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The structure of the ITS2-proximal stem is required for pre-rRNA processing in yeast

BRENDA A. PECULIS¹ and CHRIS L. GREER²

¹National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Genetics and Biochemistry Branch, Bethesda, Maryland 20892, USA

²Department of Biological Chemistry, College of Medicine, University of California at Irvine, Irvine, California 92697, USA

ABSTRACT

Accurate and efficient processing of pre-rRNA is critical to the accumulation of mature functional ribosomal subunits for maintenance of cell growth. Processing requires numerous factors which act *in trans* as well as RNA sequence/structural elements which function *in cis*. To examine the latter, we have used directed mutagenesis and expression of mutated pre-rRNAs in yeast. Specifically, we tested requirements for formation of an ITS2-proximal stem on processing, a structure formed by an interaction between sequences corresponding to the 3' end of 5.8S rRNA and the 5' end of 25S. Pre-rRNA processing is inhibited in templates encoding mutations that prevent the formation of the ITS2-proximal stem. Compensatory, double mutations, which alter the sequence of this region but restore the structure of the stem, also restore processing, although at lower efficiency. This reduction in efficiency is reflected in decreased levels of mature 5.8S and 25S rRNA and increased levels of 35S pre-rRNA and certain processing intermediates. This phenotype is reminiscent of the biochemical depletion of U8 snoRNA in vertebrates for which the ITS2-proximal stem has been proposed as a potential site for interaction with U8 RNP. Thus, formation of the ITS2-proximal stem may be a requirement common to yeast and vertebrate pre-rRNA processing.

Keywords: ITS2; nucleolus; processing; rRNA; snoRNA; *Xenopus*; yeast

INTRODUCTION

Ribosome biogenesis in eukaryotes involves ribosomal proteins, nonribosomal proteins and a large number of snoRNAs which facilitate the cleavage, modification, folding, and assembly of the pre-rRNA transcript (reviewed in Hadjiolov, 1985; Hill, 1990; Maxwell & Fournier, 1995; Tollervey & Kiss, 1997). In this complex processing pathway the single RNA polymerase I (Pol I) transcript is converted into three of the functional rRNAs (17/18S, 5.8S and 25/28S rRNAs) found in mature ribosomes via a series of cleavage reactions (summarized in Fig. 1 for yeast). Despite the identification of numerous *trans*-acting factors, many features of the mechanism for ribosome biogenesis, including *cis*-acting elements, remain poorly understood. The secondary structure of rRNA is remarkably conserved across evolution; the central core of the ribosome is little changed between Eubacteria, Archea and Eukaryotes (Gutell et al., 1993). Still, very little is known about

the dynamics of rRNA folding or structure formation and how these may affect rRNA processing and ribosome biogenesis. In this work, we focus on a conserved interaction between 5.8S and 25S rRNA that we show is essential for processing.

Pre-rRNA processing involves a large cast of *trans*-acting factors. The ribosomal proteins constitute one large group of such factors shown to be essential for ribosome biogenesis in yeast (Rotenberg et al., 1988; Moritz et al., 1990; Synetos et al., 1992; Alksne & Warner, 1993). In addition, the past 5–10 years have witnessed the identification of a large number of small nucleolar RNAs (snoRNAs). Because of their cellular localization they were believed to play a role in ribosome biogenesis. However, the range of roles and the extent to which they affect ribosome biogenesis was unexpected (reviewed in Bachellerie et al., 1995; Maxwell & Fournier, 1995; Peculis & Mount, 1996; Peculis, 1997a; Tollervey & Kiss, 1997).

Nearly all snoRNAs can be classified into one of two groups, distinguished by the role(s) they play in ribosome biogenesis. The first group contains a very large and diverse set of snoRNAs involved in modification of the rRNA, specifically methylation and pseudouridylation.

Reprint requests to: Brenda A. Peculis, NIH/NIDDK/GBB, 10 Center Drive, MSC 1766, Building 10, Room 8N322, Bethesda, Maryland 20892-1766, USA; e-mail: bp51h@nih.gov.

FIGURE 1. Pre-rRNA processing scheme in yeast. Schematic of the genomic organization of rDNA and processing pathways of the pre-rRNA transcript. The full-length precursor in yeast, 35S, is processed by alternative coexisting pathways, utilizing a variety of processing sites (denoted by gray letters and thin arrowheads on the precursor, 35S). Some of the first cleavages separate 18S rRNA from the 5.8S/25S. Subsequent cleavages within ITS2 yield mature 5.8S and 25S and are shown by the heavy lines with arrows. The sequence and structural requirements for these ITS2 processing events are focused upon in this work. ITS: Internal Transcribed Spacer.

ation (reviewed in Bachellerie et al., 1995; Peculis & Mount, 1996; Peculis, 1997a; Tollervey & Kiss, 1997). Curiously, while the nucleotide position of many of these modifications is conserved across evolution (Ofengand et al., 1995), neither the snoRNAs that direct these modifications, nor the modifications themselves appears to be essential for rRNA processing or cell viability in yeast (Ofengand et al., 1995; reviewed in Maxwell & Fournier, 1995; Tollervey & Kiss, 1997).

SnoRNAs that direct modification can be further divided into two classes based on both conserved sequence elements within the snoRNA and their functional role in vivo. The first class includes those C/D box snoRNAs responsible for site-specific methylation of rRNA (reviewed in Maxwell & Fournier, 1995; Tollervey & Kiss, 1997). Modification sites are specified by a region of 10 to 21 nt in the snoRNA complementary to a site within rRNA. The second class of modification-directing snoRNAs is the H/ACA group responsible for site-specific pseudouridylation of rRNA (Ganot et al., 1997; Ni et al., 1997; and reviewed in Peculis, 1997a; Tollervey & Kiss, 1997). For this class, the site of modification is thought to be directed by formation of a base-paired snoRNA/pre-rRNA structure called the pseudouridylation pocket.

In contrast to the modification-directing snoRNAs, the second group of snoRNAs are involved in cleavage events and play more critical roles in pre-rRNA processing; in yeast, several are essential for viability. Deletion or depletion of these snoRNAs inhibits or affects pre-rRNA processing (reviewed in Maxwell & Fournier, 1995; Tollervey & Kiss, 1997). This group of snoRNAs includes MRP RNA, U3, U14, U8, U22, snR10 and snR30.

Other snoRNAs implicated in cleavage events are U3 and U14, two nearly ubiquitous snoRNAs that are re-

quired for accumulation of mature 18S rRNA (reviewed in Maxwell & Fournier, 1995; Tollervey & Kiss, 1997). U3 is essential for processing at a site within the 5' ETS (Hughes & Ares, 1991; Beltrame & Tollervey, 1992) and maintains the ability to base pair to a site within the 5' ETS despite phylogenetic sequence variation (Beltrame & Tollervey, 1992; Beltrame et al., 1994). Depletion of U3, whether genetically in yeast (Beltrame & Tollervey, 1992; Beltrame et al., 1994), or biochemically in *Xenopus* oocytes (Savino & Gerbi, 1991), results in an underaccumulation or complete absence of mature 17S/18S rRNA. U14 has also been shown to be essential for cleavages that result in the accumulation of mature 17S/18S rRNA (Jarmolowski et al., 1990; Li et al., 1990).

Other snoRNAs implicated in 17S/18S processing are less well characterized, having been identified in either vertebrates or yeast, but not both. Depletion of U22 in *Xenopus* (Tycowski et al., 1994), or of snR10 or snR30 in yeast (Tollervey, 1987; Girard et al., 1992; Morrissey & Tollervey, 1993; Venema & Tollervey, 1996) affects accumulation of mature 17S/18S rRNA. The mechanisms by which these snoRNAs function are not well defined.

U8 snoRNA has been shown to be essential for pre-rRNA processing in vertebrates (Peculis & Steitz, 1993). To date it is the only snoRNA shown to affect processing of the large subunit ribosomal RNAs. Biochemical depletion of U8 snoRNA in *Xenopus* oocytes results in a lack of mature 5.8S and 28S rRNAs (Peculis & Steitz, 1993).

Recently, a model for the role of U8 snoRNA in pre-rRNA processing in *Xenopus* was proposed (Peculis, 1997b). In this model, the 5' end of U8 base pairs to the 5' end of 28S in the pre-rRNA. This interaction is proposed to be transient, serving to facilitate a subsequent, stable base pairing interaction between 28S and 5.8S rRNA sequences to form the ITS2-proximal stem. The

ITS2-proximal stem, a near perfect 15-base-pair helix in *Xenopus*, is the longest of the 3 base paired interactions between 5.8S and 28S.

This model for U8 snoRNA function and the role of the ITS2-proximal stem is based on four lines of evidence. First, extensive mutagenesis studies and phylogenetic conservation of regions within the U8 sequence implicate the 5' end of U8 in critical sequence-dependent interactions (Peculis & Steitz, 1994; Peculis, 1997b). Second, 2'-*O*-methyl oligoribonucleotides complementary to the 5' end of U8 RNA inhibit processing of the large subunit rRNAs in *Xenopus* oocytes (Peculis, 1997b). This inhibition is sequence specific, does not result in degradation of U8 RNP and can be rescued by subsequent injection of U8 snoRNA. Thus, inhibition in this oligonucleotide competition experiment is presumably because of interference with base pairing of the 5' end of U8 RNA to its target. Third, complementarity between the 5' end of U8 RNA and the 5' end of 28S rRNA is phylogenetically conserved, implicating this segment of 28S rRNA as a potential U8 target site (Peculis, 1997b). Fourth, 2'-*O*-methyl oligoribonucleotides complementary to the 5.8S or 28S rRNA sequences in the ITS2-proximal stem inhibit processing of the large subunit rRNAs (Peculis, 1997b).

While this fourth result is consistent with a requirement for the ITS2-proximal stem in processing, a direct test of this key feature of the model was lacking. To provide such a test, we have taken advantage of the observation that this stable, base paired structure involving 28S and 5.8S rRNA is conserved in mature ribosomes across evolution (see Figs. 2 and 6A). Thus, this interaction also occurs in yeast pre-rRNA, for which a genetic system to analyze mutations affecting rRNA processing is readily available (Nogi et al., 1991a,b; Henry et al., 1994). The results of our analyses in yeast demonstrate that the structure of the ITS2-proximal stem is essential for processing, whereas the sequence of the stem affects efficiency.

RESULTS

Mutagenesis of rDNA templates

To examine *cis* elements required for rRNA processing, we have taken advantage of a genetic system for inducible expression of rDNA by RNA polymerase II (Pol II) in yeast developed by Nomura and colleagues (Nogi et al., 1991a,b). The system is comprised of an rDNA expression plasmid and a yeast strain condi-

FIGURE 2. Secondary structure of a portion of the 5' half of the large subunit rRNA. Shown is the entire sequence of 5.8S rRNA in bold type, with 5' and 3' ends indicated. The ITS2 proximal stem is indicated and is formed by the base pairing of sequences near the 3' end of 5.8S with those near the 5' end of 25S. The ITS2 sequence, represented here by dashed lines, is 234 nt in yeast and joins the 3' end of 5.8S and the 5' end of 25S in pre-rRNA. For a schematic of the predicted ITS2 structure, see Figure 6B. The sequence of the unique tag (shown in italics; after Musters et al., 1989) and the structural alterations induced in that stem by the presence of the tag inserted into the *KpnI* site is shown below. Structure is modeled after that proposed by Gutell et al. (1993).

tional for RNA polymerase I activity. Transcription of the plasmid-borne rDNA gene is driven by Pol II and is repressed by growth in glucose and induced in response to galactose. The Pol II transcripts from this construct are processed accurately and efficiently and incorporated into ribosomes which are fully functional (Nogi et al., 1991a,b; Henry et al., 1994).

The yeast strain (NOY504) used in these experiments carries a disruption in the *rrn4* (*rpa12*) gene encoding a Pol I subunit (A12.2) (Nogi et al., 1993), which renders this strain temperature sensitive for Pol I transcription and cell growth. Use of this strain has two advantages. First, processing of plasmid-encoded rRNA transcripts can be studied under conditions of reduced competition with endogenous, chromosomal rDNA transcripts. Second, the ability of mutated rDNA genes to support processing, ribosome assembly, and translational function can be readily tested by their ability to confer galactose-dependent growth at a restrictive temperature (37 °C) (Nogi et al., 1991a,b; Henry et al., 1994).

A third component of the system used here was a unique; 18-base-tag sequence inserted within the mature 25S coding sequence (Musters et al., 1989; Beltrame & Tollervey, 1992) for use in Northern analyses and primer extensions. The sequence of the tag is indicated in Figure 2 and the insertion site is depicted schematically in Figure 3A. Although it had been shown previously that the tag inserted at this site had no effect on processing or translational function (Musters et al., 1989), we have analyzed tagged and untagged versions of each of the mutations described below. The results were identical for the two versions and only the results for tagged constructs are presented below.

This system was used to directly test the proposed requirement for the 5.8S:25S interaction in the ITS2-proximal stem. This was carried out by constructing single and compensatory double-clustered mutations in the plasmid-encoded yeast rDNA template (pNOY102) as outlined in Figure 3A. Sequences for each of the mu-

FIGURE 3. Mutagenesis of yeast rDNA template. **A:** Schematic showing the mutagenesis strategy. Using PCR directed mutagenesis (as described in Materials and Methods) with yeast rDNA plasmid pNOY102 as the template, four constructs were generated. The WT rDNA construct encoded wild-type rRNA, with the addition of a unique tag sequence to allow detection of plasmid transcripts independent of the endogenous rRNA background. 5.8Sm contained a 16-nt alteration at the 3' end of 5.8S (indicated by ■) to prevent formation of the ITS2-proximal stem. 25Sm encoded a 16-nt mutation at the 5' end of 25S (indicated by ■) which also prevented formation of the ITS2-proximal stem. The 2X mutation combined both single mutations and restored the structure of the ITS2-proximal stem. The location of the construct-specific probes used for Northern blot analysis is indicated by solid rectangles. **B:** Sequences of the mutations generated. The wild-type sequence of the ITS2-proximal stem is shown, as well as the nucleotide composition of the two single and the 2x compensatory mutation. Solid line represents ITS2 and vertical lines indicate base pairs across the ITS2-proximal stem.

tations and their predicted effect on the ITS2-proximal stem are shown in Figure 3B. The single, clustered mutations were designed to fully disrupt pairing in the stem. The 5.8Sm construct altered the 3' end of 5.8S rRNA; all 16 nt comprising this stem were changed to their Watson–Crick complements. Similarly, the 25Sm construct changed 16 nt at the 5' end of 25S to their Watson–Crick complements. Finally, a double, compensatory mutation (designated 2X) was generated by combining the mutations in the 5.8Sm and the 25Sm constructs. This double mutation alters 32 nt of the mature rRNA sequence while restoring normal base pairing potential for the ITS2-proximal stem (see Figure 3B).

Effects of rDNA mutations on cell growth rates

The three rDNA constructs described above, 5.8Sm, 25Sm, and 2X, and the wild type ITS2-proximal stem (WT) reference construct were transformed into yeast strain NOY504. Growth of strains at permissive and restrictive temperatures was then compared (data not shown). Each of the single and double constructs grew similarly to the WT transformant at the permissive temperature in the presence of galactose, indicating there was no dominant negative phenotype; the synthesis of the mutated rRNA itself did not prevent cell growth or prevent the endogenous transcripts from producing functional ribosomes. The WT transformant grew at 37 °C in the presence of galactose while the untransformed strain did not, confirming that growth at the restrictive temperature was dependent on expression of the plasmid-borne rDNA. Neither of the single mutations (5.8Sm, 25Sm) allowed growth at 37 °C indicating that these mutations blocked one or more essential steps in ribosome biogenesis or translational function. The double mutation (2X) showed galactose-dependent growth at the restrictive temperature similar to that for the WT strain. These results demonstrated that complementarity and the potential to form the ITS2-proximal stem, rather than the conserved primary sequences of these 5.8S and 25S segments, were essential for viability.

Northern analyses of processing defects

Northern hybridization analysis was used to examine the effects of single and double mutations on rRNA processing. For this purpose, each of the four transformants and the untransformed parental strain (NOY504) were grown at the permissive temperature in galactose. Logarithmically growing cultures were then shifted to 37 °C in galactose for 6 h to allow continued expression of the plasmid-borne genes while reducing expression of the Pol I-dependent endogenous rDNA genes. Total RNA was harvested from the five cell lines and equivalent amounts of RNA were resolved on denaturing agarose–formaldehyde, or polyacrylamide gels.

Northern blots were performed using four different rRNA-specific probes. The distribution of these probes is indicated schematically at the bottom of Figure 3A. Additionally, membranes were probed for the RPB4 mRNA (RPB4 encodes a subunit of Pol II) as a control for loading. For example, the comparable intensity for RPB4-hybridized bands seen in the lower panel of Figure 4A indicates that all lanes were similarly loaded. Thus, differences in the intensity of bands seen with other probes reflects differences in steady-state RNA levels due to differences in stability or pre-rRNA processing.

To examine 25S rRNA processing, membranes were probed with a deoxyoligonucleotide complementary to the tag sequence inserted into the plasmid-encoded rRNA (Fig. 4A, top panel). The specificity of the tag sequence probe for plasmid-derived rRNA transcripts was illustrated by a comparison of hybridization to RNA from untransformed cells (Fig. 4A, lane 1) with that for the WT transformant (Fig. 4A, lane 2). The trace signal seen in lane 1 was presumably due to weak cross reactivity with the large amounts of endogenous, mature 25S rRNA present in all of the samples. In contrast, substantial hybridization to mature 25S rRNA was detected for the WT transformant confirming that plasmid-derived transcripts were efficiently processed. Hybridization to 25S rRNA in the 5.8Sm and 25Sm transformants (Fig. 4A, lanes 3 and 4) was reduced to background levels, indicating these single mutations, designed to disrupt the ITS2-proximal stem, prevented accumulation of mature 25S rRNA. Furthermore, the ratio of 27S precursor to mature 25S rRNA was elevated in the single mutations, providing evidence for a processing defect. In contrast to the single mutations, significant accumulation of mature 25S rRNA was observed for the 2X double mutation, suggesting that the ability to form the ITS2-proximal stem was critical for processing or stability. However, the level of mature 25S in the 2X construct was significantly reduced relative to that in the WT construct. Additionally, the ratio of 35S precursor to mature 25S rRNA was elevated for the 2X construct. These results suggest that the efficiency of processing was reduced for the double, compensatory mutation. Although the ability to form a base-paired stem is minimally required for processing, the sequence of the ITS2-proximal stem may affect efficiency.

To provide further evidence for a defect in 25S rRNA processing, membranes were hybridized with a probe complementary to the mutated region within 25S rRNA (Fig. 4B). The specificity of the probe was confirmed by lack of hybridization to rRNA from untransformed cells (Fig. 4B, 504, lane 1) and WT transformants (Fig. 4B, lane 2), which lack the mutated 25S target sequence. Little or no mature 25S rRNA was detected for the 25Sm transformant, confirming that this single mutation, which disrupted the ITS2-proximal stem, prevented the accumulation of mature 25S rRNA (Fig. 4B,

lane 4). The accumulation of trace amounts of 27S intermediate was detected for this transformant, consistent with a 25S processing defect. In contrast, significant hybridization to mature 25S rRNA was observed for the 2X transformant (Fig. 4B, lane 3), indicating the compensatory double mutation restored at least minimal 25S processing. Both the 35S precursor and 27S intermediate were detected with this probe as de-

scribed above for the tag sequence probe (Fig. 4B). However, since this probe does not detect WT transcripts, a comparison of processing efficiency cannot be made here.

We next examined processing of 5.8S rRNA. For this purpose, membranes were hybridized with probes specific for the mutated region in 5.8S rRNA (Fig. 4C). Lack of hybridization of the mutated sequence probe to rRNA from untransformed cells (Fig. 4C, 504, lane 1) and WT transformants (Fig. 4C, lane 2) confirmed the specificity of this probe. Hybridization to 5.8Sm RNA was limited to 35S precursor and 27S intermediate with little or no mature 5.8S mature RNA detected (Fig. 4C, lane 3). Accumulation of the 27S intermediate (produced by ITS1 cleavage, see Fig. 1) and the absence of mature 5.8S suggests the single mutation produced an ITS2-specific processing defect. In contrast to the single mutation, mature 5.8S rRNA was detected for the 2X mutation. In addition to mature 5.8S, 35S precursor and 27S and 7S (a precursor of 5.8S, seen in Fig. 4C as the upper band of the doublet at the bottom of this agarose gel) were detected with the mutated sequence probe.

To examine ITS2 versus ITS1-specific effects on processing, the ratio of 5.8S_S to 5.8S_L forms was examined in the 2X mutation. These two forms of 5.8S rRNA are produced by differential processing within ITS1 in yeast (see Fig. 1, also Morrissey & Tollervy, 1993, Allmang et al., 1996a,b). The 5.8S_L rRNA contains an additional 7 nt upstream of the 5.8S mature 5' terminus. Both forms of 5.8S accumulate in cells with an

FIGURE 4. Northern blot analysis. Total RNA was isolated from the five yeast strains and, except where noted, was resolved on denaturing agarose-formaldehyde gels as described in Materials and Methods. Lanes contain 8 μ g of total RNA from the mutants as indicated: 504: RNA from untransformed NOY504 cells; WT: RNA from cells transformed with a tagged plasmid encoding the wild-type sequence of the ITS2-proximal stem; 5.8Sm: RNA from cells transformed with a tagged plasmid encoding the 5.8Sm mutation in the ITS2 proximal stem; 25Sm: RNA from cells transformed with a tagged plasmid encoding the 25Sm mutation in the ITS2 proximal stem; 2X: RNA from cells transformed with the tagged plasmid encoding the compensatory, double mutation in the ITS2 proximal stem. **A:** Top panel: Tag probe. This membrane was probed with a kinased deoxyoligonucleotide complementary to the tag sequence inserted into the plasmid. Lower panel: Rpb4 probe. Shown is part of a membrane probed with a radiolabeled RNA transcript complementary to the mRNA encoding Rpb4, a subunit of Pol II, used here as a control to compare sample loading. **B:** Mutated region in 25S. This membrane was probed with a kinased deoxyoligonucleotide (25S mutant region oligo) complementary to the mutated region within 25S in the ITS2-proximal stem. **C:** Mutated region in 5.8S. This membrane was probed with a kinased deoxyoligonucleotide (5.8S mutant region oligo) complementary to the mutated region within 5.8S in the ITS2-proximal stem. **D:** Polyacrylamide gel, hybridized for 5.8S. Total RNAs were resolved on an 8% polyacrylamide-urea gel and electrophoretically transferred to membrane. The membrane was cut and halves were probed with kinased deoxyoligonucleotides complementary to either wild-type 5.8S (lanes 1–5) or specific for the altered region within 5.8S (lanes 6–10). The 5.8S signal appears as a doublet due to the variation in processing pathways leading to the accumulation of both 5.8S_L and 5.8S_S.

approximately 9:1 ratio of 5.8S_S to 5.8S_L, and both appear to be equally functional within ribosomes. Figure 4D (left panel) shows part of a polyacrylamide gel hybridized with a wild-type 5.8S rRNA probe (lanes 1–5). The ratio of 5.8S_S to 5.8S_L, indicated by the ratio of the two bands in the observed doublet, was similar in all of the samples, as expected because this probe detects primarily the large pool of stable, endogenous 5.8S rRNA. The right panel (lanes 6–10) was hybridized with a probe specific for the mutated region within 5.8S. The specificity of the probe was confirmed by the absence of hybridization to rRNA from untransformed cells (Fig. 4D, 504, lane 6), WT (Fig. 4D, lane 7) and 25Sm (Fig. 4D, lane 9) transformants. The absence of hybridization observed for the 5.8Sm mutation (Fig. 4D, lane 8) further confirmed the processing defect in this single mutation. Accumulation of mature 5.8S rRNA was observed for the 2X mutation (Fig. 4D, lane 10). Further, the ratio of 5.8S_S to 5.8S_L forms was indistinguishable from that of the endogenous 5.8S pool (compare Fig. 4D, lane 10 to lanes 1–5). This result suggests the apparent effects on efficiency observed for the 2X mutation are limited to ITS2-related events.

Mapping of 5' ends in processed products

The results of Northern hybridization analyses suggested that while the single mutations blocked ITS2-

related processing, the compensatory double mutation was minimally sufficient to restore accurate, albeit less efficient, processing. To examine directly whether processing at the 5' end of 25S and the 5' end of 5.8S rRNAs was accurate in the 2X construct, the termini were mapped via reverse transcription. Since ribosomes are very stable macromolecules, the endogenous rRNA is present in very large amounts even in the cells grown at the nonpermissive temperature for 6 h. To ensure specificity of the reverse transcription priming, the reaction was performed using oligonucleotides complementary to regions of rRNA unique to the plasmid-encoded copy.

First, the 5' end of 5.8S rRNA containing the mutation was mapped in the 2X construct using a deoxyoligonucleotide complementary to the mutated region (at the 3' end of 5.8s) (Fig. 5A, lane 1). The size of this product was compared with the size of reverse transcription products from 2X RNA (Fig. 5A, lane 2) or WT RNA (Fig. 5A, lane 3) using a deoxyoligonucleotide primer complementary to the wild-type 5.8S rRNA sequence. This wild-type primer was of identical position and length as the 5.8S mutant primer used in the reaction shown in Figure 5A, lane 1, but would be expected to prime only from unmutated RNA transcripts, including the endogenous templates. The reverse transcription products of all three samples comigrated indicating the 5' ends were

FIGURE 5. Mapping 5' termini via reverse transcription. Total RNA was used as a template to map the 5' termini of processed rRNA by reverse transcription. **A:** The 5' end of 5.8S was mapped in RNA from WT or 2X. Lane 1: deoxyoligonucleotide complementary to the mutated region within 5.8S, and RNA from the 2X cells. This reaction would map the 5' end of the plasmid-derived mutated 5.8S. Lanes 2–7: deoxyoligonucleotide complementary to the wild-type sequence of 5.8S. In lane 2, RNA from the 2X cells, mapping the residual endogenous RNA. In lanes 3–7, RNA from WT cells which would map both the endogenous 5.8S as well as the plasmid-derived transcripts. Lanes 4–7 each contain one deoxyoligonucleotide present in the reaction to produce the sequencing ladder indicated to the right of lane 7. The +7nt denotes the 7-nt extension present on 5.8S_L. **B:** The 5' end of 25S rRNA derived from the plasmid was mapped using a primer complementary to the tag sequence. Total RNA from cells carrying the wild-type (tagged) plasmid (lanes 1–5) or from cells harboring the 2X mutation was used in the reverse transcription reaction. Corresponding sequencing ladders are shown at the right edge of each panel. In all panels, upper case letters indicate nucleotides which can easily be read from the ladder. The lower case letters indicate nucleotide positions which are not clearly resolved on this gel, with this exposure.

identical. Reverse transcription performed in the presence of dideoxyligonucleotides using reactions identical to that depicted in Figure 5A, lane 3, provided a sequencing ladder (lanes 4–7) to confirm the identification of 5' ends. These results demonstrated that accurate processing of the 5' end of 5.8S rRNA was restored for the 2X mutation.

Next, the 5' end of 25S rRNA was mapped using a deoxyligonucleotide complementary to the tag sequence within the plasmid-encoded 25S rRNA. This primer is specific for plasmid-derived transcripts. Total RNA was used as a template for reverse transcription from cells containing the WT plasmid (Fig. 5B, lanes 1–5) or the 2X plasmid (Fig. 5B, lanes 6–10). In both cases, the reverse transcription reactions were carried out in the presence of dideoxynucleotides to produce sequencing ladders for precise identification of 5' ends. Further, the sequencing ladders shown in Figure 5B confirmed the sequence of the WT and mutated 5' end of 28S. This and additional mapping experiments (data not shown) demonstrated that the position of the 5' end of the 25S rRNAs in the WT and the 2X mutated RNAs was identical. Thus, the double, compensatory mutation was sufficient to restore accurate 5' processing.

DISCUSSION

Previously one of us (B.A.P.) proposed that one role for the 5' end of U8 snoRNA in pre-rRNA processing in *Xenopus* oocytes was to transiently interact with pre-rRNA to facilitate formation of the ITS2-proximal stem between 5.8S and 28S rRNA (see Fig. 2; Peculis, 1997b). A key feature of this model is that proper formation of the ITS2-proximal stem is a prerequisite for the processing events within ITS2 that lead to the accumulation of mature 5.8S and 28S rRNA (Peculis, 1997b). Here we directly tested this feature of the model using the well characterized genetic system available in yeast for studying rRNA processing (Nogi et al., 1991a,b; Henry et al., 1994).

Two single, clustered mutations were made in which each of the 16 nt on one side of the ITS2-proximal stem was changed to disrupt the stem. Both single mutations prevented accumulation of mature, large subunit rRNAs. In contrast, a double, compensatory mutation that changed the primary sequence but preserved the base pairing potential of the stem did not prevent processing. The double mutation also rescued a rRNA expression defect in strain NOY504, indicating it did not block other essential steps in ribosome biogenesis or translation. These data demonstrate that the secondary structure of the ITS2-proximal stem is required for processing whereas the primary sequence is not essential for accurate processing, ribosome assembly, or translational function.

ITS2-proximal stem structure is required for processing accuracy, but its sequence affects efficiency

Primer-extension analysis of rRNA from 2X transformants demonstrated that processing of the 5' end of 25S was accurate to the nucleotide. Furthermore, 3' processing of 5.8S rRNA also appeared to be accurate since the correct 5' ends were confirmed by primer extension and the mature 2X products comigrated with their WT 5.8S counterparts. Thus, accuracy was retained despite mutational changes to 32 nt immediately flanking the mature sequence ends. This result suggested that either ITS2 sequence dictates the site of mature end formation or that RNA structure alone is sufficient for accurate processing.

Although processing was accurate for the 2X construct, efficiency was reduced. Northern hybridization with a tag-specific probe showed reduced levels of mature 25S rRNA in comparison to the WT control. In addition, accumulation of rRNA precursors was detected with both tag-specific and mutated sequence probes for the 2X construct in comparison to WT. Presumably this is because the 2X pre-rRNA was less efficiently processed but not destabilized relative to the WT control.

The simplest interpretation of these data is that the secondary structure of the ITS2-proximal stem is essential for accurate processing in yeast. However, the efficiency of processing is affected by the primary sequence of the stem. At this time we cannot rule out the possibility that the 2X mutant pre-rRNA may fold into an alternative structure that is processed less efficiently.

ITS2-proximal stem formation and rRNA processing: Implications for U8 function

The results of the analyses presented here for ITS2-related processing in yeast suggest some interesting parallels as well as some potential differences with the model proposed for ITS2 processing and U8 function in *Xenopus* oocytes. A requirement for formation of the ITS2-proximal stem appears to be common to yeast and *Xenopus*. Furthermore, the potential for formation of this stem (i.e. complementarity between 3' 5.8S and 5' 25/28S sequences) is highly conserved. Figure 6A illustrates this conserved complementarity for a broad sampling of eukaryotic sequences. The phylogenetic covariation evident in this comparison suggests the ITS2-proximal stem may be broadly conserved and might play a common role in ITS2-related processing events in all eukaryotes.

The results of the present analysis of the yeast ITS2-proximal stem also raise the possibility of some potential functional differences between the yeast and *Xenopus* systems. The model proposed for *Xenopus*

Conceptually, this proposed function might be limited to that of an RNA “chaperone”—directing folding of an RNA so as to enhance formation of a critical structure. In such a limited model, U8 would primarily contribute to efficiency, acting to increase the fraction of molecules in the active conformation or reduce the time required to achieve that conformation. Another feature of an RNA chaperone model is that the requirement for U8 function would be inversely related to the inherent tendency of a given rRNA precursor to form the ITS2-proximal stem in competition with other possible structures. Since this inherent tendency may vary among different rRNAs, the requirement for U8 RNA might similarly vary among different species. For example, if folding of yeast ITS2 promotes efficient formation of the proximal stem, relative to that of *Xenopus* ITS2, then the requirement for U8 function in yeast rRNA processing might be less stringent than that in *Xenopus* (Peculis & Steitz, 1993) and other vertebrates. Predicted folding of ITS2 segments and implications for ITS2-related processing are discussed in more detail below.

Applicability of the U8 model to yeast

Accurate processing of the 2X construct in yeast is not consistent with an essential, sequence-specific interaction with the 5' end of 25S rRNA as predicted by the model for U8 function in *Xenopus*. There are three possible scenarios for which the U8 model might not apply to yeast. First, the U8 model as described for *Xenopus* may not be correct: U8 may not interact with the 5' end of 28S rRNA. Second, yeast rRNA processing may differ fundamentally from *Xenopus* such that yeast does not require a U8 homologue. The third scenario is that yeast may require the functional equivalent of the U8 RNP but may not have a distinct U8 snoRNA ortholog.

Is the vertebrate U8 model valid?

There is no doubt that U8 is critical for pre-rRNA processing in vertebrates (Peculis & Steitz, 1993, 1994; Peculis, 1997b). In *Xenopus* there is considerable evidence showing that the sequence of the 5' end of U8 is critical for function (Peculis, 1997b). The phylogenetic conservation of the first 15 nt of U8 snoRNA among those species where U8 has been sequenced identifies a well conserved domain in U8. Furthermore, both indirect (in vivo competitions), and direct evidence (mutagenesis of U8) supports a functional U8:28S interaction (Peculis & Steitz, 1994; Peculis, 1997b). Nonetheless it remains possible that the base pairing of U8 with 28S may not be absolutely required for function. Direct tests for an essential U8:28S interaction, including both crosslinking and mutagenesis, are currently underway.

Is pre-rRNA processing in yeast fundamentally different?

It is not likely that yeast and vertebrates have evolved two completely different mechanisms for 5.8S/25S processing. Nonetheless, a yeast homolog of U8 snoRNA has not yet been identified on the basis of sequence similarity and predicted complementarity despite the availability of the complete yeast genome sequence (Goffeau et al., 1996).

There are a few examples of apparent differences in snoRNA requirements for pre-rRNA processing between the yeast and vertebrate systems. The U22 snoRNA was identified in human cells and shown in *Xenopus* oocytes to be involved in 18S maturation (Tycowski et al., 1994), though it has no known yeast homologue. The converse can also be found: the snR10 and snR30 snoRNAs in yeast play specific roles in pre-rRNA processing, but no functional vertebrate orthologs have been identified (Morrissey & Tollervey, 1993). RNaseMRP may provide an example of an snoRNP present in both yeast and vertebrates but with different primary functions in these two systems. Depletion of MRP in yeast affects 5.8S pro-

cessing (Chu et al., 1994; Lygerou et al., 1994, 1996), whereas in vertebrates, no clear role in pre-rRNA processing has been assigned. While it is possible these (and perhaps other) snoRNAs have evolved in a species-specific manner, it is equally likely that the functional homologues simply have not been identified.

It should be noted that while the 5' end of U8 is essential in *Xenopus*, it clearly is not the only important region. The remainder of the U8 RNA/RNP is involved in processing and/or complex formation and/or recruitment of cleavage factors (Peculis, 1997b). While this work raises the possibility that the U8:25S interaction may not be conserved in yeast, it neither eliminates nor confirms the prospect that there is a yeast U8 homologue to provide other (as yet unidentified) U8-mediated functions.

Is the U8:28S functional interaction fulfilled by another entity in yeast?

An alternative interpretation of these data is that yeast and *Xenopus* process pre-rRNA via equivalent though mechanistically distinct pathways. It is possible that vertebrates require U8 snoRNA to base pair with 28S to align 5.8S with 28S rRNAs, whereas this U8 function might be provided by another entity in yeast. One candidate for such an entity is ITS2 structure acting *in cis*. Specifically, the secondary and/or tertiary structure of ITS2 might result in juxtaposition of the 5.8S and 25S sequences thereby promoting formation of the ITS2-proximal stem.

Previous analyses of ITS2 in yeast have revealed an evolutionarily conserved secondary structure shown to be critical for rRNA processing (Yeh & Lee, 1991; van der Sande et al., 1992; van Nues et al., 1995), illustrated schematically in Figure 6B for *Saccharomyces cerevisiae*. The structure consists of a series of hairpins that bring together the 3' end of 5.8S and the 5' end of 25S. Interestingly, the functional role of ITS2 does not appear to be entirely structural; ITS2 sequences from divergent yeast species are not functionally interchangeable and deletions of some but not all of the smaller hairpins affect processing (van der Sande et al., 1992; van Nues et al., 1995). Complete elimination of the ITS2 sequence from pre-rRNA also inhibits processing (Musters et al., 1990).

Predicted structures for ITS2 have also been analyzed for angiosperms and insects (Yeh & Lee, 1991; Wesson et al., 1992; Fritz et al., 1994; Bakker et al., 1995; Hershkovitz & Zimmer, 1996). In nearly every case, ITS2 has the potential to form a series of extended hairpin structures; the 3' end of 5.8S and the 5' end of 28S align to form the ITS2 proximal stem. However, the location of the mature rRNA termini within the hairpin structures varies, as does the structure of ITS2 itself.

We have examined predicted secondary structures for the *Xenopus* ITS2 segment using the MFOLD pro-

gram of Zuker and colleagues (Zuker & Jacobson, 1995). The *Xenopus* ITS2 sequence can also adopt an extended hairpin stem loosely reminiscent of that for the yeast ITS2 sequences (data not shown). However, the excess of C over G in the *Xenopus* ITS2 sequence precludes the formation of a tight secondary structure. This might result in a more stringent requirement for U8 in *Xenopus* ITS2 processing. A caveat is that these are only predicted structures. Other structures that may not have the lowest predicted free energy may in fact be the most energetically stable in vivo (HersHKovitz & Zimmer, 1996) and radically different secondary structures can have similar free energy values (HersHKovitz & Zimmer, 1996).

Summary

We have shown that formation of the ITS2-proximal stem is essential for ITS2-related rRNA processing events in yeast. Conservation of 5.8S:25/28S sequence complementarity raises the possibility that this requirement might be common to rRNA processing in all eukaryotes. Several questions are raised by this work. Is there a functional U8 homologue in yeast? Is the U8:28S interaction in vertebrates required for efficiency or accuracy in processing? Is accurate cleavage in ITS2 due entirely to ITS2 (or more distal) primary sequences or is this specified by RNA secondary structure alone? Combined use of the yeast and *Xenopus* systems provides an effective means for addressing these key questions.

MATERIALS AND METHODS

Cloning—deoxyoligonucleotides and methodology of mutagenesis

A fragment of the yeast rDNA plasmid, pNOY102 was subcloned using unique *SacII* and *KpnI* sites. This construct,

pNOY-SK, was grown in strain, GM2163, (*dam*[−]) and served both as the replacement cassette for assembling all mutagenic fragments and as the template for PCR-directed mutagenesis. Eight deoxyoligonucleotides were synthesized to induce various mutations, using the scheme indicated in Figure 7. Deoxyoligonucleotide sequences were:

Oligo 1, 5' TGGTTCTCGCATCGATGAAGAACGCAGCG 3';
Oligo 2, 5' AATGTTTGAGAAGGAAAAGTGCAGTTTGTCCATGGCCCCCTGGAATACCAAGGGGC 3';
Oligo 3, 5' GCCCCTTGGTATTCCAGGGGGCCATGGACAACTCGCAGTTTTCTTCTCAAACATTCTG 3';
Oligo 4, 5' GATACTCTTGGAGTTAACTTGAAATTGC 3';
Oligo 5, 5' CGCTCTCTTCTTATCGATAACGTTCCAATA 3';
Oligo 6, 5' GCTTAAGTTCAGCGGGTACTCCATGGACTAACTCCAGTAACTTTAAGAACATTGTTCCGCC 3';
Oligo 7, 5' GCGAACAATGTTCTTAAAGTTACTGGAGTTTAGTCCATGGAGTACCCGCTGAACTTAAGC 3';
Oligo Kpn, 5' GCCCTCTCCAAATTACAACCTCGGGCACCG 3'.

Mutagenesis was accomplished using deoxyoligonucleotides and PCR. Deoxyoligonucleotide pairs, and the PCR fragment products they produced were oligo 1 and oligo 2 to form fragment 12, oligo 3 and oligo 5 to produce fragment 35, oligo 4 and oligo 6 to form fragment 46, and oligo 7 and oligo Kpn to form 7Kpn. These PCR products were gel purified then digested with *NcoI*. The digested DNA was run over a spin column (Qiagen) to remove the small fragments. *NcoI*-digested fragment 12 was ligated to *NcoI*-digested fragment 35 to generate the 5' mutagenic half, and *NcoI*-digested fragment 46 was ligated to *NcoI*-digested fragment 7Kpn to generate the 3' mutagenic half fragment. A fragment with the wild-type sequence was generated via PCR using oligos 1 and Kpn. The 2X mutagenic fragment was generated by digesting 1235 with *Clal* and *HpaI*, and ligating it to fragment 467Kpn digested with *HpaI* and *KpnI*. These fragments were then ligated into *Clal*- and *KpnI*-digested pNOY-SK. The 5.8Sm construct was generated by digesting fragment 1235 with *Clal* and *HpaI* and ligating it to the wild-type fragment *HpaI*/*KpnI* in the pNOY-SK vector. The 25Sm construct was generated by

FIGURE 7. Mutagenesis and cloning scheme. Shown is a schematic, not to scale, indicating the relative placement of deoxyoligonucleotides used in the mutagenesis scheme. Also shown are the restriction sites generated by the mutagenic oligonucleotides (above) or encoded within the rDNA repeat that were used to assemble and insert the mutagenic fragments into the rDNA plasmid. Deoxyoligonucleotides indicated by a straight line were perfectly complementary to the plasmid; deoxyoligonucleotides indicated by a notched line encode a mutation, so this region was not complementary to the plasmid. The tag sequence is shown as an insertion into the *KpnI* site above the schematic of the plasmid. Insertion of the tag destroyed the *KpnI* site, but provided a new unique *XhoI* site.

using the *Clal/Hpa1* fragment from the wild-type PCR fragment and ligating it to the *Hpa1/Kpn1* fragment from 467Kpn, cloned into *Clal/Kpn*. The *SacII/Kpn1* inserts from the three pNOY-SK plasmids carrying the mutations were then cloned back into the parental pNOY102 plasmid, generating the 2X, 5.8S, and 28S constructs.

To be able to distinguish the transcripts derived from the plasmid from those made from the endogenous rDNA templates, the plasmids were tagged by insertion of a unique deoxyoligonucleotide into the 25S coding region as per Musters et al. (1989). Two deoxyoligonucleotides were synthesized, then annealed to each other. The sequences were yst25-tag-a, 5'TGAAGCTCTCGAGTGTAC 3', and yst25-tag-b, 5'ACTCGAGAGCTTCAGTAC 3'. The annealed deoxyoligonucleotides formed a short, double-stranded DNA fragment having *Kpn1*-compatible termini and contained a unique and diagnostic *Xho1* site. This was ligated into (and destroyed) the unique *Kpn1* site used in the previous cloning steps. The annealed fragment was ligated into the three *Kpn1*-digested mutagenized templates, as well as into an unmutagenized template, which would serve as the wild-type rDNA plus tag control. Tagged versions of the plasmids were sequenced to insure the tag was present and in the correct orientation.

Yeast transformations and growth conditions

The yeast strain NOY504 *MAT α rm4::LEU2 ade2-101 trp1-1 leu2-3,112 his3-11 can1-100* (Nogi et al., 1993) was a generous gift of Dr. M. Nomura. Cells were initially grown at 25°C in YPD. Yeast cells were made competent essentially as described by Adams et al. (1997). The four tagged plasmids were transformed into the NOY504 cells and were grown on SD + glucose plates (Adams et al., 1997) supplemented with the essential amino acids, then transferred to and maintained on SD + galactose plus essential amino acids. Cells were grown in liquid media (SD + galactose) at 25°C to a density of 0.1 OD₆₀₀. The cultures were then shifted to 37°C for 6 h. While at this elevated temperature, the cells continued to divide. Cells were harvested at the end of the 6-h period and total RNA was isolated essentially as previously described (Lin et al., 1996).

Northern blot analysis and probes

Eight micrograms of total RNA was resolved on denaturing 1.2% agarose-formaldehyde gels, or 8% polyacrylamide gels. After electrophoresis, the RNA was transferred to membrane (ZetaProbeGT, BioRad) by capillary action (agarose gels) or electroblotted (acrylamide gels). Membranes were hybridized with probes specific for each of the altered regions.

Probes for the various regions are as follows: tag-region oligo, yst25-tag-b (sequence above); 5.8S wild-type oligo, 5' AGGAAATGACGCTCAAACAGGCATGCCCCC 3'; 5.8S mutant-region oligo, 5' AGGAAAACCTGCGAGTTTGTCCATGCCCCC 3'; 25S mutant-region oligo, 5' CATGGACTAACTCCAGTA 3'.

The Rpb4 cDNA was generated via PCR using deoxyoligonucleotides rpb4Hin5' (5' CAGAAGCTTTCAAACAAGACG GAGAAGATT 3') and rpb4Pst3' (5' GCGCTGCAGGTGTCA CAGGCCAAAGAACC 3') with total genomic DNA. These

deoxyoligonucleotides encoded unique *HinDIII* and *PstI* sites, respectively, which were used to clone the PCR product directionally to produce clone pRpb4. The resulting cDNA clone was sequenced to verify its identity.

Deoxyoligonucleotide probes were kinased with $\gamma^{32}\text{P}$ -ATP and polynucleotide kinase, whereas the RNA transcript that would detect the RPB cDNA was made as a run-off T7 transcript of linearized plasmid pRpb4 described above.

Reverse transcription

Reverse transcription was performed using AMV (Pharmacia) essentially as previously described (Peculis & Steitz, 1993). Kinased deoxyoligonucleotides corresponding to the 5.8S wild-type oligo, 5.8S mutant-region oligo and yst25S-tag-b (sequences provided above) were used to prime total RNA. Dideoxynucleotides were added individually to parallel tubes to provide a sequencing ladder. After extraction with phenol and precipitation with EtOH, the reactions were resolved on a denaturing 10% polyacrylamide gel.

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