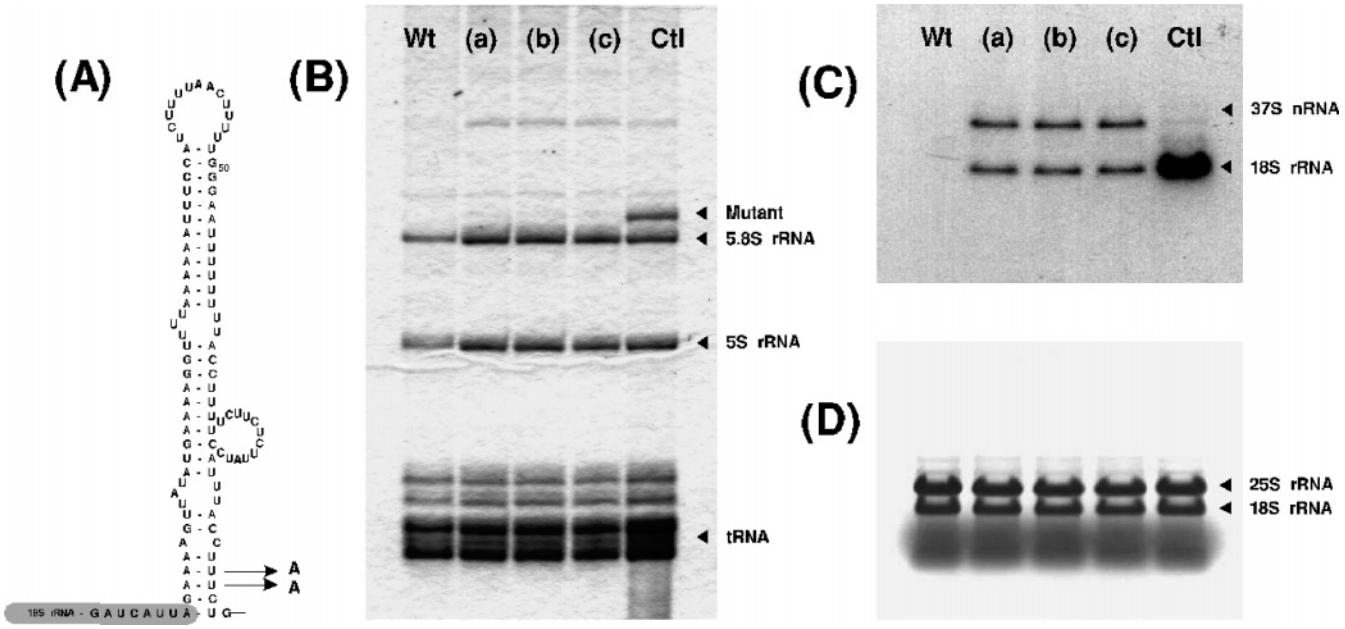

FIGURE 5: Effect of the 5′-end ITS1 junction structure on the processing and stability of the *S. pombe* pre-RNA. Whole cell RNA was extracted from untransformed *S. pombe* cells (Wt), transformed cells expressing normal "tagged" rDNA (Ctl), or three transformants (a−c) expressing tagged rDNA containing a disrupted junction structure as indicated in the predicted structure (A). Aliquots of RNA were fractionated on a 10% polyacrylamide sequencing gel for direct 5.8S rRNA analyses (B) or a 0.8% (w/v) agarose gel and transferred to nylon for hybridization analyses (C and D) as described in Figure 2. The 5.8S rRNA was detected directly with methylene blue stain; the positions of the normal (5.8S rRNA) and plasmid-derived RNAs (Mutant) are indicated on the right. A ^{32}P-labeled oligomer probe, specific for the plasmid-derived tagged 18S rRNA, was used for precursor analyses; the positions of the mature 18S rRNA as well as the 37S nucleolar transcript are indicated at the right. The positions of the mature 18S and 25S rRNAs, as initially detected by methylene blue stain (D), also are indicated at the right.

in Figure 5D, the RNA loading was uniform and clearly not the reason for the changes that were observed with this mutant.

The results shown in Figure 5 raise an intriguing question regarding the separation of the subunit RNAs. Past reports on pathways for pre-rRNA in eukaryotic cells generally have indicated that the separation begins with a cleavage within the central region of the ITS1 sequence. Indeed, a study on the rRNA maturation pathway in *S. pombe* (*48*), based on pancreatic/T1 ribonuclease protection studies and primer extension analyses, indicated several internal cleavages in ITS1, a cleavage (A3) in the central extended hairpin, and a somewhat stronger cleavage (A2) about 85 nucleotides from the 3′-end of the 18S rRNA as well as several short extensions to the 5′-end of the 5.8S rRNA. Since all of these cleavage sites appeared significant with respect to the present mutational analyses and only restriction DNA fragments markers had been used to approximate their positions, in this study, a more accurate localization of the A2 site was undertaken using S1 mapping together with sequencing reaction fragments as nucleotide markers. As shown in Figure 6, the new results generally were consistent with the previous report, accurately localizing the A2 and drawing attention to one additional area of cleavage. In Figure 6, the 3′-end of the 18S rRNA is clearly evident, and the A2 cleavage site is more accurately mapped at nucleotide A_{83}. As anticipated, the A3 site was not observed, since this cleavage occurs after the A2 cleavage has released the distal portion of the ITS1 sequence. The additional putative terminal ending at A_{24} was more diffuse in the previous pancreatic/T1 nuclease digestion map (*48*) and, as it also was less separated on a low percentage gel, was thought to be the result of incomplete digestion with respect to the 3′-end of the 18S rRNA. With the higher level of resolution, in this study, it represented a separately mapped site.

Given the influence of the terminal structure on ITS1 processing (Figure 5) and the similarity with intermediate termini in the 3′ETS region, a further possibility was examined. As noted earlier, in bacteria, RNase III is known to initiate the processing of the pre-rRNA, and in vitro studies with the 3′ETS sequence of *S. pombe* cells have shown that the RNase III-like Pac1 nuclease cleaves the conserved extended hairpin structure at two known intermediate cleavage sites (*43*, *45*) and completely releases the 3′ETS sequence in the presence of the RAC protein complex (*25*). Since the structure and the mapped termini at the 3′-end of the 18S rRNA seemed similar, the ITS1 sequence also was subjected to Pac1 nuclease cleavage. As shown in Figure 7, the results again resembled those that were previously obtained with the 3′ETS RNA. A single specific site of cleavage at A_{24} corresponded exactly with the additional putative terminal mapped in Figure 6. As observed with Pac1 cleavages in the 3′ETS (*26*), other minor cleavages were noted at different enzyme concentrations, but the specificity for A_{24} was outstanding.

DISCUSSION

Taken together, the mutational analyses in the present study indicate that the ITS1 spacer region in *S. pombe* cells contains both cis-acting elements, which are critical to rRNA maturation, and general structural features, which affect the stability of the precursor molecules and, in turn, can dramatically affect the yield of the mature RNAs. For example, in the presence of normal rRNA transcripts, the deletion of hairpin II (see Figure 2) reduced the yield of both

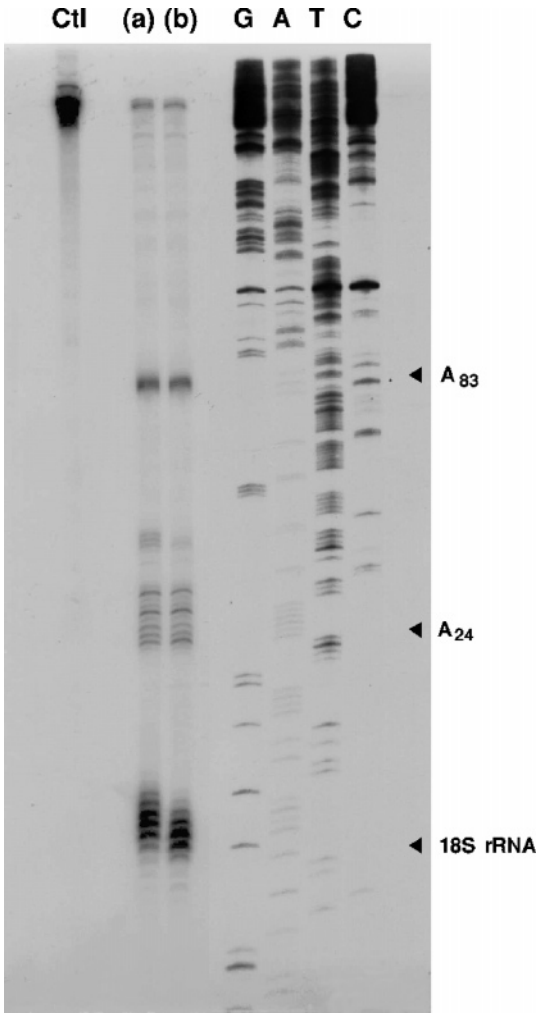

FIGURE 6: S1 nuclease protection analysis of termini in the ITS1 region of the *S. pombe* pre-rRNA. The termini associated with the 3′-end region of 18S rRNA were mapped using a single-stranded RNA probe which was prepared in vitro by T7 transcription using an *Eco*RI−*Eco*RI clone of this gene region and labeled at the 3′-end. Total cellular *S. pombe* RNA was prepared and hybridized with 250 000 cpm of probe RNA and then digested with the addition of 200 (a) or 400 (b) μg/mL S1 nuclease. Control lanes were without nuclease and without RNA. The reactions were fractionated on a standard 6% poylacrylamide/TBE sequencing gel. A control lane (Ctl) without nuclease was included together with standard dideoxy sequencing reaction (*23*) mixtures (G, A, T, and C) as chain length markers. The positions of the major termini are indicated on the right.

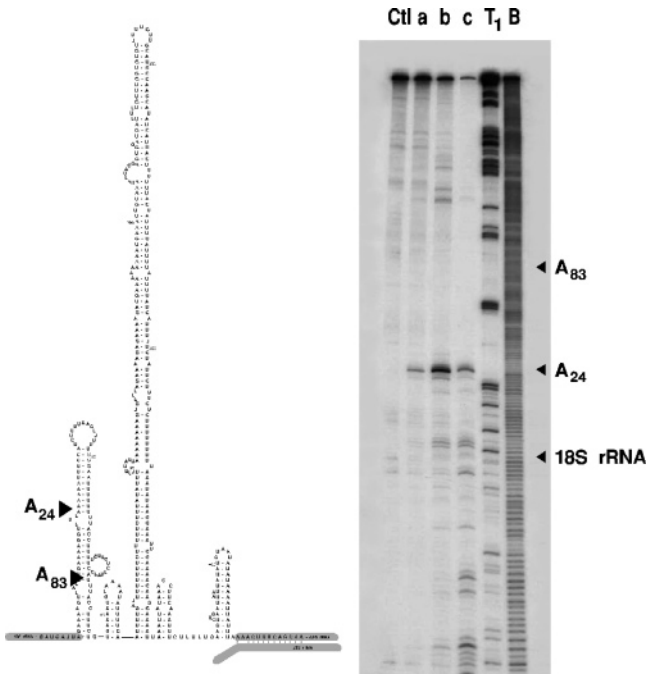

FIGURE 7: Pac1 RNase cleavage of a 5′-labeled ITS1 substrate from the *S. pombe* pre-rRNA. An ITS1 substrate was prepared in vitro by transcription with T7 RNA polymerase, labeled at the 5′-end (Ctl), and aliquots (10 000 cpm) were digested with 0.4 (a), 1.2 (b), or 3.6 (c) ng of Pac1 RNase. Digests were fractionated on a 6% denaturing polyacrylamide gel, and the fragments were detected by autoradiography. Labeled substrate, which was partially digested with T_1 ribonuclease (T_1) or mild base (B), was included as nucleotide markers. The major known termini as determined previously (*48*) or shown in Figure 6 are indicated with arrowheads.

the 5.8S and 18S rRNAs by 80%. As indicated earlier, past studies in our laboratory on rRNA maturation in *S. pombe* suggested that rRNA processing, at least in part, represents a quality control mechanism to exclude mutant RNAs from mature ribosomes. The new observations provide further evidence for this notion. All the ITS1 deletion mutants resulted in greatly reduced levels of mature RNA or even eliminated plasmid-derived RNAs entirely.

Since recognized more than 4 decades ago, the role of the transcribed nonconserved spacers in the pre-rRNA of eukaryotic cells and even the need for rRNA processing have been the subject of much speculation (see ref *21*). Despite the wide variation in size, composition, and structure among the spacer regions of the many divergent organisms which have been examined, studies in recent years (*11*−*14*, *49*−*51*) have indicated conclusively that important cis-acting elements are present within these spacers which either are critical to rRNA processing or which dramatically affect the efficiency of rRNA processing. Most functional studies on the ITS1 region have been limited to *S. cerevisiae*, where extensive studies by Planta, Raue, and co-workers have mapped both the sites of cleavage and a number of regions which are important for these cleavages (*11*−*14*, *49*−*51*). In parallel, studies in many laboratories have identified numerous trans-acting factors which are equally critical to the cleavages or ribosomes biogenesis, in general (*3*, *4*). For most steps, specific requirements have not been defined, but in some instances, absolute requirements are becoming apparent. For example, in a recent study (*52*), two RIO proteins were shown to be absolutely required for the maturation of the 20S pre-rRNA in *S. cerevisiae*. A large ribonucleoprotein complex, the small subunit processome, also has been shown to be required for the biogenesis of the 18S rRNA (53). This contains the U3 snoRNA and at least 40 proteins including 5 small ribosomal subunit proteins. Despite such extensive study, the removal of the ITS1 sequence is not well-understood, and even major aspects of the enzymology remain unclear. Roles for the Xrn1p 5′−3′ exonuclease (*54*, *55*) and for RNase MRP (*56*, *57*) with respect to trimming or degradation steps have been established, but the initial cleavage, which separates the subunits, or that which forms the mature 3′-end of the 18 S rRNA remains unclear.

As indicated earlier, recent studies on the 3′ ETS and its release in both *S. cerevisiae* and *S. pombe* have established the need for an RNase III-like endonuclease (*58*, *59*) and at least one major conserved cis-acting structural element, an

extended hairpin structure at the 3′-end of the mature 25 S rRNA sequence (*15*, *59*). In both yeasts, this structural element is essential, not only for the removal of the 3′ETS but even for the processing of the ITS2 region which is located more than 3000 base pairs upstream. At least in *S. pombe*, these events appear to be mediated by the RAC protein complex (*24*), which has been shown to bind specifically to the upper region of the conserved hairpin structure and, at least in vitro, to direct the complete removal of the 3′ETS by the Pac1 RNase III-like endonuclease (*25*).

We believe the present study on the ITS1 sequence raises interesting parallels with the processing of the 3′ETS. While all regions of the ITS1 sequence appear to play a role in precursor stability and efficient rRNA processing, Figures 2−5 indicate that in *S. pombe* two structural features are critical to RNA cleavage as indicated by the absence of mature plasmid-derived RNA or elevated levels of precursor RNA. This includes the upper helical region in the central extended hairpin structure where changes can eliminate plasmid-derived rRNA entirely (Figure 4) and has been shown to interact with the RAC protein complex (*24*). The other is the helical structure, which immediately follows the 3′-end of the mature 18S rRNA sequence. In the second case, a change can substantially elevate levels of precursor RNA (Figure 5). As was the case in the 3′ETS, protein binding to the central extended hairpin structure appears to direct cleavage at the 3′-end of the mature 18S rRNA sequence, which is essential not only for the maturation of the 18S rRNA but also for the subsequent maturation of the 5.8S rRNA.

In general, on the basis of the numerous studies of Planta and co-workers (*11*, *13*, *14*, *51*), Raue suggests (*5*) that, in *S. cerevisiae*, the critical cis-acting elements of ITS1 are predominately confined to the immediate neighborhood of the cleavage sites and that they are recognized directly by the processing nucleases. He also concludes that maturation at the 3′-end of the 18S rRNA probably requires no more than the adjacent six spacer nucleotides and that most of the remaining spacer can be deleted without significantly affecting the production of the mature rRNA species. Two of three highly conserved sequence elements were reported to have an influence on the known intermediate cleavage sites, and in general, the ITS1 appeared organized into two functionally and structurally distinct halves (*13*). Initially, many of the observations in the present study appeared to contradict the previous results in *S. cerevisiae*, but a more careful examination leads us to suggest that they result from differences in the experimental approaches as well as real differences between the organisms such as the positions of intermediate cleavage sites and even the cellular location of the cleavage event. As noted earlier (*44*), unlike the present studies in *S. pombe*, most of the studies in *S. cerevisiae* were based on the use of a low copy number shuttle plasmid with mutant transcripts being present in very small amounts and only detectable by sensitive hybridization assays. Under these conditions, the assays are likely to be largely qualitative, and since plasmid-derived transcripts represent only a very small portion of the total rRNA population, the very minor population of mutant transcripts does not utilize a significant portion of the processing machinery or have effects on it. In contrast, 50−60% of transcripts were plasmid-derived in our studies in *S. pombe*, and the quantitative effects of the mutations were clearly evident. In the case of the 5.8S rRNA, the actual amounts of RNA could be determined by direct staining. More important, in *S. cerevisiae*, unlike *S. pombe* or the higher eukaryotes, the 18S rRNA finally matures in the cytoplasm, probably relying on alternative cytoplasmic nuclease activity. Changes in the central extended stem of the ITS1 sequence in *S. cerevisiae*, which is adjacent to a cleaved site (A2) that releases the nucleolar pre 18S rRNA, also correspond to a region which could be considered analogous to the protein binding site in *S. pombe*. It also is possible to estimate other stem−loop structures in which the six nucleotides adjacent to the 3′-end of the mature 18S rRNA sequence pair with more internal ITS1 sequences, and so changes in these nucleotides could disrupt the stem as observed in *S. pombe* (Figure 5). Since the 3′-end of the 18S rRNA of *S. cerevisiae* is known to mature in the cytoplasm, the difference in this case also may actually be species-specific. Deletions I and II (Figure 2) did have much less effect on the production of the 5.8S rRNA, so as observed in *S. cerevisiae* (*13*), to some extent, this ITS1 also is organized in two functional halves. Whatever the case, the conclusions in either organism cannot be considered final or universal, and further comparative analyses will be essential to resolve the present possibilities.

Although RNase III is an important enzyme in the initial separation of the subunit RNAs of *E. coli* (*46*, *47*), in general, previous studies in eukaryotic examples have not linked RNase III-like activities with ITS1 processing. Genetic analyses indicate that a deletion of the RNase III gene (Rnt1p) in *S. cerevisiae* does not block ITS1 cleavage, although cleavages in the 5′ETS and ITS1 (A0, A1, and A2) are significantly delayed (*59*). This delay has been interpreted as an indirect effect. In contrast, the present data, particularly Figure 7, raise a different possibility. Although studies in *E. coli* have clearly documented the primary role of the RNase III enzyme in the processing of the 16S and 23S rRNAs from the primary 30S precursor (*46*, *47*), they also have documented redundancies in the rRNA processing mechanisms, and deletions in the gene encoding RNase III (rnc) have led not to cell death but to an accumulation of 30 S rRNA precursors (*60*). Since this observation is really very similar to those which, more recently, were made in *S. cerevisiae* (*59*), it raises the possibility that the yeast RNase III is equally important in the processing of the eukaryotic rRNAs. As a result of the present studies, both the requirement for a stem−loop structure (Figure 5) and the observed specificity of the Pac1, RNase III-like enzyme, in vitro (Figure 7), provide actual evidence for such a possibility. The RNase III-like enzymes in the yeasts could again have a primary role, but redundancies in the processing mechanisms may result in the delayed phenotype rather than cell death. Indeed, evidence for alternate pathways for processing in the ITS1 region of the pre-rRNA in *S. cerevisiae* has been reported previously. For example, when one of the processing sites (A2) is impaired by mutation, separation of the subunit rRNAs occurs further downstream (*61*), and yet, another novel site, A4, recently was demonstrated with a mutation in the gene encoding the RRP5 protein factor (*62*). Further genetic studies to isolate any redundant pathways should clarify this possibility in *S. pombe*.

In summary, therefore, the present study not only documents critical cis-acting elements in the processing of the

ITS1 region in the *S. pombe* pre-rRNA but also raises the possibility of important parallels with the processing of the 3′ETS and even the bacterial homologues. Clearly, further study will be required to fully establish any role of the yeast RNase III in ITS1 processing and perhaps the influence of trans-acting factors such as the RAC proteins. In the interim, the current observations may provide the first hint as to the actual enzymology which initiates the separation of the subunit RNAs and also a working model for ITS1 processing in *S. pombe* and perhaps all eukaryotic cells.

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