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# RNase MRP is required for entry of 35S precursor rRNA into the canonical processing pathway

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## ABSTRACT

RNase MRP is a nucleolar RNA–protein enzyme that participates in the processing of rRNA during ribosome biogenesis. Previous experiments suggested that RNase MRP makes a nonessential cleavage in the first internal transcribed spacer. Here we report experiments with new temperature-sensitive RNase MRP mutants in *Saccharomyces cerevisiae* that show that the abundance of all early intermediates in the processing pathway is severely reduced upon inactivation of RNase MRP. Transcription of rRNA continues unabated as determined by RNA polymerase run-on transcription, but the precursor rRNA transcript does not accumulate, and appears to be unstable. Taken together, these observations suggest that inactivation of RNase MRP blocks cleavage at sites A0, A1, A2, and A3, which in turn, prevents precursor rRNA from entering the canonical processing pathway (35S > 20S + 27S > 18S + 25S + 5.8S rRNA). Nevertheless, at least some cleavage at the processing site in the second internal transcribed spacer takes place to form an unusual 24S intermediate, suggesting that cleavage at C2 is not blocked. Furthermore, the long form of 5.8S rRNA is made in the absence of RNase MRP activity, but only in the presence of Xrn1p (exonuclease 1), an enzyme not required for the canonical pathway. We conclude that RNase MRP is a key enzyme for initiating the canonical processing of precursor rRNA transcripts, but alternative pathway(s) might provide a backup for production of small amounts of rRNA.

**Keywords:** rRNA processing; *XRN1*; exonuclease 1; rRNA turnover

## INTRODUCTION

In eukaryotes, the 18S, 5.8S, and 25S–28S rRNA molecules are transcribed as a single precursor molecule by RNA polymerase I (Pol I), while the 5S rRNA is transcribed separately by RNA polymerase III. The Pol I transcript contains external (ETS) and internal transcribed spacers (ITS) in addition to the sequences that become part of functional ribosomes (Fig. 1). Ribosome biogenesis requires removal of both ETS and ITS sequences, modification of specific nucleotides, and association of ribosomal proteins (Fatica and Tollervey 2002). These processes involve the participation of about 200 proteins and small RNAs that are essential for ribosome biogenesis, but are not components of mature functional ribosomes (Fatica and Tollervey 2002; Fromont-Racine et al. 2003). Many of these accessory factors are organized into RNA–protein particles and pro-

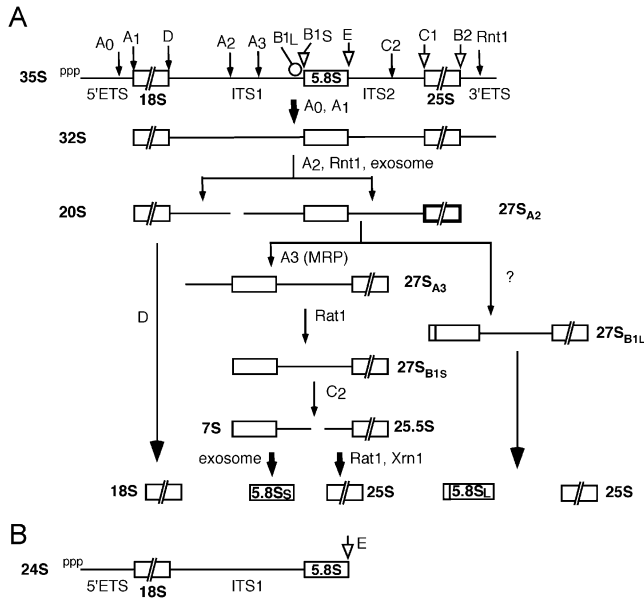
tein complexes that assemble into processomes to facilitate the construction of ribosomal particles (Dez and Tollervey 2004; Granneman and Baserga 2004; Staley and Woolford 2009).

The yeast *Saccharomyces cerevisiae* has become a model organism for studying eukaryotic rRNA processing and ribosome biogenesis. Identification of intermediates in the pathways from the primary Pol I rRNA transcript to mature rRNA entities allows deduction of at least seven endonucleolytic cleavages (Fig. 1) and a number of exonucleolytic trimmings (Fatica and Tollervey 2002). Genetic experiments permit assignment of several of the trimming reactions to the 5' > 3' RNases Xrn1p (exonuclease 1) and Rat1p and to the exosome complex of 3' > 5' RNases. However, to date, only two of the endonucleolytic reactions have been assigned to specific enzymes. These are the cleavages at A3 by RNase MRP (Schmitt and Clayton 1993; Chu et al. 1994; Lygerou et al. 1994; Lygerou et al. 1996) and cleavage within the 3' ETS by the RNase III-like enzyme encoded by *RNT1* (Abou Elela et al. 1996; Kufel et al. 1999). In contrast, the early endonucleolytic cleavages generating the major intermediates 32S, 27S, 20S, 25.5S, and 7S RNA are poorly understood.

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**FIGURE 1.** (A) Schematic representation of the canonical rRNA processing pathways. Processing sites are identified by standard nomenclature (Venema and Tollervey 1999). Arrows with solid heads indicate processing events that can be assigned (by experiments or deduction) to endonucleolytic cleavage. Arrows with open heads indicate endpoints of exonucleolytic trimming. The circle at the B1L site indicates that the nature of processing has not been determined. (B) Structure of the 24S molecule.

RNase MRP, an RNA–protein complex with extensive homology with RNase P (Walker and Engelke 2006), is located almost exclusively in the nucleolus (Reimer et al. 1988; Gill et al. 2006) (note that RNase MRP RNA in the early literature was called 7-2 RNA). However, the enzyme has also been implicated in generating RNA primers for mitochondrial DNA replication (mitochondrial RNA processing [MRP]) and in degrading the mRNA for mitosis-specific cyclin B (Chang and Clayton 1987; Cai et al. 2002; Gill et al. 2004). Furthermore, mutations in the RNA subunit of human RNase MRP are associated with pleiotropic human conditions resulting in dwarfism and several other phenotypes (Ridanpaa et al. 2001; Hermanns et al. 2005; Thiel et al. 2005).

Previous studies of RNase MRP demonstrated a single cleavage site for this enzyme in the precursor rRNA (Henry et al. 1994; Lygerou et al. 1996). This site, named A3, is located in ITS1 (Fig. 1A) and has been implicated in the formation of the 5' end of 5.8S rRNA (Shuai and Warner 1991; Lindahl et al. 1992; Schmitt and Clayton 1993; Chu et al. 1994; Lygerou et al. 1994), but is not essential for ribosome formation (Allmang et al. 1996). Most of these earlier experiments were done with a single temperature-sensitive mutant in the gene for the RNA subunit of RNase MRP (*RRP2*, also known as *NME1*). In spite of being temperature-sensitive for growth, this mutant (*rrp2-2*, also known as P6) generates very similar rRNA processing

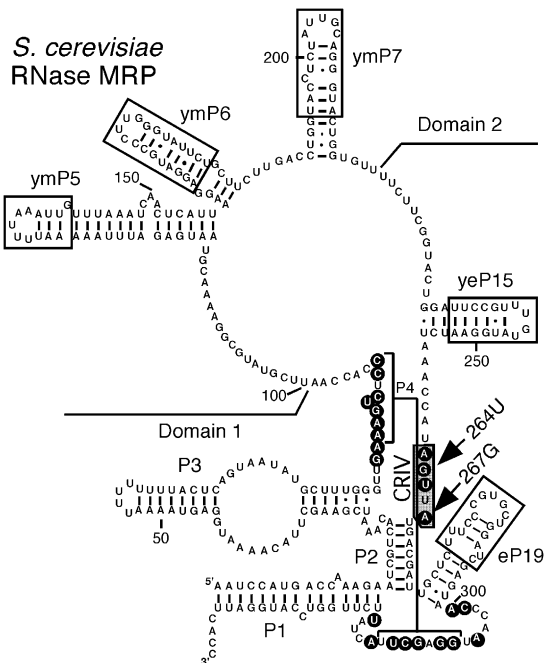
patterns at permissive and nonpermissive temperatures (Shuai and Warner 1991; Lindahl et al. 1992; Chu et al. 1994), limiting the information this mutant can contribute to our understanding of the function of RNase MRP in rRNA processing. To obtain a more complete appreciation of RNase MRP's role in rRNA processing, we have analyzed processing of the 35S pre-rRNA molecule in conditional mutants exhibiting near-normal processing at permissive temperature and complete failure of rRNA processing at nonpermissive temperature.

## RESULTS

### Inactivation of RNase MRP results in loss of 32S, 27S, and 20S intermediates

We first analyzed rRNA molecules extracted from strains carrying *Mini2*, a mutant allele of the RNase MRP RNA gene. *Mini2 rrp2* contains five deletions (Fig. 2), each of which individually has no phenotype, but collectively generate a temperature-sensitive phenotype (Li et al. 2004). The RNase MRP activity can be estimated from the types of 5.8S rRNA that accumulate. There are two forms of 5.8S rRNA, short (5.8S<sub>S</sub>) and long (5.8S<sub>L</sub>), that differ by the presence of 7 nucleotides (nt) from ITS1 in the long, but not in the short, version of 5.8S rRNA. In the presence of normal RNase MRP activity, 5.8S<sub>S</sub> accounts for about 80% of the total 5.8S rRNA, but when RNase MRP activity is reduced, 5.8S<sub>L</sub> becomes a larger fraction of the total 5.8S rRNA (Lindahl et al. 1992). Based on this criterion, the rRNA processing in the *Mini2* strain is near normal at 30°C, but severely curtailed at 37° (Li et al. 2004) and Figure 7A, below (cf. lanes 1 and 2).

To further investigate the effects of RNase MRP inactivation on rRNA processing, total RNA from the *Mini2* mutant and wild-type strains was electrophoresed through agarose gels and blotted onto membranes. The primary Pol I transcript and intermediates in rRNA processing were detected by probing these blots with oligonucleotides complementary to various portions of the external and internal transcribed spacers (Fig. 3C; Table 1). Since these regions are absent from mature rRNA, these probes specifically reveal precursor transcripts and processing intermediates. The specificity of the probes used is confirmed by the fact that hybridization patterns of each probe are in complete agreement with established endpoints of the major processing intermediates (Fig. 1). That is, probing of RNA from wild-type cells with an oligonucleotide specific for the 5' end of 5'ETS region (O1673) revealed the 35S rRNA (Fig. 3A, lanes 3,4), while oligonucleotides specific to ITS1 upstream of the A2 site (O1662) (data not shown) revealed bands for 35S, 32S, and 20S RNAs (Fig. 3A, lanes 7,8). Finally, probes specific for ITS1 sequences downstream from A2 (O1657 and O1680) or for ITS2 (O1660) generated bands for 35S, 32S, and 27S RNAs (Fig. 3A, lanes



**FIGURE 2.** Secondary structure of the RNA subunit of RNase MRP (Li et al. 2002; Piccinelli et al. 2005; Walker and Engelke 2006). Conserved nucleotides are shown in reverse contrast. The five deletions in the Mini2 allele are shown in boxed (Li et al. 2004). The arrows indicated the point mutation alleles used in Figure 3B. The region known as Conserved Region IV (CRIV), is indicated by a gray-filled box.

11,12,15,16,19,20). (We did not attempt to separate the various subforms of 27S, as this was not necessary for interpreting the results of our experiments.)

RNA prepared from the Mini2 mutant grown at 30°C was indistinguishable from wild-type RNA (Fig. 3A, cf. lanes 1 and 3, 5 and 7, 9 and 11, 13 and 15, 17 and 19). However, RNA extracted from the Mini2 mutant six hours after a shift to nonpermissive temperature (37°C) was quite different: 32S, 27S, and 20S processing intermediates were severely reduced or absent (Fig. 3A, cf. lanes 5 and 6, 9 and 10, 13 and 14, 17 and 18). Incubating the Mini2 cells for as little as two hours at 37°C resulted in some, although not total, loss of the 32S, 27S, and 20S rRNA molecules (data not shown). These results suggest one of two things: either 35S pre-rRNA synthesis is blocked after RNase MRP inactivation, or 35S pre-rRNA is formed but the canonical processing of the 35S primary transcript is blocked. A small increase in 35S was observed with the mutant at nonpermissive temperature.

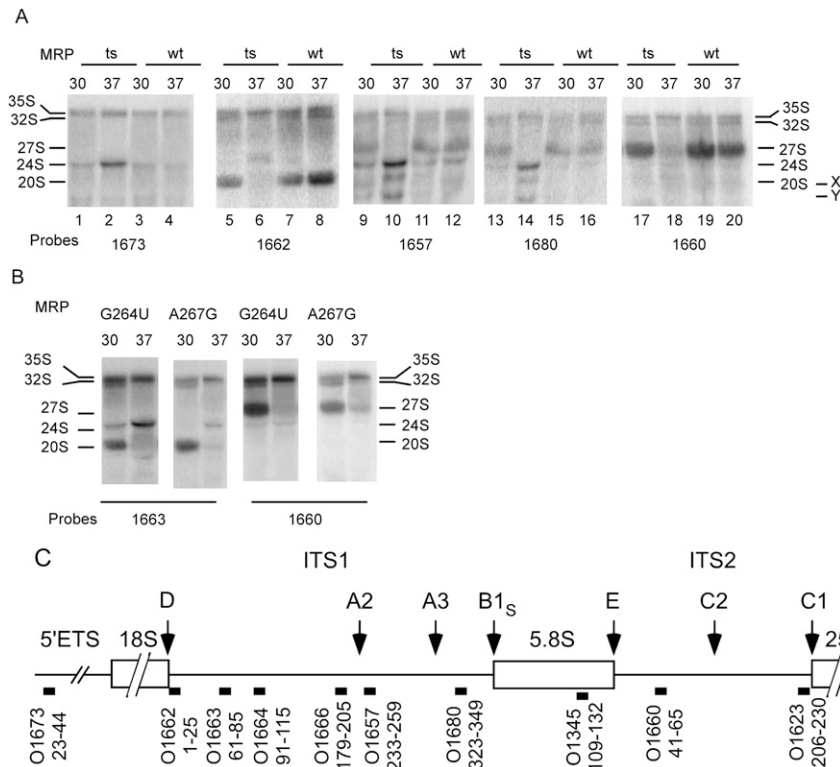
Concomitant with the loss of 32S, 27S, and 20S rRNA, we observed an increase in 24S rRNA (Fig. 3A, lanes 2,6,10,14), a transcript that was previously shown to extend from the transcription initiation region to the end of 5.8S rRNA (Fig. 1; Lindahl et al. 1992). Note that this transcript extends beyond the A3 site at the 3' end, and thus differs from the 23S transcript observed by other investigators

(e.g., Morrissey and Tollervey 1993). The 24S transcript is found in minor amounts in wild-type cells, but it is not known whether it represents an intermediate in an alternate processing pathway or is an aberrant molecule destined for turnover. The intensity of two other bands labeled X and Y also increase after the temperature shift of the Mini2 mutant (Fig. 3A, cf. lanes 9 and 10, 13 and 14). Since they only hybridize with O1657 and O1680, one of these may correspond to the 5.8S<sub>VL</sub> (in which “VL” indicates “very long”) (see, also, Fig. 5 below). Alternatively they could both result from misprocessing or intermediates in the degradation of rRNA that is not assembled into ribosomal particles (see also the following section).

### Inactivation of RNase MRP does not block rRNA synthesis

To determine if rRNA is transcribed when RNase MRP is inactivated, we performed a run-on analysis. Lysates were prepared before and 6 h after the shift to 37°C from equal amounts of cell mass, determined as OD<sup>600</sup> units of culture. The two lysates were used for run-on transcription in the presence of  $\alpha$ -<sup>32</sup>P UTP. The two lysates were equally capable of incorporating radioactive UTP into TCA-precipitable material (data not shown). Equal amounts of radioactive products from the two reactions were then hybridized to slot blots of DNA from a plasmid carrying the 35S rRNA gene, but not the nontranscribed spacers or the 5S rRNA gene. The vector used for cloning the 35S rRNA gene was used as control. The run-on products from both the 30°C and 37°C cells showed strong hybridization to the rRNA plasmid, while no significant radioactivity hybridized to the vector DNA (Fig. 4A). Furthermore, the amount of radioactivity hybridizing to the rRNA plasmid, determined by phosphoimager, was essentially the same (within 5%) for the 30°C and 37°C samples. Since the rRNA plasmid contains only the 35S gene and not the nontranscribed spacers or the 5S gene, the hybridization observed in Figure 4A must be due to transcription of the 35S pre-rRNA gene. These results show that inactivation of RNase MRP does not significantly affect the number of RNA polymerase molecules associated with the 35S rRNA transcription, indicating that either rRNA synthesis continues unabated during RNase MRP inhibition or RNA polymerases on the 35S rRNA gene are arrested in vivo and reactivated during preparation of the cell lysate. Since experiments presented below show that the formation of the long form of 5.8S is not affected by RNase MRP inactivation, we conclude that rRNA transcription continues at the nonpermissive temperature.

To further examine the processing of rRNA after RNase MRP inactivation, we analyzed newly synthesized RNA by pulse-chase experiments using <sup>3</sup>H-labeled uracil. Mini2 cells were labeled with radioactive uracil for 1.3 min and chased with nonradioactive uracil for 2, 4, and 9 min. At



**FIGURE 3.** Northern analysis of rRNA precursors in strains containing mutant RNase MRP RNA subunits. RNA from cultures incubated at 30°C or 37°C was fractionated on denaturing agarose gels, blotted to membranes, and probed with the indicated oligonucleotides. (A) Mini2 RNase MRP RNA (YLL910) and RNase MRP wild-type RNA (YLL925) strains containing wild-type *XRNI*. (B) Point mutations in positions 264 and 267 of RNase MRP RNA. (C) Map of the rRNA transcription unit indicating positions of oligonucleotide probes used in this figure and in Figure 4. Oligo O1623 was used in experiments not shown.

permissive temperature, radioactivity was incorporated into 35S rRNA and processed through the 32S, 20S, and 27S processing intermediates into mature 18S and 25S rRNA (Fig. 4B, lanes 1–3). Radioactive labeling of rRNA was severely curtailed upon inhibition of RNase MRP at nonpermissive temperature. Nevertheless, upon long exposure a 35S rRNA was visible after the 2-min chase, but this band faded with increasing chase time (Fig. 4B, lanes 4–6)

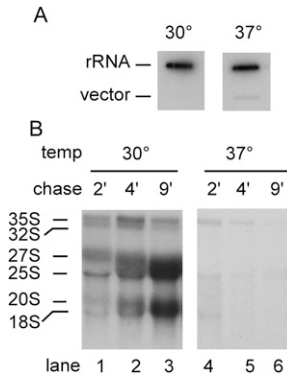
suggesting that the transcript might be degraded. Furthermore, no label was observed in the 32S, 27S, or 20S rRNA processing intermediates (Fig. 4B, lanes 4–6). This absence of radioactivity in these intermediates is compatible with the strong reduction of the major processing intermediates observed in the Northern analysis. Thus, inactivation of RNase MRP does not affect rRNA transcription (Fig. 4A), but the product is degraded rather than being processed through the canonical pathway (Figs. 3A, 4B), presumably because assembly into ribosomal particles was prevented by a block at an earlier step in rRNA processing.

### Analysis of additional RNase MRP RNA temperature-sensitive alleles

We wanted to verify that the changes observed in rRNA processing following a shift to nonpermissive temperature of the Mini2 mutant is due to RNase MRP inactivation and not to some other property of this mutant. If this were the case, the changes in the rRNA processing pattern observed after the temperature shift would be expected to be specific to the particular mutant used for the analysis. In the course of analyzing Conserved Region IV (CRIV) of RNase MRP RNA (Fig. 2; Li et al. 2004; Piccinelli et al. 2005), we systematically mutated four highly conserved nucleotides. As shown in Table 2, this screen identified lethal, temperature-sensitive, and benign mutations in RNase MRP RNA. We used the two temperature-sensitive mutants (Table 2, G264U, A267G) for additional studies of rRNA processing after RNase MRP inactivation.

**TABLE 1.** Sequence of oligonucleotides used for probing of Northern blots

Oligo	Sequence	Complementary to
O1345	TGGAATACCAAGGGGCGCAATGTG	5.8S positions 109–132
O1623	AAGAACATTGTCGCCTAGACGCTC	ITS2 positions 206–230
O1657	GCCCAAAGAAAAAGTTGCAAAGATATG	ITS1 positions 233–259
O1660	AGGCCAGCAATTTCAAGTAACTCC	ITS2 positions 42–66
O1662	CATTTTCAAATTAATAATTTCTT	ITS1 positions 1–25
O1663	CTCTTGCTTCTTGCCAGTAAAAG	ITS1 positions 61–85
O1664	CTTAAGCGCAGGCCCGGCTGGACTC	ITS1 positions 91–115
O1666	CGGTTTAATTGTCCCTATAACAAAAGC	ITS1 positions 179–205
O1673	CGTTTCAAACCTCTTTTCCAAC	5'ETS positions 23–44
O1680	CCAGTTACGAAAATTCTGTTTTGAC	ITS1 positions 323–349



**FIGURE 4.** Synthesis of rRNA after RNase MRP inactivation. (A) Run-on analysis. A culture of strain YLL910 (*rrp2*-Mini2) was grown at 30°C. Half of the culture was shifted to 37°C. Six hours later equal OD<sup>600</sup> units were harvested and lysates prepared for run-on analysis. The lysates were used for in vitro RNA synthesis in the presence of  $\alpha$ -<sup>32</sup>P UTP. Finally the radioactive RNA was hybridized to slot blots of pDK16 (Lindahl et al. 1994) and the vector used for construction of this plasmid. Note that pDK16 carries the entire 35S rRNA gene, but not the nontranscribed spacer or the 5S gene. (B) Pulse-chase labeling of RNA. Cultures of strain YLL911 (*rrp2*-Mini2) incubated at 30°C or 37°C were labeled with <sup>3</sup>H-uracil for 80 sec and chased with excess nonradioactive uracil. Total RNA was prepared 2, 4, and 9 min after the addition of nonradioactive uracil and fractionated on denaturing agarose gels.

As shown in Figure 3B, Northern analysis of RNA isolated from these two mutants using an oligo complementary to ITS1 upstream of A2 demonstrated that the 32S and 20S transcripts virtually disappeared when the strains were grown at nonpermissive temperature, while the 24S band was enhanced. Furthermore, a probe in ITS2 also showed a strong reduction in 32S and 27S rRNA. Thus, the phenotypes of these single nucleotide change mutants were identical to that of the Mini2 mutant. The consistency in the rRNA processing phenotypes between the three different temperature-sensitive RNase MRP RNA mutants strongly suggests that the change in rRNA processing following shifts to nonpermissive temperature is due specifically to the inactivation of the RNase MRP enzyme.

### Analysis of short rRNA processing intermediates

We next searched for specific processing step(s) that are blocked after RNase MRP inactivation. One of the challenges in investigating rRNA processing in vivo is the rapid turnover of products from some of the processing reactions, which may result in little or no accumulation of some intermediates. This difficulty can be overcome by introducing mutations that slow this turnover. We are not aware of any mutation that prolongs the lifetime of all processing products, but deletion of the *XRN1* gene encoding the 5'  $\rightarrow$  3' exonuclease 1 stabilizes some processing products from ITS1 (Stevens et al. 1991). Hence, we introduced this *XRN1* deletion (Stevens et al. 1991) into the Mini 2 and wild-type *RRP2* strains. Northern analysis of the

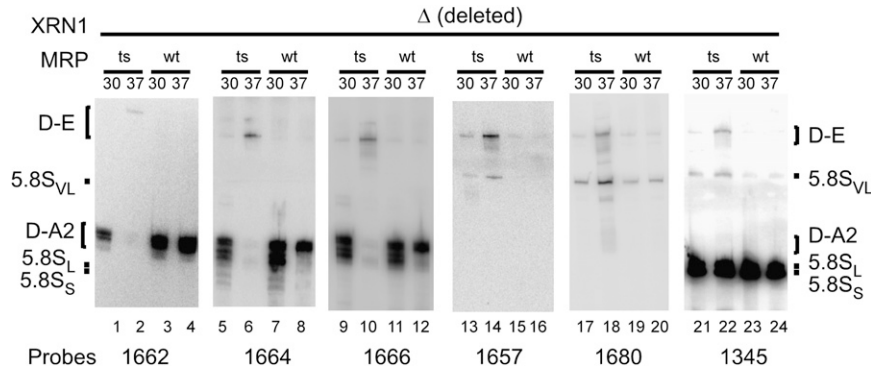
*$\Delta$ xrn1* mutants after agarose gel electrophoresis confirmed the reduction in 32S, 27S, and 20S rRNA intermediates in the Mini2 strain after inactivation of RNase MRP (data not shown). The intermediates from ITS1 processing in the  *$\Delta$ xrn1* strains were examined by polyacrylamide gel electrophoresis followed by Northern blotting. As expected (Stevens et al. 1991), we observed RNA fragments derived from the D-A2 interval when *XRN1* was deleted (Fig. 5). These fragments were seen at both 30°C and 37°C in the RNase MRP wild-type background (Fig. 5, lanes 3,4,7,8,11,12; see Fig. 3C for a map of the probes). In the Mini2-*rrp2*  *$\Delta$ xrn1* double mutant strain, the D-A2 fragments were found at permissive but not nonpermissive temperature in the RNase MRP-Mini2 strain (Fig. 5, cf. lanes 1 and 2, 5 and 6, 9 and 10). Furthermore, we observed enhancement of a band with higher molecular weight that hybridized to all probes across ITS1 and 5.8S rRNA (Fig. 5, cf. lanes 1 and 2, 5 and 6, 9 and 10, 13 and 14, 17 and 18, 21 and 22). This larger fragment corresponds to all or most of ITS1 connected to 5.8S rRNA (D-E) (see Fig. 1). The presence of the ITS1-5.8S fragment at nonpermissive temperature suggests that some processing at or close to the D site and a site in ITS2 (see below) can occur even after inactivation of RNase MRP. The disappearance of the D-A2 fragments simultaneously with the enhancement of the ITS1-5.8S molecule strongly suggests that processing at A2 is abolished upon RNase MRP inactivation.

Finally, probes complementary to ITS1 sequences downstream from A2 revealed the existence of the very long form of 5.8S rRNA extending from A2 to the E site at the end of 5.8S (5.8S<sub>VL</sub> has also been referred to as 5.8S B) (Lindahl et al. 1992). Note that the very long 5.8S rRNA is a normal,

**TABLE 2.** Phenotypes of strains carrying mutations in the conserved region CRIV of RNase MRP RNA

Wild-type nucleotide	Mutant nucleotide	16°C	25°C	30°C	35°C	Growth phenotype
A263 to	G	+	+	+	+	wt
	C	+	+	+	+	wt
	U	+	+	+	+	wt
G264 to	A	–	–	–	–	Lethal
	U	+	+	+	–	ts
	C	–	–	–	–	Lethal
U265 to	C	+	+	+	+	wt
	A	+	+	+	+	wt
	G	+	+	+	+	wt
A267 to	G	–	–	–	–	Lethal
	U	+	+	+	–	ts
	C	+	+	+	+	wt

Cells were streaked on plates containing 5-fluoroorotic acid and incubated at 16°C, 25°C, 30°C, and 35°C; wt, wild type; ts, temperature sensitive. See Materials and Methods for details.



**FIGURE 5.** Northern analysis of low molecular weight rRNA processing products prepared from the  $\Delta xrn1$  strains containing Mini2 (YLL930) or wild-type *RRP2* (YLL931) alleles. RNA from cultures incubated at 30°C or 37°C was fractionated on a denaturing acrylamide gel, blotted to membranes, and probed with the indicated oligonucleotides. For positions of probes, see Figure 3C.

stable molecule (Lindahl et al. 1992). Since the disappearance of the D-A2 products suggests that A2 cleavage is blocked during RNase MRP inactivation, the very long 5.8S rRNA observed at 37°C in Mini2 must be due to molecules accumulated prior to the temperature shift.

### Small subunit processome assembly

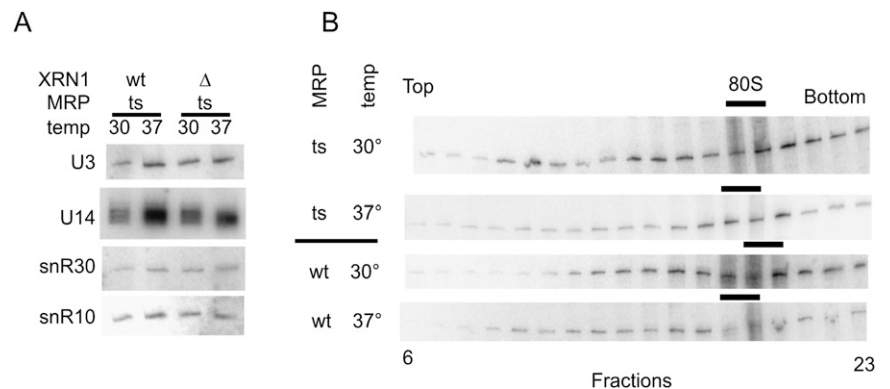
Our experiments cannot distinguish between a direct role of RNase MRP in 35S processing and an effect due to indirect mechanism(s). We investigated two obvious possibilities for indirect effects. First, RNase MRP could play a role in maturing one or more of the snoRNAs (U3, U14, snr10, or snR30) that are necessary for A2 cleavage (Venema and Tollervey 1999). To test this possibility, we fractionated total RNA from RNase MRP-Mini2 strains with *XRN1* or  $\Delta xrn1$  on long acrylamide gels providing single nucleotide resolution. These gels were blotted and probed with oligos specific for each of the four snoRNAs implicated in A2 cleavage. No difference between mutant and wild type was seen as a result of temperature-induced RNase MRP inactivation (Fig. 6A). We conclude that the effect of RNase MRP inactivation is not due to changes in snoRNA processing.

Another possibility is that RNase MRP inactivation could block assembly of the small subunit processome (SSU), or 90S particle, which has been implicated in normal separation of the RNA components destined for incorporation into each of the subunits (Dragon et al. 2002; Osheim et al. 2004). The 90S

particle is assembled from several sub-particles and Rrp5, but only depletion of Rrp5 blocks processing at all sites in 5'ETS and ITS1 (Perez-Fernandez et al. 2007). During depletion of Rrp5, U3 shifts to mono-particles rather than being distributed between monoparticles and larger assemblies. To test if inactivation of RNase MRP converts the distribution of U3 particles to monoparticles, lysates from RNase MRP Mini2 and RNase MRP wild-type cells were fractionated on sucrose gradients. RNA prepared from the indicated fractions was subjected to Northern analysis to locate U3. Results from these experiments showed that U3 RNAs were distributed over a large range of sedimentation rates with significant amounts in particles in the 60S–90S range (Fig. 6B). Furthermore, inactivation of RNase MRP did not result in conversion of U3 particles to monoparticles. Based on the currently known assembly pathways (Perez-Fernandez et al. 2007), we conclude that the effect of RNase MRP inactivation is unlikely due to problems with 90S assembly.

### Role of Exonuclease 1 (Xrn1p) in rRNA processing in the absence of RNase MRP activity

We have previously shown that the ratio of long to short 5.8S rRNA in the RNase MRP-Mini2 mutant increases after



**FIGURE 6.** Abundance of snoRNAs and sedimentation of U3-containing particles after inactivation of RNase MRP. (A) Total RNA from cultures of YLL930 (*rrp2*-Mini2  $\Delta xrn1$ ) and YLL931 (*rrp2*-Mini2 *XRN1*) incubated at 30°C or 37°C was fractionated on denaturing polyacrylamide gels to provide single nucleotide resolution, and blotted to membranes. SnoRNAs affecting A2 cleavage were visualized by probing with  $^{32}$ P-labeled oligonucleotides corresponding to their mature sequences. (B) Total lysates prepared from the indicated strains incubated at 30°C or 37°C were fractionated on sucrose gradients. RNA was prepared from each sucrose gradient fraction and analyzed by Northern blotting of denaturing polyacrylamide gels. The position of the 80S peak is indicated with bars over each blot. Sedimentation is from left to right. The five fractions closest to the top of the gradient are not shown, but contained little U3 RNA.

a temperature shift, suggesting that long 5.8S rRNA (5.8S<sub>L</sub>) accumulates in preference to short 5.8S rRNA (5.8S<sub>S</sub>) when RNase MRP is inactivated (Fig. 7A, lanes 1,2; Lindahl et al. 1992; Li et al. 2004). This suggests that the synthesis of 5.8S<sub>L</sub> rRNA continues in the absence of RNase MRP activity, while the accumulation of the 5.8S<sub>S</sub> rRNA does not. Unexpectedly, we did not see this shift in the ratio of long to short 5.8S rRNA when the *XRN1* gene, encoding exonuclease I, was deleted (Fig. 7A, lanes 3,4). Introduction of a plasmid carrying a wild-type *XRN1* gene to complement the deletion restored the shift in the long to short ratio (Fig. 7A, lanes 7,8). These results suggest that the observed shift between the forms of 5.8S rRNA after inactivation of RNase MRP requires an active *XRN1* allele.

To test our hypothesis that exonuclease I affects the formation of the long form of 5.8S rRNA, we labeled RNA synthesized at 30°C and 37°C with <sup>3</sup>H uracil in strains carrying the Mini2 mutation combined with the  $\Delta xrn1$  or wild-type *XRN1* alleles, and analyzed the RNA by acrylamide gel electrophoresis (Fig. 7B). As expected, the *rrp2* mutant strain carrying a wild-type *XRN1* allele produced both short and long 5.8S rRNA at permissive temperature, but only the long form at nonpermissive temperature (Fig. 7B, lanes 1–4). In contrast, the *rrp2* mutant strain with the *xrn1* deletion allele makes long and short 5.8S rRNA at permissive temperature (Fig. 7B, lanes 5,6), but no detectable 5.8S rRNA is made at 37°C, even though 5S and tRNA

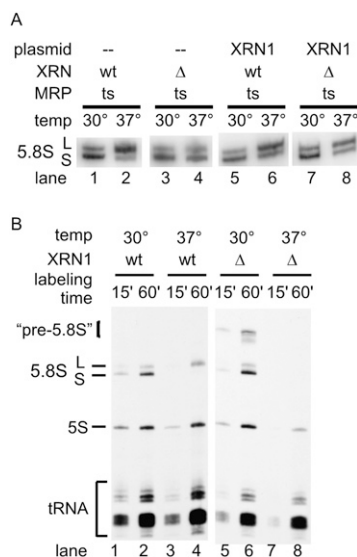
are still synthesized (Fig. 7A, lanes 7,8). The slower moving bands labeled “pre-5.8S” seen in the *RRP2*  $\Delta xrn1$  strain (Fig. 7B, lanes 5,6) presumably represent intermediates that remain untrimmed in the absence of Xrn1p, but since these RNAs are not seen in Northern blots, we assume that these molecules are unstable. The amount of incorporation into 5S rRNA is reduced, possibly because the specific activity of pyrimidine triphosphate pools increases more slowly due to reduced consumption of UTP when rRNA accumulation is reduced. In any case, compared with the intensity of the top tRNA band, the long 5.8S rRNA band is more intense in all situations except the double mutant labeled at 37°C. We conclude that synthesis of long 5.8S rRNA requires exonuclease I (Xrn1p) in the absence of RNase MRP activity, but not if RNase MRP is functional. The formation of 5.8S<sub>L</sub> rRNA must therefore follow different pathways in the presence and absence of RNase MRP activity.

## DISCUSSION

It has long been known that mutations in components of RNase MRP alter the formation of the 5' end of 5.8S rRNA (see above). This effect has been associated with cleavage at a single site, the A3 site in ITS1. Here we have shown that inactivation of RNase MRP using three different temperature-sensitive mutants has much more extensive effects on rRNA processing. At nonpermissive temperature, the abundance of the “normal” intermediates, 32S, 20S, and 27S, is drastically reduced (Fig. 3), suggesting that processing at multiple sites, not just at A3, is arrested by the inactivation of RNase MRP. (Minor changes in the amount of 20S and 27S and a reduced rate of 35S processing were observed in early reports describing the G122A mutant of *rrp2*, but implications of this observation for rRNA processing were not examined [Shuai and Warner 1991; Lindahl et al. 1992].)

We considered the possibility that the reduction of these intermediates was due to inhibition of rRNA synthesis rather than blocked processing. However, measurements of run-on transcription demonstrated that there is no significant difference in the occupancy by RNA polymerase I molecules on the 35S rRNA genes (Fig. 4A), suggesting that rRNA synthesis continues after inactivation of RNase MRP. The incorporation of radioactive uracil into 5.8S<sub>L</sub> rRNA (Fig. 7B) is also consistent with this conclusion. We also considered the possibility that the reduced accumulation of processing intermediates was due to inhibition of assembly of the 90S SSU. However, the synthesis of snoRNAs and distribution of U3 RNA in sucrose gradients argue against this.

Taken together, our experiments demonstrate that RNase MRP activity is required for pre-rRNA to enter the canonical processing pathway (Fig. 1A). No matter the mechanism, RNase MRP thus plays a key role at the very beginning of this pathway, potentially controlling the kinetics of pre-rRNA processing. Our results best fit the conclusion



**FIGURE 7.** Synthesis of 5.8S rRNA after inactivation of RNase MRP. (A) Cells were harvested from the indicated strains growing at 30°C and 24 h after shift to 37°C. RNA was analyzed by acrylamide gel electrophoresis and Northern blotting. 5.8S rRNA was visualized by probing with an oligonucleotide complementary to the last 25 nt of the mature 5.8S rRNA. (B) Long-term labeling of RNA in strains containing Mini2 mutant RNase MRP RNA. Cultures of the indicated strains growing at 30°C or 37°C were labeled with <sup>3</sup>H-uracil for 15 or 60 min. Total RNA was fractionated in denaturing polyacrylamide gels.

that RNase MRP is directly involved in cleavage of pre-rRNA at multiple sites, rather than affecting rRNA maturation in some indirect way. Examination of processing products from ITS1 shows that processing at A2 is blocked after inhibition of RNase MRP. The disappearance of the 32S, 27S, and 20S intermediates is accompanied by an increase in a 24S transcript with boundaries at the 5' end of the 5'ETS and the 3' end of 5.8S. The simplest explanation for this observation is that the 24S molecule is generated by premature cleavage at C2, followed by 3' > 5' exosome trim-back to the E site at the 3' end of 5.8S (Briggs et al. 1998). This suggests that only cleavage of A0, A1, A2, and A3 is inhibited while C2 cleavage may continue in the absence of RNase MRP activity. Interestingly, this is exactly what was observed after depletion of Rrp5 (Venema and Tollervey 1996; Gill et al. 2004). In fact, the pattern of rRNA fragments found after Rrp5 depletion are very similar to those we found after RNase MRP inactivation. It is therefore attractive to speculate that Rrp5 and RNase MRP in some way collaborate in promoting cleavage at the A0, A1, A2, and A3 sites. Final proof for this model would require in vitro cleavage experiments. RNase MRP cleavage in vitro at A3 has been reported (Lygerou et al. 1996), but a more accurate reflection of the in vivo process would require preparation of the natural substrate for RNase MRP, i.e., not naked rRNA, but rather rRNA decorated with multiple complexes of small RNAs and proteins. Preparing such substrates is likely to be a challenging task.

The modest accumulation of 35S transcript observed by radioactive labeling and Northern analysis suggests that, in the absence of active RNase MRP, 35S pre-rRNA is mostly degraded. Degradation of rRNA during stalled ribosome assembly or export has also been observed (Gorenstein and Warner 1977; Warner 1989; Kuai et al. 2004; Dez et al. 2006).

The introduction of the *XRN1* deletion led to a surprising observation. It has long been known that strains containing mutations that debilitate RNase MRP continue to accumulate the long 5.8S rRNA, but not the short 5.8S rRNA. Here we have shown that accumulation of long 5.8S rRNA after RNase MRP inactivation requires Xrn1p, even though Xrn1p is not required for synthesis of long 5.8S rRNA when RNase MRP is active. Accumulation of short 5.8S rRNA requires the other known 5' > 3' exonuclease, Rat1p (El Hage et al. 2008). These two nucleases are found in separate compartments of the cell: Rat1p has been localized in the nucleus while functional Xrn1p has been seen only in the cytoplasm (Johnson 1997; El Hage et al. 2008). Our results therefore suggest that long 5.8S rRNA is produced in the cytoplasm after RNase MRP inactivation. Perhaps transcripts with mature 5.8S 3' ends (site E) can be exported to the cytoplasm even if processing at the 5' end is unfinished. Xrn1p-dependent processing of the 5' end in the cytoplasm might follow cleavage at the D-site at the end of 18S rRNA, which occurs in the cytoplasm

(Udem and Warner 1973; Moy and Silver 1999). Such a mechanism could provide a backup pathway for synthesis of 5.8S rRNA. El Hage et al. (2008) suggested that production of short 5.8S rRNA must occur in a window of opportunity, or else only long 5.8S rRNA is produced. We hypothesize that if RNase MRP inactivation precludes canonical rRNA processing, nuclear production of both short and long 5.8S rRNA is blocked, but an alternative cytoplasmic route to production of long 5.8S rRNA opens as a backup.

In summary, the significance of the experiments reported here is twofold. First, RNase MRP is required for entry of pre-rRNA into the normal processing pathway, most likely because RNase MRP is directly involved in cleavage at A0, A1, A2, and A3. Second, inactivation of RNase MRP reveals an alternative pathway that may represent a backup process for ribosome production, and/or pathways for degradation of rRNA when normal rRNA processing is blocked.

## MATERIAL AND METHODS

### Strains and plasmids

*S. cerevisiae* strains YLL910 (Mini2-*rrp2* *XRN1*) and YLL925 (*RRP2* *XRN1*) were constructed by transforming derivatives of the Ycplac22 (*TRP1*) plasmid carrying Mini2-*rrp2* and wild-type *RRP2*, respectively, into strain YLL302 (MATa *ade2-1 his3Δ200 ura3-52 leu2-3,112 trp1Δ1 rrp2Δ::HIS3/pTOP4 URA3 RRP2*) (Lindahl et al. 2000) followed by 5-fluoroorotic acid elimination of the pTOP4 plasmid (Boeke et al. 1987) carrying wild-type *RRP2* in YLL302. The construction of the Mini2-*rrp2* mutant (Fig. 2) has been described previously (Li et al. 2004). *S. cerevisiae* strains YLL930 (Mini2-*rrp2* *xrn1-Δ*) and YLL 931 (*RRP2* *xrn1-Δ*) were derived from YLL910 and YLL925, respectively, by transforming with a PCR fragment containing the *xrn1* ΔBgl1::*URA3* allele (Larimer and Stevens 1990) and selecting for uracil prototrophy. YLL911 was prepared from YLL910 by transforming with Ycplac33 (*URA3*). Plasmids were amplified using *Escherichia coli* strain DH5α.

Point mutations in the *RRP2* gene were introduced by Quik-change mutagenesis into plasmid pDK49, a derivative of Ycplac22 harboring the wild-type *RRP2* gene. After confirmation of the desired mutation by sequencing the entire gene and flanking regions, the plasmid was transformed into YLL302 by selection on plates containing complete medium lacking tryptophan. Colonies were then streaked on plates with the same medium supplemented with 5-fluoroorotic acid in order to eliminate the pTOP4 plasmid, and incubated at 16°C, 25°C, 30°C, and 35°C.

### Media and growth conditions

Yeast cultures were grown at 30°C in SD-complete medium (Sherman et al. 1974) lacking tryptophan (YLL310 and YLL325) or lacking tryptophan and uracil (YLL330 and YLL331). When the OD<sup>600</sup> of the culture reached ~0.8 (1–2 × 10<sup>7</sup> cells per mL), half of the culture was shifted to 37°C. Incubation of both the 30°C and 37°C aliquots was continued for 6 h, at which time the cells



were harvested for preparation of purified RNA or lysates for sucrose gradient analysis.

### RNA preparation and Northern analysis

RNA was prepared by harvesting 10 mL of culture, resuspending the cells in 0.3 mL TSEI buffer (20 mM Tris-HCl at pH 7.4, 200 mM NaCl, 40 mM Na-EDTA), and extracting nucleic acids by shaking with glass beads, hot phenol, and SDS (Lindahl et al. 1992). For Northern analysis of large RNA molecules, 5  $\mu$ g total RNA was electrophoresed through glyoxal agarose gels (Ambion) and transferred to Nytran N (Schleicher and Schuell) by the vacuum method. For analysis of low molecular weight molecules, 3  $\mu$ g total RNA were electrophoresed through urea-polyacrylamide gels and electroblotted onto Hi-Bond N membranes (Amersham) (Zengel et al. 1990; Lindahl et al. 1992). Precursor ribosomal RNA molecules were visualized by hybridizing the blots with  $^{32}$ P 5'-end labeled oligonucleotides (Fig. 3C; Table 1).

### Sucrose gradients

Sucrose gradient analysis was performed essentially as described (Deshmukh et al. 1993). Briefly, 10 mg cycloheximide was added to 200 mL culture at OD<sup>600</sup> = 1. After 30 sec, cells were harvested, washed with 10 mL lysis buffer (50 mM Tris-HCl at pH 7.5, 30 mM MgCl<sub>2</sub>, 100 mM NaCl, 50  $\mu$ g/mL cycloheximide, and 200  $\mu$ g/mL heparin), resuspended in 1.25 mL lysis buffer containing 10 mM Ribonucleoside Vanadyl Complex, and then vortexed at maximal speed with 3 g glass beads (0.5 mm diameter) for 30 sec four times separated with 1 min periods on ice. Glass beads and debris were removed by centrifugation. Finally, 30 A<sup>260</sup> units were layered on a 10%–50% sucrose gradient and centrifuged for 4 h at 40,000 rpm in an SW 40 Beckman rotor. Gradients were collected in an ISCO Foxy Jr. gradient collector.

### Radioactive labeling of RNA

Labeling was initiated at time 0 min by adding 125  $\mu$ Ci (47 Ci/mmol) to 5.2 mL culture shaking at 30°C or 37°C (see above). At time = 1.3 min, nonradioactive uracil was added to a final concentration of 15  $\mu$ g/mL. At time = 3, 5, and 10 min, additional 1-mL samples were harvested. The radioactive RNA was electrophoresed through glyoxal agarose or acrylamide gels as indicated. After electrophoresis, the gels were soaked for 10 min in Fluorohance (Research Products International, Mount Prospect, IL), dried, and exposed to Kodak X-Omat Blue XB-1 X-ray film for 2 days to a month. Figures show photographs of the autoradiograms illuminated from the back.

### Run-on transcription

Run-on transcription was carried out as described by Elion and Warner (1986).

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