The EMBO Journal Vol.16 No.2 pp.417–429, 1997

Pop3p is essential for the activity of the RNase MRP and RNase P ribonucleoproteins *in vivo*

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RNase MRP is a ribonucleoprotein (RNP) particle which is involved in the processing of pre-rRNA at site A_3 in internal transcribed spacer 1. Although RNase MRP has been analysed functionally, the structure and composition of the particle are not well characterized. A genetic screen for mutants which are synthetically lethal (sl) with a temperature-sensitive (ts) mutation in the RNA component of RNase MRP *(rrp2-1)* identified an essential gene, *POP3*, which encodes a basic protein of 22.6 kDa predicted molecular weight. Overexpression of Pop3p fully suppresses the ts growth phenotype of the *rrp2-1* allele at 34°C and gives partial suppression at 37°C. Depletion of Pop3p *in vivo* results in a phenotype characteristic of the loss of RNase MRP activity; A_3 cleavage is inhibited, leading to underaccumulation of the short form of the 5.8S rRNA ($5.8S_S$) and formation of an aberrant 5.8S rRNA precursor which is 5′-extended to site A_2. Pop3p depletion also inhibits pre-tRNA processing; tRNA primary transcripts accumulate, as well as spliced but 5′- and 3′-unprocessed pre-tRNAs. The Pop3p depletion phenotype resembles those previously described for mutations in components of RNase MRP and RNase P (*rrp2-1*, *rpr1-1* and *pop1-1*). Immunoprecipitation of epitope-tagged Pop3p co-precipitates the RNA components of both RNase MRP and RNase P. Pop3p is, therefore, a common component of both RNPs and is required for their enzymatic functions *in vivo*. The ubiquitous RNase P RNP, which has a single protein component in Bacteria and Archaea, requires at least two protein subunits for its function in eukaryotic cells.

Keywords: *POP3* gene/RNA processing/RNase MRP/RNase P/*Saccharomyces cerevisiae*

Introduction

Primary RNA transcripts generally undergo a series of post-transcriptional processing reactions before they obtain their mature, functional form. Pre-mRNAs are matured by the excision of intron sequences by the spliceosome (Moore *et al.*, 1993), 3′ end processing and polyadenylation (Wahle and Keller, 1996). Pre-tRNAs are 5′ and 3′ end processed (Altman *et al.*, 1993; Deutscher, 1995) and introns, when present, are removed by endonucleolytic cleavage and ligation reactions (Westaway and Abelson, 1995). The rRNAs are transcribed in the form of a large precursor RNA, and the mature rRNAs are released by many endonucleolytic and exonucleolytic processing activities (Lafontaine and Tollervey, 1995). Many of these RNA processing reactions involve ribonucleoprotein particles (RNPs). The RNase MRP and RNase P RNPs are the only stable RNA–protein complexes identified to date which have been shown to function as endoribonucleases. Their activities are required for the processing of pre-rRNA and pre-tRNAs, respectively.

RNase MRP was identified as an *in vitro* enzymatic activity which cleaves RNA complementary to the mitochondrial origins of replication in vertebrate cells (Chang and Clayton, 1987). Although the role of RNase MRP in mitochondrial DNA replication has not yet been demonstrated unambiguously *in vivo*, this *in vitro* assay allowed the isolation of RNase MRP RNA from several eukaryotes including yeast (Chang and Clayton, 1989; Topper and Clayton, 1990; Bennett *et al.*, 1992; Schmitt and Clayton, 1992; Stohl and Clayton, 1992; Paluh and Clayton, 1995). The cloning of protein subunits was not, however, achieved by biochemical methods. At least 90% of the total RNase MRP content of a cell is located in the nucleolus (Reimer *et al.*, 1987; Yuan *et al.*, 1989; Kiss and Filipowicz, 1992; Topper *et al.*, 1992), and temperature-sensitive (ts) mutations in the MRP RNA component (Shuai and Warner, 1991; Lindahl *et al.*, 1992; Chu *et al.*, 1994), as well as depletion of yeast MRP RNA *in vivo*, (Schmitt and Clayton, 1993) showed that RNase MRP is involved in processing of pre-rRNA.

In eukaryotic cells, three of the four rRNA species are co-transcribed as a single precursor by RNA polymerase I. The processing of the large precursor rRNA does not comprise simple endonucleolytic cuts at the ends of the mature rRNAs, but involves a very complex series of reactions (Figure 1B; for recent reviews, see Eichler and Craig, 1994; Venema and Tollervey, 1995). Two independent processing pathways lead to the production of the major, short form of 5.8S rRNA ($5.8S_S$) and the minor, long form ($5.8S_L$) (Henry *et al.*, 1994), the latter having seven additional nucleotides at the 5′ end; formation of $5.8S_S$, but not $5.8S_L$, requires cleavage of the pre-rRNA at site A_3 in internal transcribed spacer 1 (ITS1). Screening of a bank of ts-lethal yeast strains for mutants which had an altered ratio of $5.8S_S$:$5.8S_L$ led to the identification of Pop1p, a protein subunit of RNase MRP (Lygerou *et al.*, 1994). The direct involvement of RNase MRP in the formation of $5.8S_S$ rRNA, by endonucleolytic cleavage of the pre-rRNA at site A_3 (Figure 1B), has been demonstrated (Lygerou *et al.*, 1996a). Employing immunoaffinity-purified, epitope-tagged Pop1p to purify RNase MRP, synthetically transcribed pre-rRNA substrates could be processed faithfully *in vitro* at cleavage site A_3. Multicopy suppression of a ts mutant in the RNA component of RNase MRP led to the identification of a second protein component of RNase MRP, Snm1p (Schmitt and Clayton, 1994).

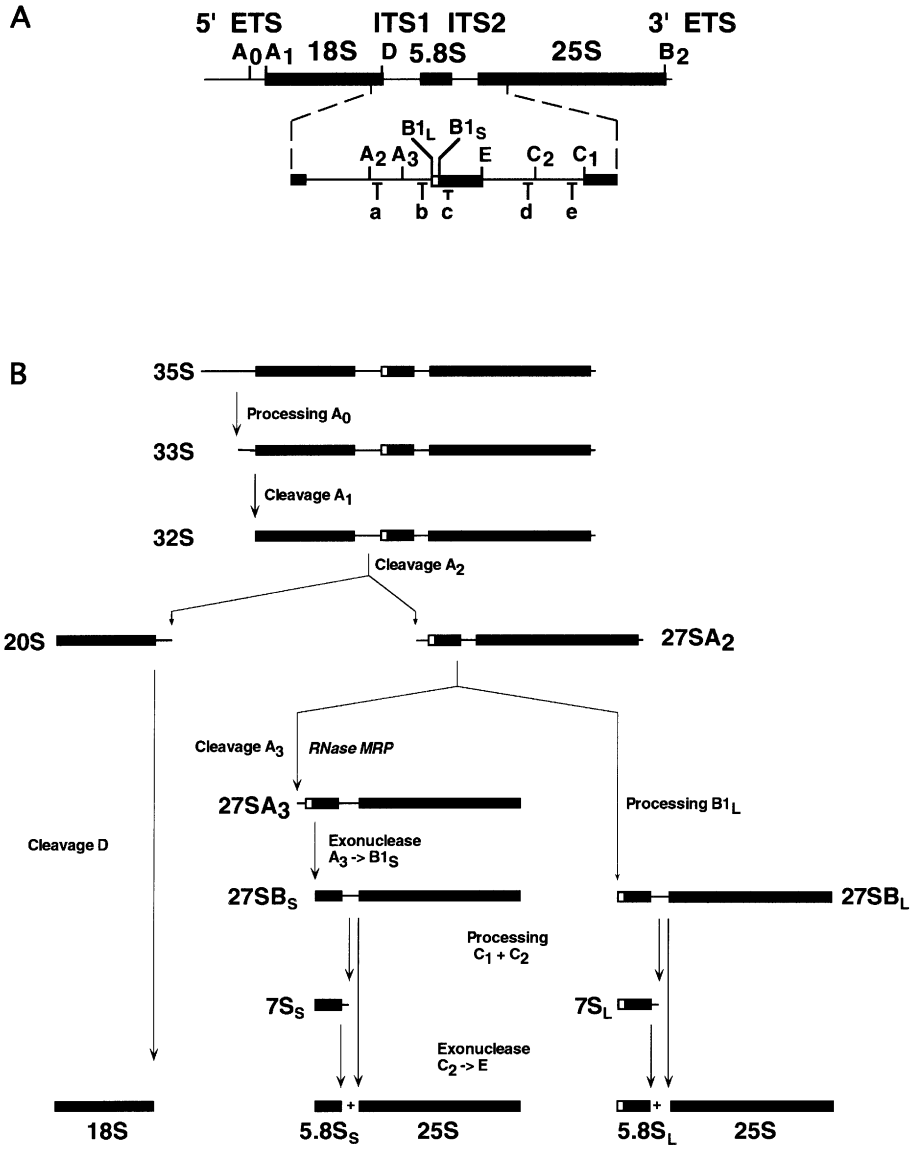

Fig. 1. Structure and processing of the yeast pre-rRNA. (**A**) Structure of the 35S pre-rRNA. Locations of the pre-rRNA cleavage sites (upper case) and the oligonucleotides (lower case, a–e) used for Northern hybridization and primer extension analysis are shown. (**B**) Pre-rRNA processing pathway. The 35S pre-rRNA undergoes snoRNP-dependent cleavages at site A_0 in the 5′ ETS (generating the 33S pre-rRNA), site A_1 at the 5′ end of the 18S rRNA (generating 32S pre-rRNA) and site A_2 in ITS1 (generating the 20S and $27SA_2$ pre-rRNAs). A_2 cleavage separates the pre-rRNA destined to form the small and large ribosomal subunits. The 20S pre-rRNA is cleaved, probably endonucleolytically, to generate the 18S rRNA. The $27SA_2$ pre-rRNA can be processed by two alternative pathways. In the major pathway, $27SA_2$ is cleaved by RNase MRP at site A_3 to generate the $27SA_3$ pre-rRNA. A_3 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, $27SB_S$. An alternative pathway leads to cleavage at site $B1_L$, the 5′ end of the $27SB_L$ pre-rRNA. The processing of both 27SB species appears to be identical. Processing at sites C_1 and C_2 separates the mature 25S rRNA from the 7S pre-rRNA, the latter then undergoes processing by 3′→5′ exonuclease digestion to produce the mature 3′ end of 5.8S rRNA.

In contrast to RNase MRP, which has only been identified in eukaryotes, RNase P is found in all phylogenetic domains. RNase P produces the mature 5′ ends of all tRNAs by endoribonucleolytic cleavage of 5′-extended tRNA precursors (for review, see Altman *et al.*, 1993). Biochemical purification of this activity led to the cloning of RNase P RNA components from many different sources, including *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Xenopus laevis* and HeLa cells (Krupp *et al.*, 1986; Bartkiewicz *et al.*, 1989; Doira *et al.*, 1991; Lee *et al.*, 1991). A 100 kDa protein was identified in association with purified RNase P from *S.pombe* (Zimmerly *et al.*, 1993), but the corresponding gene has not been cloned. The *S.cerevisiae* (Lygerou *et al.*, 1994) and human *POP1* genes have been cloned and the putative *Caenorhabditis elegans* homologue identified (Lygerou *et al.*, 1996b); the overall conservation of these proteins is very low (23% identity between human and *S.cerevisiae*).

The bacterial RNase P enzymes contain only a single protein, C5, which also shows comparably low homology (Altman *et al.*, 1993). Despite this, RNase P activity can be reconstituted with the heterologous RNA and protein components from *Escherichia coli* and *Bacillus subtilis* in functional, hybrid holoenzymes (Guerrier-Takada *et al.*, 1983). This indicates that both the tertiary structure and the function of the protein subunits are conserved. The RNA

component of the bacterial enzyme shows *in vitro* catalytic activity in the absence of the protein subunit (Guerrier-Takada *et al.*, 1983). However, the presence of the protein co-factor stimulates the enzymatic activity considerably *in vitro* and is essential for viability *in vivo*. In contrast, the RNA components of archaeal (Nieuwlandt *et al.*, 1991; LaGrandeur *et al.*, 1993) and eukaryotic RNase P enzymes do not show *in vitro* catalytic activity on their own, indicating a crucial requirement for protein subunits.

RNase MRP and RNase P share structural and functional features. The RNA components of both particles show similar secondary structural elements, in particular the so-called 'cage domain', a pseudo knot structure which contains many of the conserved nucleotides and may represent the catalytic core of the molecule (Forster and Altman, 1990). In yeast and humans, the particles share at least one protein subunit, the Pop1p protein (Lygerou *et al.*, 1994, 1996b). Pop1p has also been shown to contain an antigenic determinant which is recognized by autoimmune sera derived from patients suffering from the connective tissue diseases systemic lupus erythematosus (SLE) and scleroderma (Lygerou *et al.*, 1996b). These Th/To autoimmune sera immunoprecipitate two RNA species which have been shown to be identical to the RNA components of RNase MRP and RNase P (also referred to as 7–2 and 8–2 RNAs, respectively) (Gold *et al.*, 1988, 1989; Yuan *et al.*, 1991), together with a number of proteins, including hPop1p (Lygerou *et al.*, 1996b). Furthermore, RNase P also processes both *in vitro* substrates of RNase MRP, the yeast pre-rRNA (Lygerou *et al.*, 1996a) and the RNA complementary to the mitochondrial D-loop region (Potuschak *et al.*, 1993). Based on the common features of both particles, it has been proposed that RNase MRP arose from RNase P by duplication of the RNase P RNA gene in an early eukaryote (Morrissey and Tollervey, 1995).

We were interested in the identification of new components of RNase MRP and we therefore screened for mutants which are synthetically lethal (sl) with a ts mutation (*rrp2-1*) in the RNA component of RNase MRP (Shuai and Warner, 1991; Lindahl *et al.*, 1992). The *rrp2-1* allele shows a wild-type phenotype when yeast cells are grown at 18°C. At 25°C the mutant strain is viable but cleavage of the pre-rRNA at site A_3 is strongly inhibited (Lygerou *et al.*, 1994). The *POP3* gene was identified by complementation of the sl phenotype of the obtained mutant strains. We show that Pop3p is a protein subunit not only of RNase MRP but also of RNase P, and that Pop3p is required for the function of both enzymes *in vivo*.

Results

Screen for mutants which are synthetically lethal with a temperature-sensitive mutation in the RNA component of RNase MRP

To screen for mutants which are sl with a ts point mutation in the RNA component of RNase MRP (*rrp2-1*) (Shuai and Warner, 1991), we produced a haploid *rrp2-1* yeast strain (YBD1) which contains a centromeric plasmid (pBD1) carrying the *RRP2* gene together with the *URA3* and *ADE3* marker genes. The presence of a functional *ADE3* gene in an *ade2/ade3* mutant background results in a red phenotype of the yeast cells, whereas the strain has a white phenotype in the absence of the *ADE3* marker. Loss of the pBD1 plasmid can, therefore, be monitored by the appearance of white sectors in the yeast colonies (Koshland *et al.*, 1985; Kranz and Holm, 1990).

In order to generate the sl mutant strains, the YBD1 strain was plated on YPD medium, UV irradiated to ~10% survival and grown at 24°C (see Materials and methods). At this temperature, the non-irradiated strain shows a sectoring phenotype, demonstrating that the cells do not depend on the pBD1 plasmid. Approximately 1×10^5 cells survived the mutagenesis, of which 332 displayed a non-sectoring phenotype. To screen for truly plasmid-dependent mutants, these strains were tested for their ability to grow on plates containing 5-fluoro-orotic acid (5-FOA) which counter-selects strains containing a functional *URA3* gene. Twenty-one strains did not grow on 5-FOA plates, indicating that they are truly plasmid dependent. The 21 yeast strains were transformed with a plasmid containing the *RRP2* gene and carrying *LEU2* as a selectable marker (pRRP2-LEU2) or with the corresponding empty vector as a control. Fifteen mutant strains regained sectoring only when transformed with pRRP2-LEU2 and, therefore, carry mutations which are synthetically lethal with *rrp2-1*.

Cloning of the POP3 gene

In order to avoid the isolation of the *RRP2* gene as a complementing activity, we took advantage of the ts-lethal phenotype of *rrp2-1*. Strain SL311 was transformed with a centromeric yeast genomic library and colonies which showed a sectoring phenotype were isolated. The white sectors of these colonies (which have lost the pBD1 plasmid) were tested for the ts-lethal phenotype. Library plasmids carrying the *RRP2* gene are expected to support growth in the white sectors at 37°C, the non-permissive temperature for *rrp2-1*. White sectors from four of the nine independent transformants tested did not grow at 37°C, and the library plasmids from three of these strains were recovered in *E.coli*. All plasmids contained an overlapping region of the yeast genome, which did not include the *RRP2* gene. One library plasmid was mapped with restriction endonucleases, and deletion mutants were constructed (Figure 2A). A minimal 3.5 kb *Hin*dIII fragment complemented the sectoring phenotype (subclone 3, Figure 2A). DNA end sequencing of this fragment revealed part of the *ERG24* gene (Lorenz and Parks, 1992). Using an ordered yeast genomic library (Riles *et al.*, 1993), the 3.5 kb *Hin*dIII fragment was mapped to chromosome XIV. Since the sequence of the chromosomal locus was not available, we sequenced both strands of the 3.5 kb *Hin*dIII fragment, excluding the already published *ERG24* sequence. This region contains two open reading frames (ORFs) of 588 and 462 nucleotides, respectively. A subclone which contains the complete 462 nt ORF (subclone 5, Figure 2A) does not complement sectoring in the SL311 strain, nor does a subclone which excludes nt +446 to +588 (numbering relative to the ATG start codon) of the 588 nt ORF (subclone 4, Figure 2A). We conclude that the 588 nt ORF contains the complementing activity and designated it *POP3*, for processing of precursor RNAs 3 (the designation *POP2* having been used for a gene implicated in the regulation of sugar metabolism).

Using the same cloning strategy as described for SL311, seven plasmids which complemented the sl phenotypes of

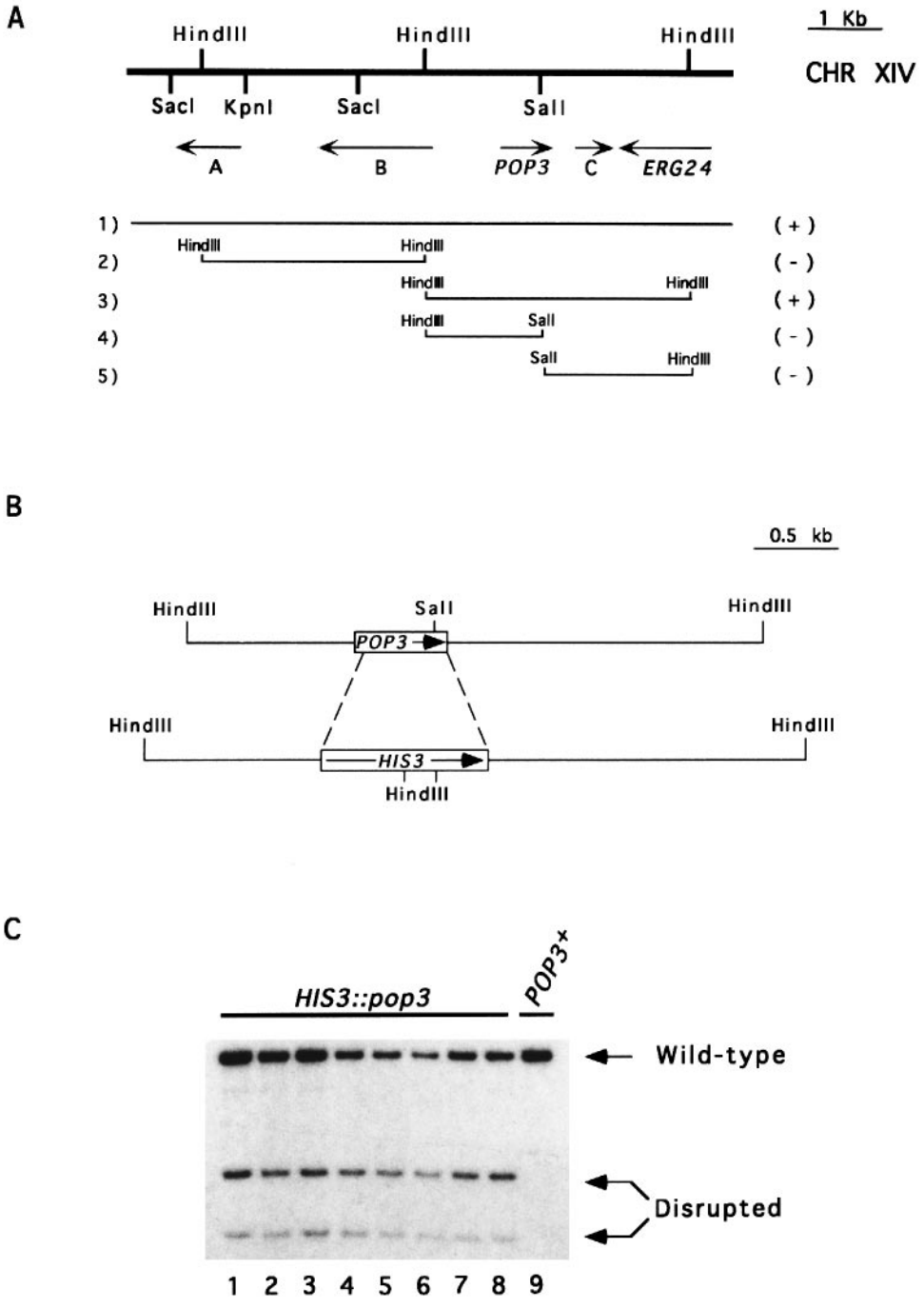

Fig. 2. Characterization of the *POP3* gene locus and disruption of the *POP3* gene. (**A**) Restriction map of the chromosomal locus containing the *POP3* gene. The direction and position of ORFs present in one of the recovered library plasmids which are derived from the complete DNA sequence of yeast chromosome XIV (P.Philippsen *et al.*, in preparation), are shown. The ORF 'A' represents YNL284c, the ORF 'B' represents YNL283c, the ORF 'C' represents YNL281w, the *ERG24* ORF corresponds to YNL280c and the *POP3* ORF corresponds to YNL282w, according to the nomenclature of the complete DNA sequence of yeast chromosome XIV (P.Philippsen *et al.*, in preparation). The lines below the restriction map represent (1) a full-length library plasmid, (2), (3), (4) and (5) subclones which were generated and tested for their ability to restore sectoring to SL311. The ability of the subclones to restore sectoring is indicated by (+) and (–), respectively. (**B**) Schematic representation of the *POP3* gene locus. Employing a one-step PCR method, the *POP3* gene was disrupted by insertion of the *HIS3* marker gene at the *POP3* chromosomal locus. (**C**) Southern blot analysis of diploid *POP3*/*pop3::HIS3* strains (lanes 1–8) and an isogenic wild-type strain (lane 9). *Hin*dIII-digested chromosomal DNA obtained from these strains yielded the expected wild-type and disrupted fragments (a 187 bp *Hin*dIII fragment of the *HIS3* gene is not shown), as indicated.

three other strains (SL88, SL125 and SL194) were isolated. In each case, the complementing plasmids were found to contain *POP3*. We therefore determined whether *POP3* also complements the sectoring phenotype of the remaining 11 sl strains. Ten of the 11 remaining strains had a sectoring phenotype when transformed with the *POP3* gene (shown for SL311 in Figure 3A and data not shown).

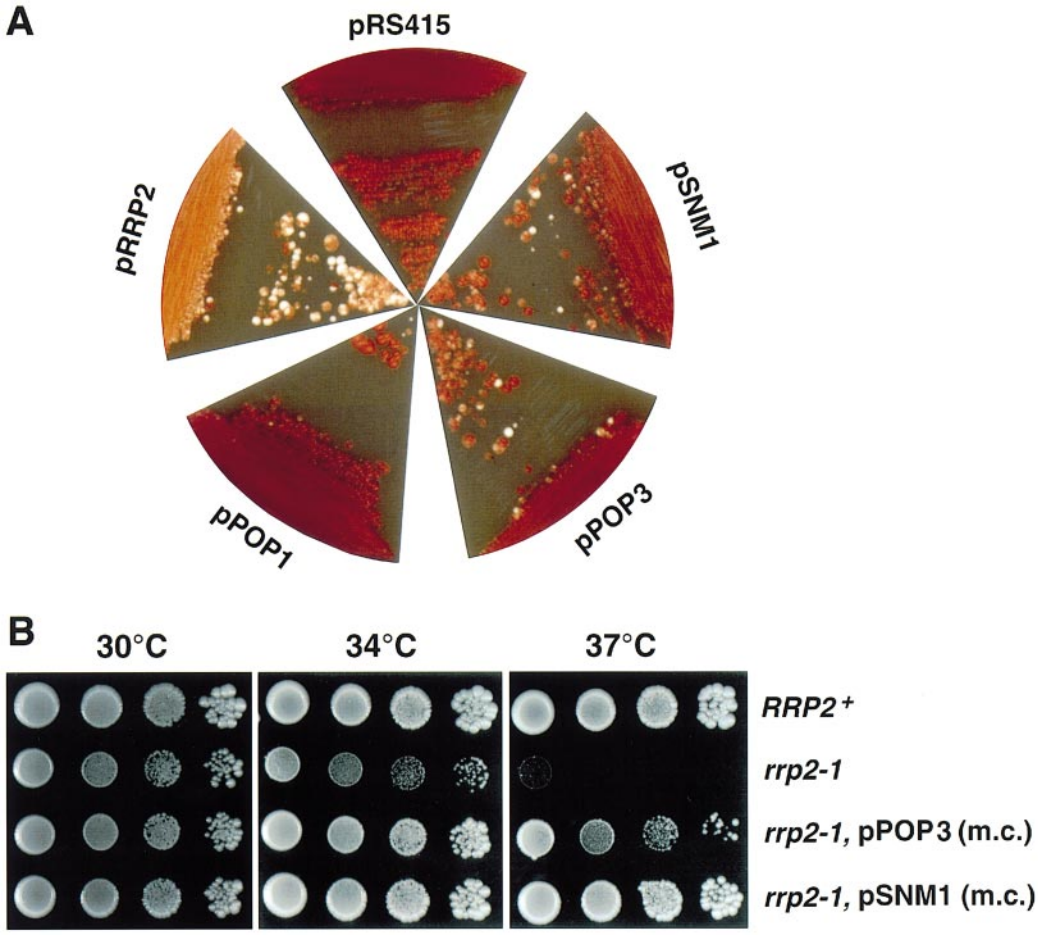

Fig. 3. *POP3* restores sectoring in SL311 and suppresses the ts growth phenotype of the *rrp2-1* allele. (**A**) The sl strain SL311 was transformed with the single copy plasmids pRS415, pRS415-LEU2-RRP2 (pRRP2), pRS415-LEU2-POP1 (pPOP1), pRS415-LEU2-POP3 (pPOP3) or pMES194-LEU2-SNM1 (pSNM1), streaked on YPD (4% glucose) plates and incubated at 24°C for 10 days. (**B**) Serial dilutions (10-fold dilutions from left to right) were spotted onto SD-leu plates and incubated at 30, 34 or 37°C. *RRP2*$^+$ (YBD3) is an otherwise isogenic wild-type strain of the *rrp2-1* mutant strain (YBD2); both strains contain the empty pRS425 vector. pPOP3 (YBD20) and pSNM1 (YBD21) indicate *rrp2-1* strains (YBD2) in which these proteins are overexpressed from multicopy plasmids (m.c. = multicopy).

POP3 encodes a 22.6 kDa protein which is essential for cell viability

The *POP3* gene encodes a basic protein (pI 9.64) of 22.6 kDa predicted molecular weight. Comparison of the *POP3* sequence with sequences from databases did not reveal clear similarity to any known protein. We used a one-step PCR method (see Materials and methods; Baudin *et al.*, 1993) to disrupt the *POP3* gene with the *HIS3* marker gene in a diploid yeast strain which carries a total deletion of the *HIS3* locus (Figure 2B). Correct integration of the *HIS3* marker gene at the *POP3* locus was confirmed by PCR (data not shown) and Southern blot analysis (Figure 2C). From one heterozygous diploid strain, 22 tetrads were dissected, all of which gave rise to only one or two viable spores (data not shown). All viable progeny were His$^-$. When the disrupted diploid strain was transformed with the wild-type *POP3* gene on a centromeric plasmid carrying the *URA3* marker, the tetrads gave rise to mostly three or four viable spores. All progeny which showed a His$^+$ phenotype were non-viable on medium containing 5-FOA, showing that the haploid strains carrying the *pop3::HIS3* allele are plasmid dependent. *POP3* is, therefore, essential for cell viability.

POP3 suppresses the ts growth phenotype of the rrp2-1 allele

To determine whether the sl mutation in strain SL311 lies in *POP3*, chromosomal DNA from this strain was used as a template to PCR amplify and clone the *POP3* gene. The gene was sequenced fully in DNA obtained from two independent *E.coli* transformants. No identical mutations were present in the clones; two altered nucleotides were observed only in a single clone and can be attributed to infidelities during PCR amplification of the gene (data not shown). We conclude that in strain SL311, the chromosomal *POP3* gene is wild-type and the *POP3* gene present on the *CEN* plasmid is acting as a low copy number suppressor of the sl phenotype. In has not yet been determined whether this is also the case for the remaining sl strains.

The ability of *POP3* to suppress the ts-lethal phenotype of the *rrp2-1* mutation was also tested. When present on a low copy plasmid, *POP3* confers only very weak suppression of the *rrp2-1* growth phenotype at 34°C (data not shown). Expression of *POP3* from a high copy plasmid fully suppresses the lethality of *rrp2-1* for growth at 34°C, but only partially suppresses lethality at 37°C (Figure 3B).

In contrast, the *SNM1* gene, which was identified as a multi-copy suppressor of a mutation in *RRP2* (Schmitt and Clayton, 1994), suppresses the growth defect to wild-type level at 37°C (Figure 3B). Overexpression of Pop3p, therefore, partially suppresses the effects of a point mutation in the RNA component of RNase MRP.

The Pop1p and Snm1p proteins have been shown previously to be components of RNase MRP (Lygerou *et al.*, 1994; Schmitt and Clayton, 1994). To determine whether these genes complement the sl mutations, the mutant strains were transformed with centromeric plasmids carrying the *LEU2* marker and either the *POP1* or *SNM1* gene. All sl strains maintained the red phenotype when transformed with the *POP1* plasmid, indicating that the sl phenotypes are not due to mutations in *POP1*. In contrast, all 14 sl strains which are complemented by *POP3* also regained the sectoring phenotype when transformed with the *SNM1* plasmid. The *SNM1* gene would not have been recovered as a clone which complements the sl mutations since it suppresses the ts-lethality of *rrp2-1*, even when present on a single copy plasmid (data not shown), and would, therefore, have been discarded (see above). It is has not yet been determined whether the sl mutations lie in *SNM1* or whether it is also acting as a low copy number suppressor.

Pop3p is a common protein component of the RNase MRP and RNase P ribonucleoproteins

The ability of the *POP3* gene to suppress the ts lethality of the *rrp2-1* allele suggested a possible physical interaction between the gene products. To test this, we constructed a chimeric *POP3* fusion gene encoding two IgG binding domains of protein A from *Staphylococcus aureus* fused in-frame with the N-terminus of the *POP3* ORF. This fusion gene (*ProtA–POP3*) was expressed on a centromeric plasmid (carrying the *TRP1* marker gene) under the control of the homologous *POP3* promoter in a *POP3/pop3::HIS3* diploid yeast strain. Tetrad dissection resulted in mostly three or four viable spores. All spores which showed a His^+ phenotype were also Trp^+, showing that the *ProtA–POP3* construct is able to complement the *pop3::HIS3* disruption. No growth difference was observed between the wild-type strain and a strain in which the chromosomal *pop3::HIS3* allele is complemented by the plasmid-borne *ProtA–POP3* gene, showing the ProtA–Pop3p fusion protein to be fully functional. Western blot analysis confirmed that the fusion protein is expressed with the expected apparent mol. wt of 37 kDa (data not shown).

Immunoprecipitation experiments were performed (see Materials and methods) using cell lysates derived from the *ProtA–POP3* strain (YBD32). As controls, a *ProtA–POP1* strain (BSY414), a *ProtA–NOP1* strain and a non-tagged wild-type strain were used. Total RNA was extracted from equivalent amounts of mock-depleted total lysates (Figure 4, lanes 1, 4, 7 and 10), immune supernatants (Figure 4, lanes 2, 5, 8 and 11) and precipitated IgG–agarose pellets (Figure 4, lanes 3, 6, 9 and 12) and then analysed by Northern hybridization. Approximately 50% of the RNase MRP RNA was co-precipitated in the pellet fraction derived from the ProtA–Pop3p lysate (Figure 4, lane 3). Since the RNase MRP and RNase P RNPs have been shown to share the Pop1p component (Lygerou *et al.*, 1994), we also tested for the association

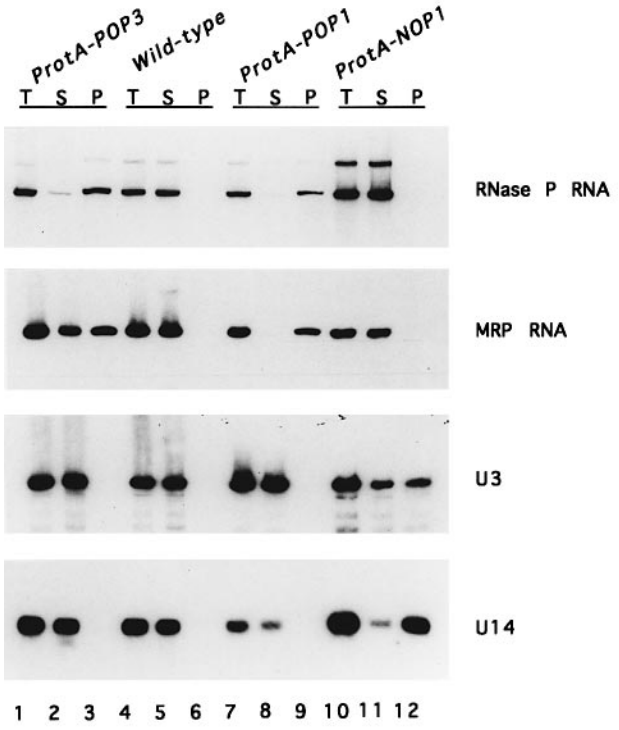

Fig. 4. Co-precipitation of the RNase P and RNase MRP RNAs in the *ProtA–POP3* strain. Cell lysates were prepared from strains expressing ProtA–Pop3p (YBD32), ProtA–Pop1p (BSY414), ProtA–Nop1p and a wild-type strain. Immunoprecipitation experiments were performed as described in Materials and methods and total RNA was extracted from equivalent amounts of mock-depleted total lysates (lanes 1, 4, 7 and 10), immuno-supernatants (lanes 2, 5, 8 and 11) and IgG–agarose-precipitated pellets (lanes 3, 6, 9 and 12). RNA was separated on a polyacrylamide gel and transferred for Northern hybridization. Oligonucleotides used for hybridization correspond to the RNAs indicated on the right of the figure. The upper band in the RNase P RNA panel represents a 5′- and 3′-extended form of RNase P RNA which is probably the precursor of the mature RNase P RNA.

of Pop3p with RNase P RNA. More than 90% of the RNase P RNA was co-precipitated from the ProtA–Pop3p lysate (Figure 4, lane 3). The 5′- and 3′-extended, putative precursor of RNase P RNA (Lee *et al.*, 1991) was also co-precipitated (Figure 4, lane 3). In contrast, we did not detect precipitation of snoRNAs U3, U14, U24, snR10 or snR30 (Figure 4, lane 3 and data not shown) with ProtA–Pop3p. As expected, RNase MRP RNA and RNase P RNA were co-precipitated efficiently and specifically from the ProtA–Pop1p lysate (Figure 4, lane 9) (Lygerou *et al.*, 1994), while the snoRNAs were co-precipitated only with ProtA–Nop1p (Figure 4, lane 12 and data not shown) (Schimmang *et al.*, 1989). Some variation in the efficiency of co-precipitation of the U3 and U14 snoRNAs with ProtA–Nop1p was observed (Figure 4, lane 12); variation in the efficiency of precipitation of different snoRNAs with anti-Nop1p antibodies has also been reported (Schimmang *et al.*, 1989). No precipitation of any RNA species was observed in the lysate derived from the non-tagged strain. These data show clearly that ProtA–Pop3p specifically and efficiently co-precipitates the RNase MRP and RNase P RNAs. We conclude that Pop3p is a common protein component of RNase MRP and RNase P.

Construction of a conditional POP3 allele

In order to assess whether Pop3p is required for the function of RNase MRP and RNase P, we constructed a

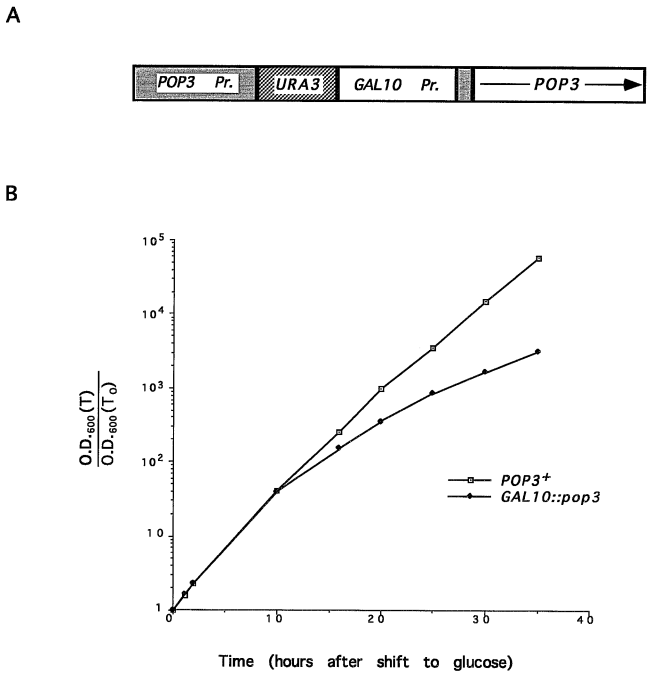

Fig. 5. Construction and growth curve of a conditional *GAL10::pop3* strain. (**A**) Representation of the chromosomal *POP3* gene locus (not to scale) in the *GAL10::pop3* strain. A *URA3–GAL10* promoter cassette was inserted 20 nucleotides upstream of the *POP3* ATG start codon, using PCR-generated restriction sites. The linear fragment represented was targeted to the chromosomal *POP3* gene locus in a wild-type yeast strain (BWG1-7A). (**B**) Growth curve of the *GAL10::pop3* strain (YBD34, filled symbols) and the otherwise isogenic wild-type strain (BWG1-7A, open symbols) after shift from permissive galactose medium to repressive glucose medium.

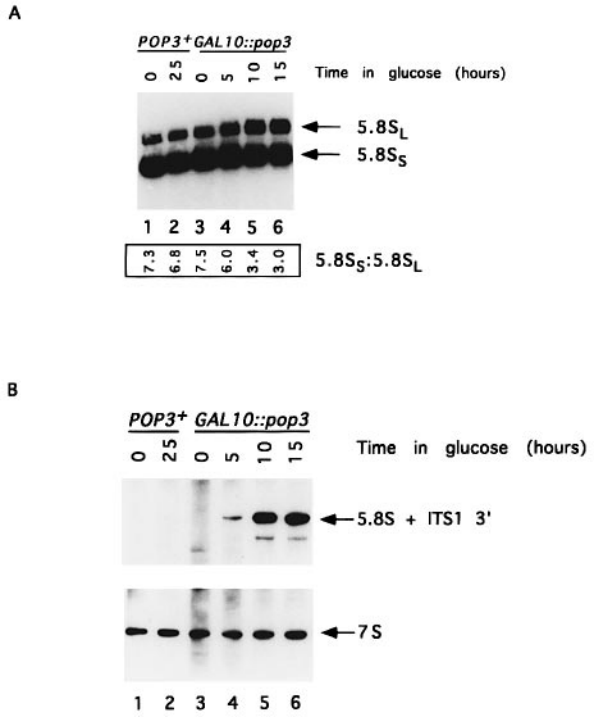

Fig. 6. Pop3p depletion inhibits pre-rRNA processing. Equal amounts of total RNA obtained from a wild-type strain (BWG1-7A) after growth on galactose medium (lane 1) or following growth for 25 h on glucose medium (lane 2), as well as RNA from the *GAL10::pop3* strain (YBD34) after growth on galactose medium (lane 3), or following growth on glucose medium for 5 (lane 4), 10 (lane 5) and 15 h (lane 6), were separated on a polyacrylamide gel and transferred for Northern hybridization. (**A**) Hybridization with oligonucleotide c (see Figure 1A) against the mature 5.8S rRNA species. The ratio of the steady-state levels of $5.8S_S$:$5.8S_L$ has been quantitated with a phosphor-imager (Molecular Dynamics) and the values obtained are indicated. (**B**) Hybridization with oligonucleotide a (see Figure 1A) complementary to a region of ITS1 between sites A_2 and A_3 (upper panel), and oligonucleotide d (see Figure 1A) complementary to the 5′ region of ITS2 (lower panel). In the upper panel, an aberrant 5.8S pre-rRNA molecule is detected which is 5′ extended to site A_2 in ITS1. The lower panel shows the normal 7S rRNA precursor which represents 5.8S rRNA 3′ extended to site C_2 in ITS2.

conditional *POP3* allele. The chromosomal *POP3* gene was placed under the control of the repressible *GAL10* promoter (Figure 5A) in a haploid yeast strain (*GAL10::pop3*, YBD34) (see Materials and methods). Correct integration of the *URA3–GAL10* fragment at the *POP3* locus was confirmed by Southern hybridization (data not shown). The *GAL10* promoter is induced when cells are grown in galactose medium, whereas transcription is repressed in glucose medium. Figure 5B shows growth curves obtained with a *GAL10::pop3* strain and an otherwise isogenic wild-type strain after shift from the permissive galactose to the repressive glucose medium. During the first 10 h after shift to glucose medium, both strains exhibited a doubling time of 2.5 h. At later time points, the doubling time of the *GAL10::pop3* strain steadily increased, while the wild-type control strain continued growth at the initial rate. After 30 h of repression, the *GAL10::pop3* strain had a doubling time of 5 h. Since the *GAL10::pop3* strain did not cease growth completely, repression appears to be leaky. Repression of *GAL*-regulated genes is generally not complete (Jansen *et al.*, 1993; Lafontaine *et al.*, 1995).

Depletion of Pop3p in vivo results in phenotypes characteristic of the loss of both RNase MRP and RNase P activities

To analyse the effects of Pop3p depletion on pre-rRNA and pre-tRNA processing, yeast cells from the *GAL10::pop3* strain and the isogenic wild-type strain were harvested at regular intervals following the shift from galactose to glucose medium. Total RNA was extracted and analysed by Northern hybridization and primer extension.

A probe complementary to the mature 5.8S rRNA species reveals a clear change in the $5.8S_S$:$5.8S_L$ ratio in the *GAL10::pop3* strain during growth in glucose medium (Figure 6A). The ratio of these rRNA species in wild-type strains is ~7:1 but decreases in the *GAL10::pop3* strain to 3:1 after 15 h in glucose medium, showing under-accumulation of the major, short form of 5.8S rRNA ($5.8S_S$). An oligonucleotide which hybridizes between sites A_2 and A_3 (oligonucleotide a, see Figure 1A) detects an aberrant precursor of 5.8S rRNA which is 5′ extended to site A_2 in ITS1, after 5 h of Pop3p depletion (Figure 6B, upper panel, lanes 4–6). The accumulation of this rRNA species would be predicted if pre-rRNA processing at site A_3 is inhibited while cleavage at site A_2 and processing in ITS2 continue. In contrast, no changes in the levels of the normal 7S precursor (5.8S rRNA which is 3′-extended to site C_2 in ITS2, see Figure 1B) were observed in the *GAL10::pop3* strain (Figure 6B, lower panel). These results indicate that A_3 cleavage is inhibited in the Pop3p-depleted strain. To evaluate the levels of

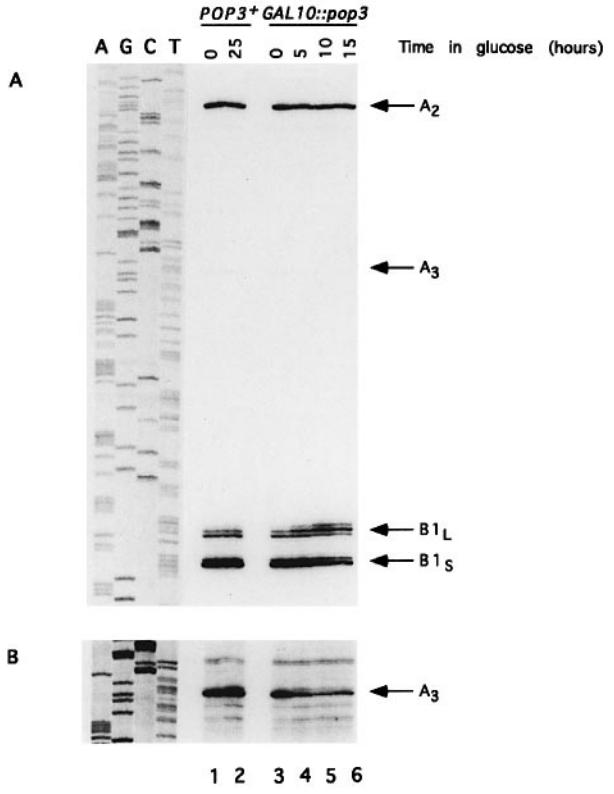

Fig. 7. Primer extension analysis through site A_3. (**A**) Primer extension analysis was performed, employing oligonucleotide d (see Figure 1A) which hybridizes 5′ of site C_2 in ITS2. Total RNA derived from the *GAL10::pop3* strain and the isogenic wild-type strain was used as described in the legend of Figure 6 (in the same lane order). The processing sites revealed as major primer extension stops are indicated on the right. DNA sequencing reactions on a wild-type rDNA plasmid using the same primer are indicated. (**B**) Over-exposure of the autoradiogramm presented above, in order to visualize the weaker primer extension stop at site A_3.

A_3 cleavage during Pop3p depletion, primer extension analysis was performed using oligonucleotide d (see Figure 1A) on total RNA from the *GAL10::pop3* strain and the otherwise isogenic wild-type strain. Oligonucleotide d is complementary to the pre-rRNA in ITS2, 5′ of cleavage site C_2, and primer extension products show the levels of pre-rRNAs processed at $B1_L$, $B1_S$ and at cleavage sites within ITS1. Figure 7 shows that pre-rRNA processing at site A_3 is reduced several-fold after 10 h of growth in glucose medium (Figure 7B, lanes 3–6). The primer extension stop at site $B1_S$ is also reduced (Figure 7A, lanes 3–6), in agreement with the under-accumulation of $5.8S_S$. The stop at site $B1_L$ is clearly increased after 10 h in glucose (Figure 7A, lanes 3–6) and a displacement by one nucleotide 5′ is observed (Figure 7A, lanes 3–6). The basis of this displacement is unclear, but it is also observed in strains carrying mutations in *POP1* and *RRP2* and is, therefore, diagnostic of a loss of RNase MRP activity. In contrast, the primer extension stop at site A_2 is not affected in the *GAL10::pop3* strain (Figure 7A, lanes 3–6). No alteration in the level of pre-rRNA cleaved at A_0 was observed and the levels of the other pre-rRNA species, 35S, 32S, $27SA_2$ or 20S and mature rRNAs 18S and 25S were unaffected by Pop3p depletion (data not shown). We conclude that Pop3p is required specifically for the cleavage of the pre-rRNA at site A_3. The pre-rRNA processing phenotype of the Pop3p-depleted strain resembles that observed in strains depleted of the RNA component of RNase MRP or carrying the *rrp2-1* mutation (Shuai and Warner, 1991; Lindahl *et al.*, 1992; Schmitt and Clayton, 1993) and in strains carrying the *pop1-1* mutation (Lygerou *et al.*, 1994).

The ability of the overexpression of Pop3p and Snm1p to suppress the A_3 cleavage defect of the *rrp2-1* mutation was assessed (Figure 8). The level of the $27SA_3$ pre-rRNA was analysed by primer extension on RNA extracted from an *rrp2-1* strain in which the *POP3* gene is present on a high copy number plasmid, following growth at 34°C (Figure 8A). Although the *rrp2-1* growth defect is fully suppressed by *POP3* at this temperature (Figure 3B), the level of the $27SA_3$ is not increased detectably. Similarly, the ratio $5.8S_S$:$5.8S_L$ is also only slightly increased in the *rrp2-1* strain by overexpression of *POP3* (Figure 8B). This confirms the previous report (Henry *et al.*, 1994; reviewed by Tollervey, 1996) that the essential function of RNase MRP is not the cleavage of pre-rRNA at site A_3. In contrast, overexpression of Snm1p does increase the level of the $27SA_3$ pre-rRNA (Figure 8A) and substantially increases the ratio $5.8S_S$:$5.8S_L$ (Figure 8B).

The effects of Pop3p depletion on pre-tRNA processing were also assessed. A Northern blot was probed with an oligonucleotide complementary to the mature form of $tRNA_3^{Leu}$ (Figure 9). As early as 5 h after transfer to glucose medium, the pre-$tRNA_3^{Leu}$ primary transcript strongly accumulates, as does the processing intermediate which is spliced but 5′ and 3′ unprocessed (+5′, +3′); the lower band which also accumulates may represent a 5′-unprocessed, 3′-trimmed species. In contrast, the pre-tRNA species which is 5′ and 3′ mature but non-spliced (+IVS) is depleted (Figure 9A). Following 10 h of growth in glucose medium, the level of mature $tRNA_3^{Leu}$ is also reduced in the *GAL10::pop3* strain (Figure 9B). The reduction in the level of mature $tRNA_3^{Leu}$ is comparable with the reduction in the ratio of mature $5.8S_S$:$5.8S_L$ rRNA (Figure 6A).

We also analysed two other spliced tRNAs ($tRNA_{CCA}^{Trp}$ and $tRNA_{UGG}^{Pro}$), two non-spliced tRNAs ($tRNA_{UGC}^{Ala}$ and $tRNA_{GCG}^{Gly}$) and a dimeric tRNA transcript ($tRNA^{Arg}$–$tRNA^{Asp}$). The processing defects observed for these tRNA species were consistent with the results obtained from $tRNA_3^{Leu}$ (data not shown), showing that the phenotype observed in the *GAL10::pop3* strain is not limited to a certain tRNA species. We conclude that Pop3p is required for the 5′ and 3′ processing of pre-tRNAs. The pre-tRNA processing phenotype of the Pop3p-depleted strain resembles that observed in strains carrying mutations in the RNA component of RNase P (Lee *et al.*, 1991) and in the Pop1p component (Lygerou *et al.*, 1994).

Components of RNPs are sometimes required for the biogenesis or stability of the complex. The snRNPs involved in pre-mRNA splicing require the common Sm proteins for their biogenesis (for review, see Mattaj *et al.*, 1993) and the U6 snRNA requires the snRNP proteins Prp3p, Prp4p, Prp6p and Prp24p for its stability (Blanton *et al.*, 1992). We therefore determined the levels of RNase MRP and RNase P RNA during Pop3p depletion (Figure 10). No decrease in the levels of the RNA components of RNase MRP or RNase P was observed. Constant levels of the snoRNAs U3 and U14 were also observed in the

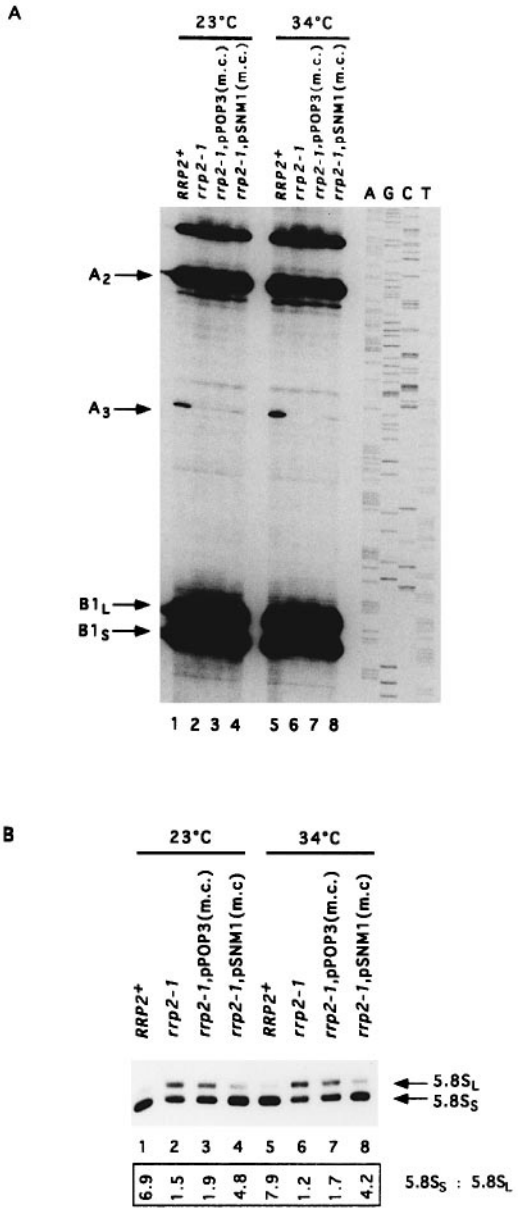

Fig. 8. *POP3* is not a suppressor of the A_3 processing defect of *rrp2-1*. (**A**) Primer extension analysis through site A_3 using oligonucleotide d (see Figure 1A) on total RNA derived from the indicated yeast strains which were grown at 23°C (lanes 1–4) and shifted to 34°C for 6 h (lanes 5–8) respectively. *RRP2*$^+$ (YBD3, lanes 1 and 5) is an otherwise isogenic wild-type strain of the *rrp2-1* mutant strain (YBD2, lanes 2 and 6). pPOP3 (YBD20, lanes 3 and 7) and pSNM1 (YBD21, lanes 4 and 8) indicate the overexpression (m.c. = multicopy) of these proteins in the *rrp2-1* mutant strain (YBD2). The processing sites revealed as major primer extension stops are indicated on the left. (**B**) Total RNA derived from yeast strains YBD3 (lanes 1 and 5), YBD2 (lanes 2 and 6), YBD20 (lanes 3 and 7) and YBD21 (lanes 4 and 8), as described in (A), was separated on a polyacrylamide gel, transferred to a Hybond N^+ filter and probed with an oligonucleotide against the mature form of 5.8S rRNA (oligonucleotide c, Figure 1A). The ratio of the steady-state levels of $5.8S_S$:$5.8S_L$ has been quantitated with a phosphor-imager (Molecular Dynamics) and the obtained values are indicated.

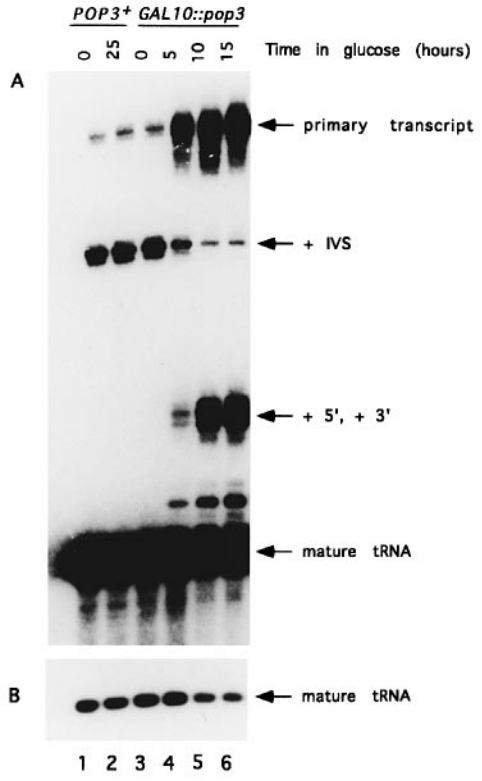

Fig. 9. Pop3p depletion inhibits pre-tRNA processing. (**A**) Northern hybridization using an oligonucleotide complementary to the mature $tRNA_3^{Leu}$. Total RNA derived from the *GAL10::pop3* strain and the isogenic wild-type strain was used as described in the legend of Figure 6 (in the same lane order). The RNA was separated on a polyacrylamide gel, transferred to a Hybond N^+ filter and hybridized. The positions of the primary transcript of $tRNA_3^{Leu}$, as well as the 5′- and 3′-processed but non-spliced precursor (+ IVS), the spliced but 5′- and 3′-unprocessed precursor (+ 5′, + 3′) and the mature $tRNA_3^{Leu}$ are indicated on the right. (**B**) Shorter exposure of the autoradiogram shown above in order to visualize the under-accumulation of mature $tRNA_3^{Leu}$ after 10–15 h of Pop3p depletion (lanes 5 and 6).

GAL10::pop3 strain (Figure 10). This strongly indicates that the loss of RNase MRP and RNase P functions is not a consequence of a requirement for Pop3p in the biogenesis or stability of either particle. Pop3p is, therefore, required for the function of the RNase MRP and RNase P RNPs *in vivo*.

Discussion

A genetic screen was performed in order to identify novel proteins which physically and/or functionally interact with RNase MRP RNA or the RNase MRP particle. Fifteen mutant strains were obtained which are synthetically lethal with a ts mutation (*rrp2-1*) in the RNA component of RNase MRP. To isolate genes which complement the sl phenotypes, four strains were transformed with a genomic library in a low copy number (*CEN*) vector. This led to the isolation of the *POP3* gene from all four sl strains. The *POP3* gene also complements the sl phenotype of 10 of the 11 remaining strains. One strain which is complemented by *POP3* (SL311) was chosen for detailed analysis. To determine whether the sl mutation in SL311 lies in *POP3*, the chromosomal gene was cloned and fully sequenced. No mutations were found, showing that *POP3* is acting as a low copy number suppressor of the sl phenotype in SL311. The presence of *POP3* on a low copy number plasmid also confers weak suppression of the ts-lethal phenotype of the *rrp2-1* mutation; when

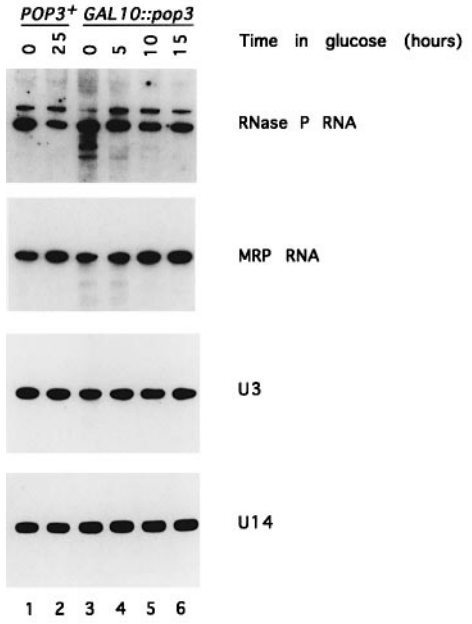

Fig. 10. Steady-state levels of RNase MRP RNA, RNase P RNA and the snoRNAs U3 and U14 during Pop3p depletion. Total RNA derived from the *GAL10::pop3* strain and the isogenic wild-type strain was used as described in the legend of Figure 6 (in the same lane order), separated on a polyacrylamide gel, transferred for Northern hybridization and probed with oligonucleotides complementary to the RNA species indicated on the right.

present on a high copy number vector, *POP3* gives good suppression of *rrp2-1* at 34°C but not at 37°C.

Pop3p is physically associated with RNase MRP *in vivo*, as shown by the co-immunoprecipitation of RNase MRP RNA with ProtA–Pop3p. Furthermore, the RNA component of RNase P, as well as a 5′- and 3′-extended putative precursor, are also co-precipitated with ProtA–Pop3p. This demonstrates that, like Pop1p, Pop3p is common to RNase MRP and RNase P. The RNase MRP RNA is co-precipitated less efficiently with ProtA–Pop3p than is RNase P RNA; this may be due to differences in the accessibility of the protein A moiety in the RNP particles. Similar variation in the efficiency of co-precipitation of other snoRNAs with ProtA–Nop1p was observed.

All of the sl strains to which sectoring is restored by the *POP3* gene, including SL311, also show a sectoring phenotype when transformed with the *SNM1* gene. *SNM1* was isolated as a multicopy suppressor of an *rrp2* mutation and Snm1p is physically associated with RNase MRP but not with RNase P (Schmitt and Clayton, 1994). In contrast, the presence on a plasmid of the *POP1* gene, which encodes another protein component of RNase MRP, does not complement the sl phenotypes of any of the mutant strains. It seems likely that both *POP3* and *SNM1* act as suppressors of the sl phenotype because increased expression of the proteins allows more efficient assembly with the mutant MRP RNA component.

Depletion of Pop3p *in vivo* inhibits the functions of both RNase MRP and RNase P. The major pre-rRNA processing pathway, which leads to the formation of the $5.8S_S$ rRNA, requires endoribonucleolytic cleavage by RNase MRP (Lygerou *et al.*, 1996a). Previously described mutants in the MRP RNA (Shuai and Warner, 1991; Lindahl *et al.*, 1992; Chu *et al.*, 1994) and the Pop1p (Lygerou *et al.*, 1994) component of RNase MRP result in inhibition of this cleavage event. Here we show that depletion of Pop3p results in a phenotype which is consistent with the inhibition of RNase MRP function. Pre-rRNA cleavage at site A_3 is specifically inhibited, resulting in an altered ratio of the $5.8S_S$:$5.8S_L$ rRNAs and the accumulation of an aberrant pre-rRNA molecule which is 5′ extended to site A_2 in ITS1. Likewise, mutants in the RNA (Lee *et al.*, 1991) or protein component (Lygerou *et al.*, 1994) of RNase P show a tRNA processing phenotype which resembles the depletion phenotype of the *GAL10::pop3* strain. Pre-tRNAs accumulate which are 5′ and 3′ unprocessed while pre-tRNA splicing continues. This demonstrates that Pop3p is not only physically associated with both particles, but is also required for the *in vivo* functions of both enzymes.

What role does Pop3p play in the complexes? Pop3p is not simply required for the stability or the maturation of the RNP complexes or RNA components, since depletion of the protein does not affect the cellular levels of either RNA component. We cannot formally exclude a role for Pop3p in the correct cellular localization of the RNPs, but this does not appear very probable.

The *POP3* gene is essential for cell viability, as is the case for all protein or RNA components which have been identified as constituents of either RNase MRP or RNase P. The role of RNase P in the maturation of the 5′ end of all tRNAs is clearly essential (Altman *et al.*, 1993). This is not the case for RNase MRP. The cleavage of pre-rRNA at site A_3 is a non-essential event, since cells which lack A_3 cleavage due to a mutation in *cis* deleting the cleavage site are viable (Henry *et al.*, 1994). The reported function of RNase MRP in mitochondrial DNA replication is also non-essential, since yeast cells lacking mitochondrial DNA are viable. Therefore, we predict that there is another, so far unidentified, function for the RNase MRP enzyme. This is supported by our observation that the overexpression of the *POP3* gene in a *rrp2-1* ts strain at 34°C restores cell viability but does not restore A_3 cleavage.

The Pop3p protein does not exhibit significant similarity to any known protein. In Bacteria and Archaea, RNase P contains a single protein of ~14 kDa (Altman *et al.*, 1993). The mitochondrial RNase P from *S.cerevisiae* also requires only a single protein component (Rpm2p) of 100.5 kDa for its activity (Morales *et al.*, 1992; Dang and Martin, 1993). In eukaryotes, however, at least two proteins are required for nuclear RNase P function (Pop1p and Pop3p). This is an unexpected result, since biochemical purification of nuclear RNase P activity from *S.pombe* resulted in the co-purification of RNase P RNA and a single 100 kDa protein (Zimmerly *et al.*, 1993), which is similar in size to yPop1p and hPop1p (Lygerou *et al.*, 1996b); a protein similar in size to Pop3p (22.6 kDa) was not observed in these experiments.

The identification of Pop3p as a common protein subunit of the RNase MRP and RNase P RNPs strongly supports the hypothesis that both particles are closely related in evolution (Morrissey and Tollervey, 1995). Although the primary sequences of the MRP and P RNAs are not well conserved, they have similar secondary structures (Forster and Altman, 1990) and the particles share at least two protein subunits (Pop1p and Pop3p). These common

Table I. Yeast strains

Strain	Genotype	Reference
YBD1	*MAT*α, *ade2, ade3, leu2, his3, trp1, ura3, rrp2-1,* [pBD1]	this work
YBD2	*MAT*α, *ade2, ade3, leu2, his3, trp1, ura3, rrp2-1,* [pRS425]	this work
YBD3	*MAT*α, *ade2, ade3, leu2, his3, trp1, ura3, RRP2,* [pRS425]	this work
YBD20	*MAT*α, *ade2, ade3, leu2, his3, trp1, ura3, rrp2-1,* [pRS425-POP3]	this work
YBD21	*MAT*α, *ade2, ade3, leu2, his3, trp1, ura3, rrp2-1,* [pRS425-SNM1]	this work
YBD30	*MAT*α/*MAT***a**, *ade2-1/ade2-1, his3-Δ200/his3-Δ200, leu2-3,112/leu2-3,112, trp1-1/trp1-1,ura3-1/ura3-1, can1-100/can1-100, pop3::HIS3*	this work
YBD32	*MAT*α, *ade2-1, his3-Δ200, leu2-3,112, trp1-1, ura3-1, can1-100, pop3::HIS3,* [pRS314-TRP1-ProtA-POP3]	this work
YBD34	*MAT***a**, *ade1-100, his4-519, leu2-3,112, ura3-52, GAL10::pop3*	this work
SL311	*MAT*α, *ade2, ade3, leu2, his3, trp1, ura3, rrp2-1, sl311,* [pBD1]	this work
BMA38	*MAT*α/*MAT***a**, *ade2-1/ade2-1, his3-Δ200/his3-Δ200, leu2-3,112/leu2-3,112, trp1-1/trp1-1,ura3-1/ura3-1, can1-100/can1-100*	Baudin *et al.* (1993)
ProtA-NOP1	*MAT*α, *ade, leu, trp, lys, ura3, nop1::URA3,* [pUN100-ProtA-NOP1]	Jansen *et al.* (1991)
BSY414	*MAT***a**, *ura3-52, arg4, leu2-3,112, ade2, trp1-289, pop1::TRP1,* [pRS415-ProtA-POP1]	Lygerou *et al.* (1994)
BWG1-7A	*MAT***a**, *ade1-100, his4-519, leu2-3,112, ura3-52*	L.Guarente (MIT, Cambridge)

structural features may explain the ability of the particles to cleave the same substrates *in vitro*; both RNase MRP and RNase P can process the RNA complementary to the mitochondrial D-loop region (Potuschak *et al.*, 1993). RNase P can also cleave the yeast pre-rRNA at site A_3 *in vitro* (Lygerou *et al.*, 1996a) but not *in vivo*. The high degree of similarity between these two enzymes raises the question of which component(s) are required for the substrate specificity of the particles *in vivo*. Identification of protein components of the particles in *S.cerevisiae* now allows us to address this question.

Materials and methods

Microbiological techniques, strains and media

Growth and handling of *S.cerevisiae* (Sherman, 1991) and *E.coli* (Maniatis *et al.*, 1982) were by standard techniques. Yeast transformations were carried out according to Gietz *et al.* (1992). Plasmid recovery from yeast into *E.coli* was performed as described in Robzyk and Kassir (1992). Table I lists the yeast strains used in this study.

For Pop3p depletion, cells growing exponentially in galactose minimal medium (SGal-ura) at 30°C were harvested by centrifugation, and resuspended in glucose minimal medium (SD-ura). During growth, cells were diluted with pre-warmed medium and constantly maintained in exponential phase.

Plasmids

The *RRP2* gene [nt –271 to +388 relative to the transcription start site (+1)] was amplified by PCR from yeast genomic DNA using the following primer pair: RRP2-5′ AAAGGATCCGTCAGGGCTCTTCAAC and RRP2-3′ AAAGTCGACTGCTAAAAAATAGTGTAA. The PCR-generated *Bam*HI and *Sal*I restriction sites were then used to clone the PCR product (which was verified by DNA sequencing and found to contain a C→G mutation at position –217) into the *Bam*HI and *Sal*I sites of plasmid pHT4467 (CEN-URA3-ADE3, Venema and Tollervey, 1996), yielding plasmid pBD1, and into the same sites of plasmid pRS415 (CEN-LEU2, Stratagene), yielding plasmid pRS415-LEU2-RRP2.

Isolation of mutant yeast strains which are synthetically lethal with rrp2-1

Before mutagenesis, the YBD1 strain was grown in liquid medium (SD-ura) to an $OD_{600\ nm}$ of ~0.6. Approximately 1×10^6 cells were plated on YPD (4% glucose) plates, UV irradiated ($\lambda = 254$ nm) for 40 s and incubated at 24°C for 7–10 days. About 1×10^5 cells survived the mutagenesis (~10%), most of which exhibited a sectoring phenotype. For the first 24 h, incubation was in the dark in order to inactivate the photoreactivation repair pathway. One thousand non-sectoring red colonies initially were picked and streaked on YPD (4% glucose). A red phenotype was retained in 332 strains when restreaked on the same medium. True plasmid-dependent growth of those strains was then tested by plating the 332 strains on medium containing 5-FOA (0.1% w/v). Twenty one strains did not grow on 5-FOA, demonstrating plasmid dependence. After test transformations with plasmids pRS415-LEU2-RRP2 and pRS415 (LEU2, CEN; Sikorski and Hieter, 1989), 15 red synthetic lethal mutants remained. Test transformation with pRS415-LEU2-POP1 did not restore sectoring in any of the sl strains, whereas transformation of the sl strains with pMES194-LEU2-SNM1 (Schmitt and Clayton, 1994) restored sectoring in 14 out of 15 strains.

Cloning of POP3, DNA sequencing and gene disruption

To clone genes which are able to complement the sl phenotype of the obtained sl strains but are different from *RRP2* and *SNM1*, one sl strain (SL311) was transformed with a yeast genomic library in pUN100 (*LEU2, CEN*; Bergès *et al.*, 1994) and plated on SD-leu medium. After 5 days, the colonies were replica plated on YPD (4% glucose) plates and grown for an additional 5 days. Out of ~5000 transformants, nine regained a sectoring phenotype. White sectors were purified and tested for growth at 37°C. Four of the white strains showed a ts phenotype, which was interpreted as indicating that they did not carry the *RRP2* or *SNM1* genes on the plasmid. Three of these strains were used to recover the library plasmids in *E.coli*.

The recovered plasmids were characterized by restriction digestion and shown to contain an overlapping region of the yeast genome, which was mapped to chromosome XIV with the use of an ordered yeast genomic library (Riles *et al.*, 1993). One library plasmid was used to construct deletion mutants which were cloned into pRS415 and tested for their ability to complement the sl phenotype. A minimal 3.5 kb *Hin*dIII complementing fragment was obtained (plasmid pRS415-LEU2-POP3), and DNA sequencing of the ends of the 3.5 kb insert using primers complementary to the polylinker region of the vector revealed the partial ORF of the *ERG24* gene (Lorenz and Parks, 1992). Both strands of the 3.5 kb *Hin*dIII fragment were sequenced by primer walking, excluding the already published *ERG24* sequence (EMBL accession No. X95844). Two complete ORFs were identified, encoding potential proteins of 195 (588 nt, designated *POP3*) and 153 (462 nt) amino acids, respectively. Deletion mutants within the 588 nt ORF (*POP3*) showed that the complementing activity could be attributed to this gene. Three other sl strains (SL88, SL125 and SL194) were similarly transformed with genomic libraries. Seven clones were recovered from these strains, all of which contained the *POP3* gene.

To construct a *POP3* null allele, a one-step PCR method was employed according to Baudin *et al.* (1993). The *HIS3* gene was PCR amplified, using the following primer pair: oligo-pro; TTTTTCCTCGCTTTCTCTGCCCACTTTTTTCTTCTGTCTTCTAGTCGTTCAGAATGACACG and oligo-term; GCAGGTGTATAAGCCCGTGCTAGACAATCCGTTCACAAACGAACTCTTGGCCTCCTCTAG. The linear PCR fragment was gel-purified and used to transform the diploid yeast strain BMA38 (Rothstein, 1991). His^+ transformants were tested for correct integration by PCR and Southern analysis. One heterozygous *POP3*/*pop3*::*HIS3* diploid strain (YBD30) was sporulated and tetrads were dissected. Twenty two dissected tetrads gave rise to either one or two viable spores, which were always His^-. The plasmid pRS316-URA3-POP3 was transformed into the strain YBD30 and the resultant strain was sporulated and tetrads were dissected. Mostly three or four viable spores were

obtained. All spores which carried the *HIS3* marker were unable to grow on plates containing 5-FOA, which selects for loss of the pRS316-URA3-POP3 plasmid.

Epitope tagging of Pop3p with protein A

To construct a *ProtA–POP3* fusion gene, a 395 bp *Nco*I–*Eco*RI fragment encoding two IgG binding domains of *S.aureus* protein A was recovered from plasmid p28NZZtrc (Grandi *et al.*, 1993) and fused to a PCR-generated *Bam*HI–*Nco*I fragment comprising the promoter region of *POP3* (nt –500 to –1, numbering relative to the ATG start codon) in pRS314 (TRP1-CEN, Sikorski and Hieter, 1989), to give the cloning intermediate pBD20. The primers used for amplification of the *Bam*HI–*Nco*I fragment are: ProtA-1 AAAGGATCCAAAAACCTGCTGTAAAT and ProtA-2 AAAAACCACGGCTACTTACCGTCTTGAT. A PCR-amplified *Eco*RI–*Kpn*I fragment containing the complete *POP3* coding sequence (nt +1 to +588) was then cloned into pBD20, in-frame, immediately before the initiation codon, to generate the *ProtA–POP3* fusion gene resulting in pBD25. The primers used for amplification of the *POP3* coding sequence are: primer ProtA-3 AAAGAATTCAATGT-CGGGCGGGTCGTTAAAA and primer ProtA-4 AAAGGTACCCTA-CTTTTGCCTCTTCTT. All PCR-amplified regions were verified by sequencing.

pBD25 was then used to transform a heterozygous *POP3*/*pop3::HIS3* diploid strain (YBD30). Subsequent sporulation and tetrad dissection yielded a haploid strain (YBD32) carrying the *pop3::HIS3* deletion complemented by the *ProtA–POP3* fusion gene.

Immunoprecipitation of ProtA–Pop3p

Yeast whole cell extracts were prepared essentially as described (Séraphin and Rosbash, 1989). Immunoprecipitation experiments were performed as previously described (Lygerou *et al.*, 1994).

Construction of a conditional GAL10::pop3 allele

To construct a conditional *GAL10::pop3* allele, a 1.5 kb *Hin*dIII–*Bam*HI fragment containing the *URA3* marker gene and the *GAL10* promoter cassette (Guarente *et al.*, 1982) was fused to the promoter region of the *POP3* gene (nt –500 to –21, numbering relative to the ATG start codon), in pBS(KS+) (Stratagene), resulting in the cloning intermediate pBD23. The *POP3* promoter region was PCR amplified as a *Kpn*I–*Hin*dIII PCR fragment, using the primer pair: primer GAL-1 AAAGGTACCAAA-AACCTGCTGTAAATG and primer GAL-2 AAAAAAGCTTCCT-CACCTTCCTTGT. A PCR-amplified *Bam*HI–*Sac*I fragment containing the complete *POP3* coding sequence (nt –20 to +588) was then cloned into pBD23, creating pBD27. The primers used for amplification of the *Bam*HI–*Sac*I *POP3* fragment (nt –20 to +588) are primer GAL-3 AAAGGATCCAATCAAGACGGTAAGTA and primer GAL-4 AAAA-GAGCTCCTGCGCGGGTAATTTTGT. All PCR-generated fragments were verified by sequencing. A linear *Kpn*I–*Sac*I fragment from pBD27 was gel purified and used to transform a wild-type haploid yeast strain (BWG1-7A). Ura^+ transformants were isolated on SGal-ura plates and correct integration of the *GAL10::pop3* construct was verified by Southern blot analysis (data not shown).

RNA extraction, primer extension and Northern hybridization

RNA was extracted as described previously (Tollervey and Mattaj, 1987). Northern hybridization (Tollervey, 1987) and primer extension (Beltrame and Tollervey, 1992) were as described previously. Oligonucleotides were used for pre-rRNA Northern hybridization and primer extension. Oligonucleotide a is ATGAAAACTCCACAGTG; oligonucleotide b is CCAGTTACGAAATTCTTG; oligonucleotide c is TTT-CGCTGCGTTCTTCATC; oligonucleotide d is GGCCAGCAATTT-CAAGT; and oligonucleotide e is AGATTAGCCGCAGTTGG. Oligonucleotides were also used for hybridization of tRNA. The mature $tRNA_3^{Leu}$ probe is GCATCTTACGATACCTG; the mature $tRNA_{UGC}^{Ala}$ probe is CTACCAACTGCGCCATG; the mature $tRNA_{GCG}^{Gly}$ probe is TACCACTAAACCACTAGC; the mature $tRNA_{UGG}^{Pro}$ probe is ACCCA-GGGCCTCTCG; the mature $tRNA_{CCA}^{Trp}$ probe is AACCTGCAA-CCCTTCGA; and the probe against the mature $tRNA^{Arg}$ of the dimeric tRNA transcript $tRNA^{Arg}$–$tRNA^{Asp}$ is GTCGAACCCATAATCTTC.

Acknowledgements

We gratefully acknowledge the gifts of plasmids and yeast strains from M.Schmitt, L.Guarente, Z.Lygerou, B.Séraphin and H.Tekotte. We also want to thank our colleagues in the laboratory, B.Séraphin, J.Valcarcel and J.Venema, for advice on the preparation of the manuscript.

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Received on August 7, 1996; revised on September 26, 1996