The *POP1* gene encodes a protein component common to the RNase MRP and RNase P ribonucleoproteins

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Two forms of the yeast 5.8S rRNA are generated from a large precursor by distinct processing pathways. Cleavage at site A3 is required for synthesis of the major, short form, designated 5.8S(S), but not for synthesis of the long form, 5.8S(L). To identify components required for A3 cleavage, a bank of temperature-sensitive lethal mutants was screened for those with a reduced ratio of 5.8S(S):5.8S(L). The *pop1-1* mutation (for processing of precursor RNAs) shows this phenotype and also inhibits A3 cleavage. The pre-rRNA processing defect of *pop1-1* strains is similar to that reported for mutations in the RNA component of RNase MRP; we show that a mutation in the RNase MRP RNA also inhibits cleavage at site A3. This is the first site shown to require RNase MRP for cleavage in vivo. The *pop1-1* mutation also leads to a block in the processing of pre-tRNA that is identical to that reported for mutations in the RNA component of RNase P. The RNA components of both RNase MRP and RNase P are underaccumulated in *pop1-1* strains at the nonpermissive temperature, and immunoprecipitation demonstrates that POP1p is a component of both ribonucleoproteins. The *POP1* gene encodes a protein with a predicted molecular mass of 100.5 kD and is essential for viability. POP1p is the first protein component of the nuclear RNase P or RNase MRP for which the gene has been cloned.

[Key Words: *POP1* gene; RNase MRP; RNase P; *S. cerevisiae*; RNA processing]

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Two ribonucleoprotein (RNP) complexes are known to have endoribonuclease (RNase) activity. These are the RNase P and RNase mitochondrial RNA processing (MRP) RNPs.

All pre-tRNAs are transcribed with 5' and 3' extensions, and some contain intervening sequences that are removed to produce the mature tRNAs. RNase P is responsible for the 5' processing of pre-tRNAs in eubacteria, archaeobacteria, and eukaryotes (for review, see Altman 1989; Darr et al. 1992; Altman et al. 1993). RNase P also processes the precursor of the 4.5S RNA in *Escherichia coli* (for review, see by Altman et al. 1993) but has not been reported to have substrates other than pre-tRNAs in archaeobacteria or eukaryotes. The RNA component of eubacterial RNase P has catalytic activity in vitro in the absence of protein cofactors. In contrast, the RNA components of archaeobacterial (Nieuwlandt et al. 1991; LaGrandeur et al. 1993) and eukaryotic RNase P have not been found to be catalytic in the absence of proteins, despite their considerable structural homology to the eubacterial RNAs. *E. coli* RNase P contains a single protein, designated C5, which is small (119 amino acids, 13.8 kD) and highly basic. This protein cofactor is required for RNase P activity in vivo and greatly stimulates activity in vitro. The protein plays important roles in the release of the product, the mature tRNA, from the

enzyme, which is the rate-limiting step for the RNA catalyzed reaction in vitro (Reich et al. 1988; Tallsjö and Kirsebom 1993), and in the binding of suboptimal substrates, including the 4.5S RNA (Peck-Miller and Altman 1991; for review, see Altman et al. 1993). The *Bacillus subtilis* RNase P contains a protein, P protein, which is of similar size to C5 but only 25% identical. Despite this low homology, C5 and P proteins will reconstitute RNase P holoenzymes with the heterologous RNAs, showing that their binding sites and functions have been conserved (Guerrier-Takada et al. 1983; for review, see Altman et al. 1993).

The major RNase P RNA in the budding yeast *Saccharomyces cerevisiae* is 369 nucleotides in length; less abundant 5' and 3' extended forms are also detected and may represent precursors (Lee et al. 1991). *S. cerevisiae* RNase P RNA is encoded by the *RPR1* gene, which is essential for viability. A temperature-sensitive mutant of *rpr1* accumulates pre-tRNAs that are spliced but 5' and 3' unprocessed (Lee et al. 1991). Analysis of the protein components of the nuclear RNase P from *S. cerevisiae* has not been reported; however, highly purified RNase P from *Schizosaccharomyces pombe* contains a single protein component of 100 kD estimated molecular mass (Zimmerly et al. 1993). RNase P from vertebrates can be immunoprecipitated by human Th autoim-

mune sera [also called To sera] (Gold et al. 1989). These sera also precipitate a protein of 40 kD estimated molecular mass (for review, see Reimer et al. 1987), as well as several other polypeptides (Gold et al. 1989). Surprisingly, anti-Th sera from many different patients also precipitate another RNP particle, RNase MRP (or Th RNP), which has been taken to show that human RNase P and RNase MRP share at least one antigenic epitope or common polypeptide (Gold et al. 1989).

RNase MRP was identified and purified from both vertebrates and yeast using an *in vitro* assay for the cleavage of RNA transcribed from the mitochondrial DNA at a position close to the site of formation of the primer for mitochondrial DNA replication (Chang and Clayton 1987; Stohl and Clayton 1992). Both the RNA component, MRP RNA or 7-2 RNA, and the Th antigenic peptide are, however, localized predominantly in the nucleolus (Reimer et al. 1987, 1988; Yuan et al. 1989; Kiss et al. 1992; Topper et al. 1992), indicating that RNase MRP may also have a role in pre-rRNA processing. Both RNase P and endonuclease G have also been reported to cleave the mitochondrial RNA *in vitro* at some of the sites cleaved by RNase MRP (Côté and Ruiz-Carrillo 1993; Potuschak et al. 1993). The RNA components of RNase P and MRP have considerable structural homology, with a conserved core region termed the cage structure (for review, see Altman 1989; Darr et al. 1992; Altman et al. 1993; Karwan 1993; Schmitt et al. 1993). This, together with the existence of a common epitope or polypeptide, suggests that these RNPs have substantial similarities.

Yeast RNase MRP RNA is 340 nucleotides in length and is encoded by the *NME1* gene, which is essential for viability (Schmitt and Clayton 1992; Stohl and Clayton 1992). A previously identified gene, *RRP2*, has been reported recently to be allelic with *NME1* (Chu et al. 1994). Depletion of RNase MRP by repression of the *NME1* gene placed under the control of a *GAL* promoter, as well as temperature-sensitive alleles of *nme1* (*rrp2*), affect pre-rRNA processing (Shuai and Warner 1991; Lindahl et al. 1992; Schmitt and Clayton 1993).

The yeast 18S, 5.8S, and 25S rRNAs are cotranscribed as a large 35S rRNA precursor (Fig. 1A) that undergoes a series of cleavage and modification steps in the nucleolus to generate the mature rRNAs (Fig. 1B). The mature yeast 5.8S rRNA is heterogeneous at its 5' end (Rubin 1974) with a major short form, designated 5.8S(S) and a minor form, 7–8 nucleotides longer, designated 5.8S(L). The 5.8S(S) and 5.8S(L) rRNAs do not have a precursor/product relationship but are formed by distinct processing pathways (Amberg et al. 1992; Schmitt and Clayton 1993; Henry et al. 1994). The 5' end of the major 5.8S(S) RNA is generated by exonucleases that digest 5' → 3' from site A3 in internal transcribed spacer 1 (ITS1) (see Fig. 1B), and the synthesis of 5.8S(S) is therefore inhibited by mutations in the pre-rRNA that prevent A3 cleavage (Henry et al. 1994). In contrast, formation of the 5.8S(L) rRNA does not require cleavage at site A3. The *rrp2-1* and *nme1* mutants show defects in pre-rRNA processing, including the underaccumulation of the 5.8S(S) rRNA

Figure 1. Structure and processing of the yeast pre-rRNA. (A) Structure of the 35S pre-rRNA. Locations of the pre-rRNA cleavage sites (uppercase). Oligonucleotides used for Northern hybridization and primer extension (lowercase) are shown. (B) Pre-rRNA processing pathway. The 35S pre-rRNA undergoes snoRNP-dependent cleavages at site A0 in the 5' ETS (generating the 33S pre-rRNA), site A1 at the 5' end of the 18S rRNA (generating 32S pre-rRNA) and A2 in ITS1 (generating the 20S and 27SA pre-rRNAs). A2 cleavage separates the pre-rRNAs destined to form the small and large ribosomal subunits. The 20S pre-rRNA is cleaved, probably endonucleolytically, to generate the 18S rRNA. The 27SA pre-rRNA can be processed by two alternative pathways. In the major pathway, 27SA is cleaved by RNase MRP at site A3 to generate the 27SA' pre-rRNA. A3 acts as an entry site for an exonuclease activity that degrades the pre-rRNA 5' → 3' to site B1(S) generating the 5' end of the short form of the 27SB pre-rRNA, 27SB(S). This pre-rRNA is subsequently processed to the 5.8S(S) and 25S rRNAs. An alternative pathway leads to cleavage at site B1(L), the 5' end of the 27SB(L) pre-rRNA, which is processed to yield the 5.8S(L) and 25S rRNAs. For simplicity, details of the processing of 27SB to 5.8S and 25S are not shown. It is not clear whether the 33S RNA is an intermediate in the major processing pathway, and the precise timing of the cleavage of the short 3' ETS sequence is not known.

and the accumulation of a 5' extended form of 5.8S rRNA that extends into ITS1 to pre-rRNA cleavage site A2 (see Fig. 1B) (Shuai and Warner 1991; Lindahl et al. 1992; Schmitt and Clayton 1993). This suggests the involvement of RNase MRP in the formation of the 5.8S(S) rRNA.

To identify components specifically inhibited in cleavage at site A3, we screened a bank of temperature-sensi-

tive-lethal mutants for those that alter the ratio of 5.8S(L) to 5.8S(S) at the nonpermissive temperature. We obtained one mutant with this phenotype; the gene mutated, *POP1* (for processing of precursor RNAs), encodes a common component of the RNase MRP and RNase P RNPs.

Results

Isolation of *pop1-1*, a temperature-sensitive mutation causing defects in both RNase MRP and RNase P functions

A bank of temperature-sensitive yeast mutants was generated by UV-mutagenizing haploid yeast cells to 5–15% survival. Following growth at 23°C, surviving colonies were tested for growth at 37°C. A total of 246 temperature-sensitive mutants were isolated.

To identify mutants that are altered in the ratio of 5.8S(L) to 5.8S(S), RNA was extracted from the 246 temperature-sensitive-lethal strains following growth for 2.5 hr at the nonpermissive temperature. RNA was separated by polyacrylamide gel electrophoresis and transferred for Northern hybridization. The filters were hybridized with an oligonucleotide probe hybridizing within the 5.8S rRNA-coding region (oligonucleotide c, see Fig. 1A). One mutant strain was identified that underaccumulates 5.8S(S), but not 5.8S(L), and the mutation was designated *pop1-1*.

At the permissive temperature (23°C) synthesis of rRNA and tRNA by the *pop1-1* strain (Fig. 2, lane 2) is similar to that of the *POP1* strain (Fig. 2, lane 1). During growth at 37°C, the *pop1-1* strain underaccumulates the 5.8S(S) rRNA and has a mildly increased level of 5.8S(L) (Fig. 2, lanes 3–5). In the wild-type strain the ratio of 5.8S(L)/5.8S(S) is ~1:8; this falls to ~1:1 following growth of the *pop1-1* strain for 20 hr at 37°C. Hybridization shows that the *pop1-1* strain strongly accumulates a 5' extended form of the 5.8S rRNA, which includes the 3' region of ITS1 (5.8S+ITS1 3') during growth at 37°C (Fig. 2, lanes 8–10, top right). This RNA hybridizes with an oligonucleotide complementary to the region of ITS1 immediately 3' to site A2 (oligonucleotide a; see Fig. 1A) (Fig. 2, lanes 6–10, top right) and also with an oligonucleotide from within the mature 5.8S region (oligonucleotide c; see Fig. 1A) (data not shown). The size and hybridization pattern of this RNA is consistent with its extending from cleavage site A2 to the 3' end of the mature 5.8S rRNA. Such an RNA species would be generated if processing at site A3 was inhibited while processing in ITS2 continued (see Fig. 1B). This phenotype is the same as that reported for mutations in the RNA component of RNase MRP (Shuai and Warner 1991; Lindahl et al. 1992; Schmitt and Clayton 1993). The level of the 5S rRNA which, like 5.8S rRNA, is a component of the 60S ribosomal subunit, is also reduced in the *pop1-1* strain grown at 37°C. The steady-state levels of several small nucleolar RNAs (snoRNAs) and small nuclear RNAs (snRNAs) are, however, not affected by growth of the *pop1-1* strain at 37°C (see below; data not shown),

Figure 2. Effects of *pop1-1* on 5.8S rRNA and tRNA synthesis. (Lanes 1–5) Staining of RNA with ethidium bromide. Lanes 6–10, *top*) Northern hybridization with probe specific for the 3' region of ITS1 (oligonucleotide a in Fig. 1); (*bottom*) Northern hybridization with probe specific for the mature region of tRNA^{Leu}. (Lanes 1,6) RNA extracted from a wild-type *POP1*⁺ strain following growth at 37°C; (lanes 2,7) RNA extracted from a *pop1-1* strain following growth at 23°C; (lanes 3–5, 8–10) RNA extracted from a *pop1-1* strain following growth at 37°C for the times indicated. RNA extracted from 0.1 OD₆₀₀ unit of cells (~2×10⁶ cells) was loaded in each lane. The position of the 5.8S(L), 5.8S(S), and 5S rRNAs and tRNAs are indicated. Also shown are the positions of the primary transcript of tRNA^{Leu} (PT), which is 5' and 3' extended and contains an IVS, and the pre-tRNA, which is 3' extended and contains the IVS (+3' +IVS), 5' and 3' mature but contains the IVS (+IVS), spliced but 5' and 3' extended (+5' +3') and mature (tRNA^{Leu}), as well as the 5' extended form of 5.8S rRNA (5.8S+ITS1 3'). Note that the tRNAs are heterogeneous in size. The upper bands visible in the *POP1*⁺ strain are the larger tRNA species and not the pre-tRNAs, which cannot be observed by ethidium staining of RNA extracted from wild-type cells.

showing that the reduction in the levels of the mature rRNAs is not attributable to differences in RNA recovery.

In addition, the *pop1-1* strain underaccumulates mature tRNAs at the nonpermissive temperature and a number of additional RNAs accumulate, the sizes of which are appropriate for pre-tRNAs (Fig. 2, lanes 3–5). Hybridization with probes to the mature tRNA^{Leu} (Fig. 2, lanes 6–10, bottom right), intervening sequence (IVS) and 5' leader (data not shown), shows that at 23°C processing of the pre-tRNA is similar in the *pop1-1* (Fig. 2, lane 7) and *POP1* (Fig. 2, lane 6) strains. However, following growth of the *pop1-1* strain at 37°C, the 5'- and 3'-unprocessed pre-tRNA (+5' +3') accumulates to very high levels (Fig. 2, lanes 8–10). After growth of the *pop1-1* strain for 20 hr at 37°C, the 5'- and 3'-unproc-

essed pre-tRNA has accumulated to a level similar to that of the mature tRNA. In contrast, the levels of the pre-tRNA, which is unspliced but has been processed at the 5' end (+IVS +3') or 5' +3' ends (+IVS), are strongly reduced (these species are not easily visible at the exposure shown in Figure 2 and their levels were assessed on longer exposures; data not shown). The level of the primary transcript (PT) is little affected in the *pop1-1* strain. This indicates that pre-tRNA splicing is not affected by the *pop1-1* mutation, but 5' and 3' processing is inhibited. This phenotype is identical to that reported for mutations in the *RPR1* gene, which encodes the RNA component of RNase P (Lee et al. 1991). Hybridization with probes to the mature regions of two other spliced tRNAs, tRNA^{ProUGG} and tRNA^{TrpCCA}, and two unspliced tRNAs, tRNA^{AlaUGC} and tRNA^{GlyGCC}, showed that in each case the mature tRNAs are underaccumulated in the *pop1-1* strain grown at 37°C, whereas pre-tRNAs are strongly accumulated (data not shown).

From these results we conclude that the phenotype of the *pop1-1* mutation resembles a combination of the phenotypes of mutations in the RNA components of RNase P and RNase MRP.

pop1-1 and *rrp2-1* mutants are inhibited in A3 cleavage

Formation of the 5.8S(S) rRNA proceeds through cleavage at site A3, within the ITS1 region, which generates the 27SA' pre-rRNA. This intermediate is then processed exonucleolytically to generate the 27SB(S) pre-rRNA, which has the same 5' end as the mature 5.8S(S) rRNA (Henry et al. 1994; see Fig. 1B).

Primer extension was used to assess whether *pop1-1* strains are defective in synthesis of the 27SA' pre-rRNA, which would indicate a block in A3 cleavage. Oligonucleotides hybridizing to the 3' region of ITS1, the 5' region of ITS2, and the 3' region of ITS2 (oligonucleotides b, d, and e, respectively; see Fig. 1A) were used for primer extension (shown for oligonucleotide d in Fig. 3). In the *POP1* control strain (Fig. 3, lane 1) and the *pop1-1* strain following growth at 23°C (Fig. 3, lane 2), the 27SA' pre-rRNA is clearly detected. No primer extension stop at site A3 is detected in RNA transcribed in vitro (Fig. 3, lane 6), showing that this is not attributable to the secondary structure of the pre-rRNA. Following growth of the *pop1-1* strain for 2 hr at 37°C, the level of 27SA' pre-rRNA, shown by the level of the primer extension stop at site A3, is already strongly reduced (Fig. 3, lane 3). At this time point, other pre-rRNA processing reactions are not clearly inhibited in the *pop1-1* strain (Fig. 3; data not shown). Growth of the *pop1-1* strain at 37°C for 6 or 20 hr, leads to further reduction in the level of 27SA' pre-rRNA (Fig. 3, lanes 4,5). The level of 27SA pre-rRNA, which extends from A2 to the 3' end of 25S rRNA, as shown by the primer extension stop at site A2, is reduced only after prolonged growth of the *pop1-1* strain at 37°C (Fig. 3, lane 5). The ratio between the long and short forms of the 27SB pre-rRNA [designated 27SB(L) and 27SB(S), respectively; see Fig. 1B], shown by the primer extension stops at B1(L) and B1(S), is increased in the

Figure 3. Primer extension through pre-rRNA cleavage site A3 in *POP1* and *pop1-1* strains. (Lane 1) RNA extracted from a wild-type *POP1*⁺ strain following growth at 37°C; (lane 2) RNA extracted from a *pop1-1* strain following growth at 23°C; (lanes 3–5) RNA extracted from a *pop1-1* strain following growth at 37°C for the times indicated; (lane 6) pre-rRNA transcribed in vitro. Primer extension was performed using oligonucleotide d, which hybridizes to the 5' region of ITS1 (see Fig. 1). Primer extension stops at sites A2, A3, B1(L), and B1(S), corresponding to the 5' ends of the 27SA, 27SA', 27SB(L), and 27SB(S) pre-rRNAs, respectively, are indicated. Sequencing reactions on the rDNA with the same primer are also shown.

pop1-1 strain grown at 37°C (data not shown). This parallels the increase in the ratio of the mature 5.8S(L) to 5.8S(S).

Synthesis of the 27SA' pre-rRNA was also examined in an *rrp2-1* strain (Shuai and Warner 1991; generously provided by J. Warner), which carries a mutation in the RNA component of RNase MRP (Chu et al. 1994). Strains carrying *rrp2-1* or *rrp2-2* are defective in the synthesis of 5.8S(S) at 25°C but not at 18°C (Lindahl et al. 1992; Shuai and Warner 1991; data not shown). The synthesis of 27SA' pre-rRNA was therefore assessed by primer extension on RNA extracted following growth of the *rrp2-1* strain at 18°C and 25°C (Fig. 4). The 27SA' pre-rRNA is detected following growth of the *rrp2-1* strain at 18°C (Fig. 4, lane 2) at a level similar to that in an *RRP2*⁺

Figure 4. Primer extension through pre-rRNA cleavage site A3 in *RRP2* and *rrp2-1* strains. (Lane 1) RNA extracted from a wild-type *RRP2*⁺ strain following growth at 30°C; (lane 2) RNA extracted from a *rrp2-1* strain following growth at 18°C; (lane 3) RNA extracted from a *rrp2-1* strain following growth at 25°C. Primer extension was performed using oligonucleotide d, which hybridizes to the 5' region of ITS1 (see Fig. 1). The primer extension stop at site A3, corresponding to the 5' end of the 27SA' pre-rRNA, is indicated. Sequencing reactions on the rDNA with the same primer are also shown.

strain (Fig. 4, lane 1). Following growth of the *rrp2-1* strain at 25°C, no 27SA' pre-rRNA can be detected (Fig. 4, lane 3). The level of premature primer extension stops because of secondary structure in the pre-rRNA, shown by their presence in RNA transcribed in vitro, is higher in the primer extension experiment shown in Figure 4 than in Figure 3. This variation may be attributable to the batch of reverse transcriptase used in the experiment (see also Henry et al. 1994). Other pre-rRNA processing reactions are not inhibited during growth of the *rrp2-1* strain at 25°C (data not shown). Following incubation of the *rrp2-1* strain at 37°C, the nonpermissive temperature for growth, a more complex inhibition of pre-rRNA processing is seen (Shuai and Warner 1991; Lindahl et al. 1992; Schmitt and Clayton 1993; data not shown).

We conclude that cleavage of site A3 requires both the RNA component of RNase MRP and POP1p.

POP1 defines a single gene coding for an essential protein of 100 kD

To determine whether the phenotype observed in the *pop1-1* strain was attributable to a single mutation, this strain was backcrossed to a wild-type strain. The growth phenotype was found to be recessive to *POP1* and, after sporulation and dissection of the diploid, the temperature sensitivity segregated 2:2 in the progeny. The temperature sensitivity and the defect in the synthesis of

both 5.8S rRNA and tRNA (analyzed by Northern hybridization) were found to cosegregate in the progeny of four complete tetrads tested, making it likely that all of the observed phenotypes are the result of a single mutation.

The wild-type *POP1* gene was cloned from a yeast genomic DNA library by complementation of the temperature sensitivity of the *pop1-1* strain. Twelve complementing plasmids were recovered, all of which contained overlapping inserts derived from a single chromosomal locus. The restriction map of this locus and the position of the two least overlapping inserts are shown in Figure 5A. By subcloning from the original isolates, the complementing activity was localized to a 3.5-kb fragment (Fig. 5A). Sequence analysis of this fragment revealed an open reading frame (ORF) of 876 amino acids, encoding a basic protein (pI = 9.8) of 100.5 kD predicted molecular mass. The DNA sequence and the deduced amino acid sequence are shown in Figure 5B. Comparison of the DNA and protein sequences to the data bases revealed no significant homologies to any known proteins, and no motifs or repeats could be identified. The amino-terminal part of the protein is particularly basic and contains putative nuclear localization sequences.

Sequencing of the extremities of the original complementing clones revealed another putative ORF, upstream of the *POP1* gene and transcribed in the opposite orientation. When compared with the Swissprot data base of protein sequences, this ORF shows high homology (36–60%) to adenylosuccinate synthase from different organisms. In yeast, adenylosuccinate synthase is encoded by the *ADE12* gene (Dorfman 1969), which maps to the left arm of chromosome XIV (Mortimer et al. 1992). While this work was in progress, the sequence of the *S. cerevisiae ADE12* gene was submitted to the EMBL data library. It overlaps our fully sequenced, 3.3-kb fragment by 400 nucleotides. The position of the *ADE12* gene, with respect to the restriction map established for *POP1*, is shown on Figure 5A. On the basis of the linkage to the *ADE12* gene, we conclude that the *POP1* gene is localized on the left arm of chromosome XIV.

To demonstrate that the cloned DNA contained the wild-type allele of *pop1-1* and not an extragenic suppressor, the *LEU2* gene was targeted to the chromosomal locus of the cloned fragment in a wild-type haploid strain (see Materials and methods). Correct integration of the marker was verified by Southern analysis. The resultant strain was crossed to *pop1-1* and analyzed by segregation. This revealed complete cosegregation of the *LEU2* marker with the wild-type *POP1* for nine complete tetrads tested, demonstrating tight linkage between the cloned *POP1* gene and the temperature-sensitive lesion.

A null allele of *POP1* was constructed by replacing the 766 amino-terminal amino acids of *POP1* with the *TRP1* gene. The resultant *pop1::TRP1* gene was used to transform two diploid strains to Trp prototrophy. Insertion at the *POP1* locus was confirmed by Southern analysis. From each of these strains, 22 tetrads were dissected. Each tetrad yielded only two viable spores, and none of

Figure 6. Levels of the RNase P and RNase MRP RNAs in *pop1-1* strains. (Lane 1) RNA extracted from a wild-type *POP1*⁺ strain following growth at 37°C; (lane 2) RNA extracted from a *pop1-1* strain following growth at 23°C; (lanes 3–5) RNA extracted from a *pop1-1* strain following growth at 37°C for the times indicated. RNA was separated on a polyacrylamide gel and transferred for Northern hybridization. RNA extracted from 0.1 OD₆₀₀ unit of cells (~2 × 10⁶ cells) was loaded in each lane. Hybridizing RNAs are indicated; the upper band on the RNase P RNA panel is the 5' and 3' extended species, which may be a precursor to the mature RNase P RNA.

steady-state levels of both RNase MRP and the mature RNase P RNAs.

RNase P and RNase MRP RNAs coprecipitate with a ProtA-POP1 fusion protein

To test for the direct association of POP1p with the RNA components of RNase P and RNase MRP, we constructed an epitope-tagged POP1 protein. Two IgG-binding domains from the *Staphylococcus aureus* protein A were fused in-frame to POP1p at either the amino-terminal (ProtA-POP1) or the carboxy-terminal (POP1-ProtA) end. These constructs, on a single-copy plasmid, were used to transform a *pop1::TRP1/POP1* diploid heterozygous for the *POP1* deletion. After sporulation and dissection, spores disrupted for *POP1* but carrying the ProtA-POP1 fusion protein were viable and exhibited no growth defect, showing that the amino-terminal fusion protein is fully functional. In contrast, no progeny carrying *pop1::TRP1* and complemented by the POP1-ProtA construct were obtained from the dissection of 22 tetrads, and the carboxy-terminal fusion protein therefore appears not to be functional.

Interaction of POP1p with the RNase P and RNase MRP particles was tested by immunoprecipitation (Fig. 7). Whole cell extracts were prepared from two different strains expressing the ProtA-POP1 protein fusion in a

pop1::TRP1 genetic background (Fig. 7, lanes 3,4,7,8, and 11,12), and as controls, extracts from a wild-type strain (Fig. 7, lanes 2,6,10) and a strain expressing a fusion protein between protein A and the 70-kD U1 snRNP protein (Fig. 7, lanes 1,5,9) were used. The extracts were immunoprecipitated using IgG-agarose (see Materials and methods), and RNA was recovered from the total extract (Fig. 7, lanes 1–4), from the supernatant (Fig. 7, lanes 5–8), and from the immunoprecipitates (Fig. 7, lanes 9–12). RNAs from equivalent quantities of each fraction were analyzed by Northern hybridization (Fig. 7) and primer extension (data not shown). The RNA components of both RNase P and RNase MRP are recovered very efficiently in the immunoprecipitates from the strains expressing ProtA-POP1 (Fig. 7, lanes 11,12) but not from the control strains (Fig. 7, lanes 9,10). Greater than 90% of both RNAs can be coprecipitated with ProtA-POP1. For RNase P RNA, the mature RNA and

Figure 7. Coprecipitation of the RNase P and RNase MRP RNAs with POP1p. (Lanes 1–4) Total RNA from cell lysates; (lanes 5–8) RNA recovered from the immunosupernatants; (lanes 9–12) RNA recovered from the immunoprecipitates; (lanes 1,5,9) Wild-type strain of yeast; (lanes 2,6,10) Strain expressing a protein A-U1 70K protein fusion; (lanes 3,4,7,8,11, and 12) Strains expressing the ProtA-POP1 fusion. IgG agarose was used to immunoprecipitate the protein A fusions from whole cell lysates. RNA was recovered from the immunosupernatants and immunoprecipitates, and from equivalent aliquots of the total lysates, separated on a polyacrylamide gel, and transferred for Northern hybridization. Hybridizing RNAs are indicated; the upper band on the RNase P RNA panel is the 5' and 3' extended species, which may be a precursor to the mature RNase P RNA.

the larger, putative precursor RNA are immunoprecipitated with similar efficiencies. Other snoRNAs, snR10, U14 (Fig. 7), U3, and snR30 (data not shown), are not detectably immunoprecipitated. The U1 snRNA is not coprecipitated with ProtA–POP1 (Fig. 7, lanes 11,12), but is coprecipitated with the ProtA–U1 70K fusion protein (Fig. 7, lane 9).

From these experiments we conclude that POP1p is a component of both the RNase P and RNase MRP particles.

Discussion

POP1 is a component of RNase P and RNase MRP

We have isolated a mutation in a novel gene, *POP1*, from a bank of temperature-sensitive-lethal yeast mutants. The mutant allele, *pop1-1*, inhibits both the pre-rRNA processing pathway leading to the synthesis of the 5.8S rRNA and the processing of pre-tRNAs. The coprecipitation of the RNA components of RNase P and RNase MRP with ProtA–POP1, together with the loss of these RNAs on growth of the *pop1-1* strain at nonpermissive temperature, demonstrates that POP1p is a component of both RNPs.

Bacterial RNase P consists of an RNA and a single protein. The proteins, cloned from different bacterial species, are small and basic but show only limited sequence conservation (for review, see Altman et al. 1993). Some homology can be found between conserved residues in the bacterial RNase P proteins and sequences in POP1p, but it is unclear whether this is significant given the overall basic character of these proteins. Highly purified RNase P from the fission yeast *S. pombe* also contains a single protein (Zimmerly et al. 1993), of 100 kD estimated molecular mass. The similarity between this estimate and the size of POP1p (100.5 kD) suggests that this *S. pombe* protein is the homolog of POP1p. A protein component of the *S. cerevisiae* mitochondrial RNase P, encoded by the *RPM2* gene, has also been characterized (Morales et al. 1992; Dang and Martin 1993). This protein has a molecular mass of 105 kD and is essential for mitochondrial RNase P function in vivo but has no significant homology to POP1p. The protein composition of RNase P and RNase MRP from higher eukaryotes is less well defined, but they may share a protein of 40 kD, referred to as the Th protein (Gold et al. 1989; Yuan et al. 1989; Karwan 1993). The Th protein has not been sequenced or cloned but may have epitopes in common with the bacterial RNase P proteins, as antibodies against the *E. coli* protein cross-react with a 40-kD protein component of human RNase P (Mamula et al. 1989), which may well be the Th protein. The buoyant density reported for human RNase P (Bartkiewicz et al. 1989) and the sedimentation velocity of the RNase MRP (Karwan 1993) would not be consistent with the presence of only the RNA and a protein of 40 kD, indicating that other protein components are likely to be present. POP1p is the first nuclear RNase P and the only RNase MRP protein for which the gene has been cloned. It is not

clear whether POP1p is the homolog of the Th antigen or represents a different common component.

Incubation of *pop1-1* strains at the restrictive temperature leads to a decrease in the levels of the mature RNase P and RNase MRP RNAs, whereas the longer, putative precursor of RNase P RNA is much less affected. The fact that both forms of the RNase P RNA coprecipitate with the ProtA–POP1 fusion protein shows that POP1p binds efficiently to the longer as well as to the shorter RNase P RNA. POP1 protein binding might stabilize the mature RNase P and RNase MRP RNAs. Alternatively, binding of POP1p to the RNase P RNA precursor could be required for maturation to occur. It is not unusual for RNP proteins to be involved in the maturation or stability of their respective RNAs. In higher eukaryotes, the common U-snRNP Sm proteins are required for modification and transport of the newly synthesized snRNPs (for review, see Mattaj et al. 1993), and in *S. cerevisiae*, the protein components of the U4/U6 and U4/U6/U5 spliceosomal snRNPs, PRP3, PRP4, PRP6, and PRP24 contribute to U6 snRNA stability (Blanton et al. 1992).

POP1p is required for RNase P and RNase MRP function

The *pop1-1* mutation inhibits the function of RNase P and RNase MRP in vivo. Mutations in the RNA components of both RNase P and RNase MRP also inhibit enzymatic activity in vivo, and the combined phenotypes of these mutations resemble very closely that of the *pop1-1* mutation. These RNPs therefore appear to require both RNA and protein components for function. The function of the snRNAs and snoRNAs studied to date also requires an association with the cognate proteins to produce an RNP complex. In *E. coli*, the RNase P protein (C5) is required for the function of the RNP in vivo. C5 plays a role in the binding of at least some substrates (Peck-Miller and Altman 1991) and in release of the product, and also improves the discrimination between binding to the substrate and product (Reich et al. 1988; Tallsjö and Kirsebom 1993; for review, see Altman et al. 1993). In addition, binding of the protein in vitro can suppress mutations in the RNase P RNA (Lumelsky and Altman 1988), indicating that it can alter the structure of the RNA. It has been proposed that a protein component of the *S. cerevisiae* RNase P plays a role in substrate binding (Nichols et al. 1988) and the 100-kD protein that copurifies with the *S. pombe* RNase P activity can interact specifically with the pre-tRNA substrate (Zimmerly et al. 1993).

The kinetics of the inhibition of processing following transfer of *pop1-1* strains to the nonpermissive temperature shows that loss of enzymatic activity is not attributable to the underaccumulation of the RNAs. Cleavage of A3 is lost within 2 hr of transfer to nonpermissive temperature, and clear accumulation of pre-tRNA is also seen at this time. In contrast, the levels of the RNase P and RNase MRP RNAs are not strongly reduced at this time. It remains possible that the *pop1-1* mutation re-

sults in the mislocalization of the RNPs at the nonpermissive temperature, but it is more likely that POP1p has a direct role in catalysis, either by altering the conformation of the RNase P and RNase MRP RNAs or by binding directly to the substrates. The role of POP1p in binding to the pre-tRNA and/or pre-rRNA substrates can now be tested.

A requirement for RNase MRP RNA and POP1p for A3 cleavage

The processing of the 35S rRNA precursor involves a series of cleavage steps that finally generate the mature forms of the 18S, 5.8S, and 25S rRNAs (see Fig. 1B). Cleavage of the yeast pre-rRNA at different processing sites shows distinct requirements for RNPs. The early cleavages at sites A0, A1, and A2 require the activities of the essential snoRNAs, U3, U14, and snR30, together with their associated proteins, fibrillarin (NOP1), SOF1p, and GAR1p (for review, see Fournier and Maxwell 1993). However, no mutations in these components have been found to impair cleavage at site A3 (Henry et al. 1994). In contrast, we have shown here that cleavage of the pre-rRNA at site A3 requires another RNP complex, the MRP RNA and POP1 protein. Cleavage at site A3 has been shown to be required as an entry site for the exonucleases that generate the 5' end of the 5.8S(S) but is not required for the synthesis of 5.8S(L) (see Fig. 1B) (Henry et al. 1994). Consistent with these results, the *rrp2-1* (Shuai and Warner 1991; Lindahl et al. 1992; Schmitt and Clayton 1993) and *pop1-1* mutants are both inhibited in cleavage at A3 and underaccumulate 5.8S(S). In contrast, the level of the 5.8S(L) rRNA is mildly increased in these mutants. In *pop1-1* strains, cleavage at site A3 is absent 2 hr after temperature shift, before other pre-rRNA processing defects are evident and well before any growth defect is observed. This makes it probable that RNase MRP is directly responsible for A3 cleavage in vivo. RNase MRP has been purified from vertebrates and yeast using an assay for the in vitro cleavage of the cognate mitochondrial RNA sequences at the site of priming for DNA replication (Chang and Clayton 1987; Stohl and Clayton 1992). The sequence surrounding A3 is not clearly homologous to this site, perhaps reflecting differences between the specificity of RNase MRP in vitro and in vivo.

The cleavage at site A3 by RNase MRP plays a crucial role in the synthesis of yeast 5.8S rRNA. All of the available evidence shows that the processing of yeast and vertebrate pre-rRNAs is highly conserved. It will therefore be of interest to determine whether RNase MRP cleavage sites also lie upstream of vertebrate 5.8S rRNA genes.

Materials and methods

Strains and media

Growth and handling of *S. cerevisiae* and *E. coli* was by standard techniques. Table 1 lists the yeast strains used in this study.

Mutagenesis

A haploid yeast strain (BSY295) was grown to saturation and washed once with water, and appropriate dilutions were plated on YPD plates to a density of 3000 cells/plate. The plates were irradiated using the Stratalinker (Stratagene) at a dose of 1.8 mJ that results in 5–15% survival. Plates were incubated in the dark for 3 days, and the surviving colonies were replicated at 15°C, 23°C, and 37°C to test for temperature and cold sensitivity. Putative temperature-sensitive and cold-sensitive strains were colony purified and retested for growth at different temperatures and for the loss of specific markers. Of 12.5×10^3 surviving colonies 246 temperature-sensitive strains (2%) and 129 cold-sensitive strains (1%) were isolated; the rate of loss of different markers was in the range of 1.5–2%.

Cloning of POP1

To clone the *POP1* gene, the *pop1-1* temperature-sensitive strain was transformed with a genomic *S. cerevisiae* library in pUN100 (Berges et al. 1994), and cells were directly selected for growth at 37°C. Twelve colonies growing at 37°C were picked, and all were shown to contain a plasmid. Plasmids were recovered in *E. coli*, and restriction map analysis of the inserts showed that they fell into five overlapping groups, ranging in size from 5.5 to 10.5 kb and deriving from the same part of the yeast genome. To localize further the complementing activity, a 3.5-kb *HindIII*–*SacI* fragment, representing the smallest region present in all clones and two further subclones (a 1.5- and a 2.5-kb *HindIII*–*PstI* fragment; see Fig. 4A) were cloned into vector pRS415 (Sikorski and Hieter 1989) and tested for complementation of *pop1-1* temperature sensitivity. The 3.5-kb *HindIII*–*SacI* fragment was the minimal complementing subclone.

Both strands of the 3.5-kb *HindIII*–*SacI* fragment were se-

Table 1. Yeast strains

Strain	Genotype
BSY295	<i>MATa, ura3-52, arg4, leu2-3, 112, ade2, TRP1::SNR14-t*</i>
BSY360	<i>MATa, ura3-52, his3-Δ1, leu2-3, 112, ade2, ade5, TRP1::SNR14-t*</i>
BSY413	<i>MATa, ura3-52, his3-Δ1, leu2-3, 112, ade2, arg4, trp1-289, pop1::TRP1</i> [pRS415, ProtA–POP1]
BSY414	<i>MATa, ura3-52, arg4, leu2-3, 112, ade2, trp1-289, pop1::TRP1</i> [pRS415, ProtA–POP1]
BSY19	<i>MATa/MATa, ura3-52/ura3-52, leu2-3, 112/leu2-3, 112, ade2/ade2, trp1-289/trp1-289, his3-Δ1/HIS3, arg4/ARG4</i>
BSY20	<i>MATa/MATa, ura3-52/ura3-52, arg4/arg4, leu2-3, 112/leu2-3, 112, ade2/ade2, trp1-289/trp1-289</i>
BSY409	<i>MATa/MATa, ura3-52/ura3-52, leu2-3, 112/leu2-3, 112, trp1-289/trp1-289, ade2/ADE2, his3-Δ1/HIS3, arg4/ARG4, pop1::TRP1/POP1</i>
BSY410	<i>MATa/MATa, ura3-52/ura3-52, arg4/arg4, leu2-3, 112/leu2-3, 112, ade2/ade2, trp1-289/trp1-289, pop1::TRP1/POP1</i>

*SNR14-t is a copy of the U4 snRNA gene under the control of the GAL10 regulatory sequences and bearing a neutral tag.

quenced (Sanger et al. 1977) from the original as well as smaller subclones using vector and internal oligonucleotide primers.

To establish linkage of the cloned genomic fragment to *pop1-1*, one of the originally recovered plasmids, containing a 5.5-kb insert, was digested with *XhoI* and *SalI* and religated to remove the CEN-ARS part of the pUN100 vector. The plasmid derived was linearized with *BglIII* and used to transform a wild-type haploid yeast strain (BSY360). Insertion of the *LEU2* marker at the correct chromosomal location was verified by Southern analysis. The strain was mated to *pop1-1*, the diploid was sporulated, and the segregation pattern of *LEU2* and temperature sensitivity was monitored.

To construct a *POP1* null allele, a 2.3-kb *XbaI*–*BstEII* fragment containing most of the *POP1* ORF was replaced by a 854-bp *EcoRI*–*BglIII*, Klenow-treated fragment containing the *TRP1* ORF. In the clone selected, *TRP1* would be transcribed in the opposite orientation to *POP1*. A 3.5-kb linear fragment containing the *pop1::TRP1* allele was excised by digestion with *ClaI* and *SpeI* and used to transform two wild-type diploid strains (BSY19 and BSY320). *TRP*⁺ transformants were tested for correct integration events by Southern analysis. The heterozygous *POP1/pop1::TRP1* diploids obtained (BSY409 and BSY410) were sporulated and dissected. In all 22 tetrads dissected for each diploid, only two of four spores were viable and the growing colonies were always *trp1*.

Construction of the *ProtA*–*POP1* fusion

To construct a *ProtA*–*POP1* fusion, a 395-bp fragment containing two IgG-binding domains of the *S. aureus* protein A was amplified by PCR from plasmid p28NZZtrc (see Grandi et al. 1993) and fused in-frame to the *POP1*-coding sequence either immediately before the first AUG (*ProtA*–*POP1* fusion) or at the carboxyl terminus of *POP1*, after amino acid 775 of the predicted *POP1* ORF (*POP1*–*ProtA* fusion). The original *POP1* promoter and 3′-flanking sequences were retained in these constructs. Both constructs were sequenced to confirm that no mutations were introduced during the PCR amplification steps.

The *ProtA*–*POP1* and *POP1*–*ProtA* fusion genes in plasmid pRS415 were used to transform the heterozygous *pop1::TRP1/POP1* diploids BSY409 and BSY410. Haploids containing *pop1::TRP1* complemented by the fusion gene were obtained only for the *ProtA*–*POP1* construct (strains BSY413 and BSY414).

Immunoprecipitation of *ProtA*–*POP1*

Yeast whole cell extracts were prepared essentially as described (Séraphin and Rosbash 1989). For the immunoprecipitations, 200 μ l of a 50% solution of rabbit IgG agarose beads (Sigma A-2909) were washed three times in IPP150 (150 mM NaCl, 10 mM Tris-HCl at pH 8, 0.1% NP-40, 0.1% Na₂S₂O₃) and then added to 30 OD equivalents of extract brought to 400 μ l in IPP150. After incubating for 2.5 hr at 4°C, the IgG pellets were washed four times with IPP150. The RNAs were extracted from the pellets by adding 400 μ l of proteinase K buffer (100 mM Tris-HCl at pH 7.5, 125 mM EDTA at pH 8, 150 mM NaCl, 1% SDS) and 20 μ g of *E. coli* tRNA as carrier. After three phenol/chloroform extractions, the RNAs were precipitated with ethanol.

RNA extraction, primer extension, and Northern hybridization

RNA was extracted as described previously (Tollervey and Mat-taj 1987). Northern hybridization (Tollervey 1987) and primer extension (Beltrame and Tollervey 1992) were as described previously. Oligonucleotide probes were used for pre-rRNA hybrid-

ization and primer extension. Oligonucleotide a is AT-GAAACTCCACAGTG; oligonucleotide b is CCAGTTAC-GAAAATTCCTTG; oligonucleotide c is TTTCGCTGCGTCTC-TCATC; oligonucleotide d is GCCCAGCAATTTCAAGT; and oligonucleotide e is AGATTAGCCGCGAGTTGG. Oligonucleotide probes were also used for hybridization of tRNA. The mature tRNA^{Leu} probe was GCATCTTACGATACCTG; the 5′-flanking sequence probe was CCAAACAACCACTTATTTGT-TGA; the intron probe was CACAGTTAACTGCGGTC; the mature tRNA^{Ala}UGC probe was CTACCAACTGCGCCATG; the mature tRNA^{Gly}GCC probe was TACCACTAAACCAC-TAGC; the mature tRNA^{Pro}UGG probe was ACCCAGGGCCT-CTCG; and the mature tRNA^{Trp}CCA probe was AACCTG-CAACCCTTCGA.

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The POP1 gene encodes a protein component common to the RNase MRP and RNase P ribonucleoproteins.

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