The 3' end of yeast 5.8S rRNA is generated by an exonuclease processing mechanism

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Eukaryotic rRNAs (with the exception of 5S rRNA) are synthesized from a contiguous pre-rRNA precursor by a complex series of processing reactions. Final maturation of yeast 5.8S rRNA involves processing of a 3'-extended, 7S precursor that contains ~140 nucleotides of the internal transcribed spacer 2 (ITS2) region. In yeast strains carrying the temperature-sensitive (ts) *rrp4-1* mutation, 5.8S rRNA species were observed with 3' extensions of variable length extending up to the 3' end of the 7S pre-rRNA. These 3'-extended 5.8S rRNA species were observed at low levels in *rrp4-1* strains under conditions permissive for growth and increased in abundance upon transfer to the nonpermissive temperature. The *RRP4* gene was cloned by complementation of the ts growth phenotype of *rrp4-1* strains. *RRP4* encodes an essential protein of 39-kD predicted molecular mass. Immunoprecipitated Rrp4p exhibited a 3' → 5' exoribonuclease activity in vitro that required RNA with a 3'-terminal hydroxyl group and released nucleoside 5' monophosphates. We conclude that the 7S pre-rRNA is processed to 5.8S rRNA by a 3' → 5' exonuclease activity involving Rrp4p. Homologs of Rrp4p are found in both humans and the fission yeast *Schizosaccaromyces pombe* (43% and 52% identity, respectively), suggesting that the mechanism of 5.8S rRNA 3' end formation has been conserved throughout eukaryotes.

The primary transcripts[1] of RNA-encoding genes commonly undergo a series of endonucleolytic and exonucleolytic processing reactions to generate the mature RNA species. Similar or identical ribonuclease activities are also required for the degradation of functional and aberrant RNA transcripts, as well as the turnover of noncoding RNA fragments released during RNA maturation processes. Two exoribonucleases have been cloned from the yeast *Saccharomyces cerevisiae*, Xrn1p and Rat1p, both of which hydrolyze RNA in the 5' → 3' direction in vitro (Stevens 1980; Kenna et al. 1993). Mutations in *XRN1* and *RAT1* have pleiotropic effects on RNA metabolism in vivo, affecting processing of rRNA (Stevens et al. 1991; Amberg et al. 1992; Henry et al. 1994) and snoRNAs (for review, see Lafontaine and Tollervey 1995), as well as the degradation of mRNA (Larimer et al. 1992; Muhlrad et al. 1994). 3' → 5' Exoribonuclease activities have also been demonstrated to be involved in the processing and degradation of RNA in a range of eukaryotes (Xing and Worcel 1989; Åström et al. 1991; Imboden et al. 1992; Preiser et al. 1993; Kiss and Filipowicz 1995). Although a number of 3' → 5' exoribonuclease activities have been purified biochemically from different eukaryotes (Lazarus and Sporn 1967; Kwan 1977; Kumagai

et al. 1979; Eichler and Eales 1985; Åström et al. 1992; Sachs and Deardorff 1992; Min et al. 1993; Caruccio and Ross 1994; Ito et al. 1994), none of the genes encoding these activities has been identified unambiguously.

In contrast, all eight exoribonucleases isolated from *Escherichia coli* hydrolyze RNA in a 3' → 5' direction (for review, see Deutscher 1993) and mutants are available for seven of the corresponding genes. However, the assignment of specific functions for these enzymes in vivo has been hampered by their apparent functional redundancy. Strains mutant for the exoribonucleases RNase II or polynucleotide phosphorylase (PNPase) exhibit no major phenotype, whereas the doubly mutant strain is nonviable and is defective in mRNA degradation (Donovan and Kushner 1986). More remarkably, any one of at least five exoribonucleases (RNase II, RNase D, RNase BN, RNase T, and RNase PH) are sufficient to support 3'-end maturation of $tRNA^{Tyr}su^+_3$ in vivo in the absence of the remaining four activities (Reuven and Deutscher 1993). RNase T was first isolated as the activity responsible for turnover of the terminal adenosine residue of tRNA (Deutscher et al. 1984) and has recently been demonstrated to be essential for correct 3'-end maturation of 5S rRNA (Li and Deutscher 1995).

In eukaryotes, rRNAs (with the exception of 5S rRNA) are cotranscribed as a single precursor molecule (pre-rRNA) by RNA polymerase I. The structural arrange-

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ment of the nuclear rDNA repeat and the pathway by which the primary transcript is processed to the mature rRNA species have been highly conserved. The rRNA processing pathway has been studied in a number of eukaryotes but is best characterized for the yeast *S. cerevisiae* (summarized in Fig. 1). Although several pre-rRNA cleavage sites have been localized at the nucleotide level (Klootwijk and Planta 1989 and references therein; Henry et al. 1994), the mechanism of pre-rRNA processing and the factors required for specific steps in the pathway remain poorly characterized (for a recent review, see Venema and Tollervey 1995).

As is the case in other eukaryotes, the 5′ end of 5.8S rRNA in *S. cerevisiae* is heterogeneous (Rubin 1974; Henry et al. 1994). Two major forms of 5.8S rRNA are found in yeast; a predominant short form, designated 5.8S(S), and a less abundant long form containing an additional 7-8 nucleotides at its 5′ end, designated 5.8S(L). The long and short forms of 5.8S rRNA do not have a precursor/product relationship but rather the two 5′ termini are generated by distinct processing mechanisms (Henry et al. 1994) (see Fig. 1B). The 5′ end of the predominant 5.8S(S) rRNA is generated by 5′ → 3′ exonuclease activities involving Xrn1p and Rat1p after endonucleolytic cleavage at a site A3, 76 nucleotides 5′ to the 5.8S rRNA-coding region (Henry et al. 1994).

The mechanism of 5.8S rRNA 3′ end formation is less clear. Processing in ITS2 at sites C1 and C2 generates the 5′ end of the mature 25S rRNA and the 3′ ends of the 7S(S) and 7S(L) pre-rRNAs, respectively (see Fig. 1B). No previous data were available concerning either the mechanism by which the 7S pre-rRNAs are processed to mature 5.8S rRNAs, or the components required for this step.

Here we report the characterization of a temperature-sensitive (ts) lethal mutation in *S. cerevisiae*, designated *rrp4-1* (for ribosomal RNA processing). The gene mutated encodes a protein that is required for processing of
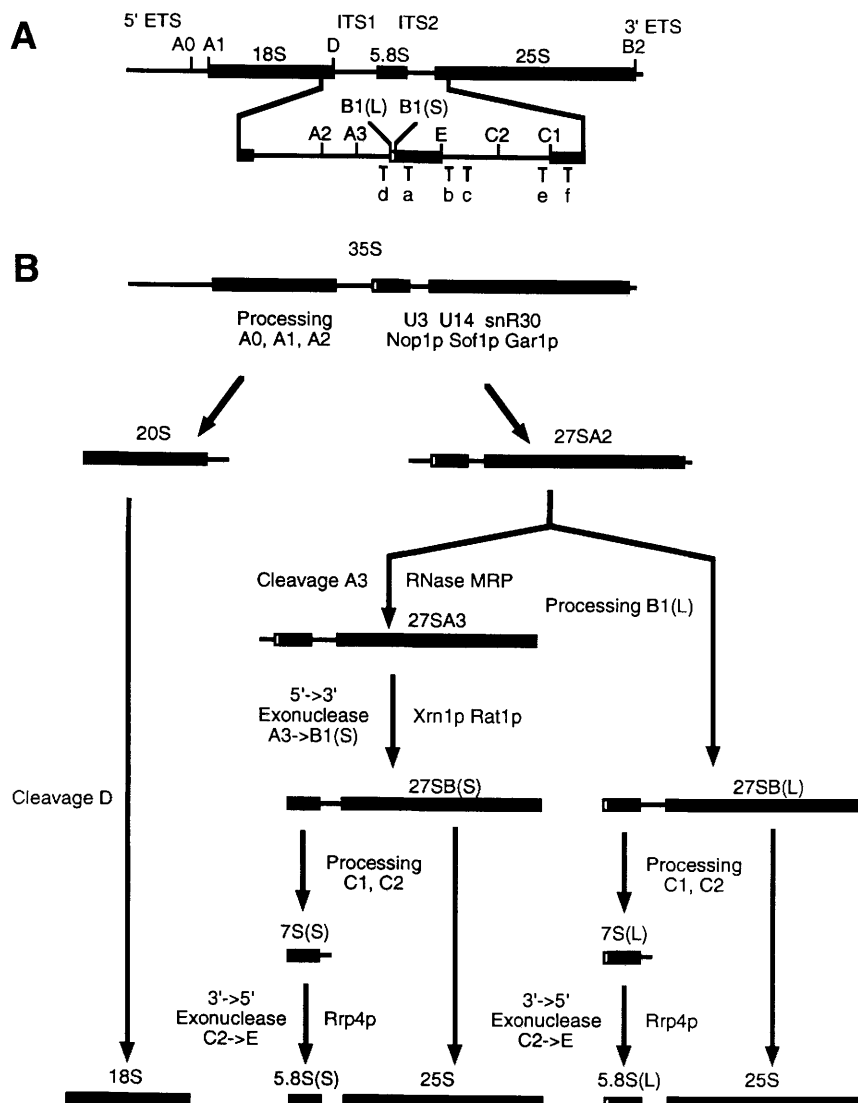

Figure 1. Structure and processing of yeast pre-rRNA. (*A*) Structure of 35S pre-rRNA. rRNA-coding regions are denoted as bars; transcribed spacer regions are denoted as lines. Processing sites are indicated in uppercase letters. Positions complementary to oligonucleotides used for Northern blot hybridization and primer extension are indicated in lowercase letters. (*B*) Yeast 35S pre-rRNA processing pathway. Components shown to be required in the processing pathway are denoted at the relevant steps. snoRNP-dependent early processing events at sites A0, A1, and A2 generate the 20S and 27SA$_2$ pre-rRNAs. The 20S pre-rRNA is cleaved endonucleolytically at site D to generate the mature 18S rRNA. Processing of the 27SA$_2$ pre-rRNA in internal transcribed spacer 1 (ITS1) proceeds by two independent pathways to generate the 27SB(S) and 27SB(L) rRNAs. In the major pathway, the 27SA$_2$ pre-rRNA is cleaved by RNase MRP at site A3, generating the 27SA$_3$ pre-rRNA. This pre-rRNA is then processed to 27SB(S) by a 5′ → 3′ exonuclease activity. Processing at site B1(L) generates 27SB(L) by an unknown mechanism. ITS2 processing of both the 27SB(S) and 27SB(L) pre-rRNAs at sites C1 and C2 generates the mature 25S rRNA and the 7S(S) and 7S(L) pre-rRNAs. Subsequently, a 3′ → 5′ exonuclease activity requiring Rrp4p processes both the 7S(S) and 7S(L) pre-rRNAs to the mature 5.8S(S) and 5.8S(L) rRNAs, respectively. For simplicity, details of the early processing steps are not shown. The precise timing of the removal of the short 3′-external transcribed spacer (ETS) sequence is unclear.

the 7S pre-rRNA to mature 5.8S rRNA and is associated with a 3′ → 5′ exoribonuclease activity in vitro. Rrp4p is the first factor demonstrated to be directly required for ITS2 processing and, to the best of our knowledge, is the first component of a characterized eukaryotic 3′ → 5′ exoribonuclease for which the gene has been cloned.

Results

rrp4-1 *strains accumulate 3′-extended forms of 5.8S rRNA*

Final maturation of 5.8S rRNA involves processing of a 7S pre-rRNA precursor containing a 3′ extension of ~140 nucleotides. To identify factors required for 5.8S rRNA 3′-end formation in *S. cerevisiae* we screened a collection of ts-lethal yeast mutants (Lygerou et al. 1994) for strains exhibiting a conditional 7S pre-rRNA processing phenotype. Total RNA was extracted from 246 mutant strains after growth for 2.5 hr at 37°C. Low molecular mass RNAs were resolved by polyacrylamide gel electrophoresis and transferred to filters for Northern blot analysis. The filters were hybridized with a probe specific for the 5′ region of the ITS2 (probe c in Fig. 1A) that, in wild-type strains, hybridizes exclusively to the 7S pre-rRNAs. In one mutant strain a number of additional fragments intermediate in length between the 7S pre-rRNAs

and 5.8S rRNAs were detected. The mutation was designated *rrp4-1*. Compared to the parental wild-type *RRP4* strain, growth of the *rrp4-1* strain was reduced at the permissive temperature (25°C) and was strongly inhibited ~3 hr after shift to the nonpermissive temperature (37°C).

To analyze the biochemical phenotype of the *rrp4-1* strain in greater detail, Northern hybridizations and primer extension analyses were performed on RNA isolated from the *rrp4-1* strain after incubation at the permissive temperature and after 2, 6, and 20 hr at the nonpermissive temperature. RNA from the parental wild-type strain grown at 37°C was analyzed in parallel. Filters were hybridized using probes complementary to sequences within the mature 5.8S rRNA and to sequences 5′ or 3′ to the 5.8S rRNA-coding region (Fig. 2). The lengths of the RNAs detected by Northern hybridization were estimated by comparing their mobilities with DNA sequencing reactions that were resolved and electrotransferred in parallel.

Northern blot analysis of total RNA from the wild-type *RRP4* strain using probe a, which is complementary to the 5.8S rRNA-coding region, detects the mature 5.8S(S) and 5.8S(L) species, as well as their 3′-extended precursors 7S(S) and 7S(L), respectively (Fig. 2A, lane 1). Under the electrophoresis conditions used (see Materials

Figure 2. An *rrp4-1* mutant accumulates 3′-extended forms of 5.8S rRNA. Northern blot analysis of total RNA from wild-type, *rrp4-1*, and *rrp2-1* strains using probes specific for 5.8S rRNA, ITS1, and ITS2. (A–D) Hybridization using probes a–d, respectively. (Lane 1) RNA extracted from a wild-type, *RRP4* strain grown at 37°C for 20 hr; (lane 2) RNA extracted from an *rrp4-1* strain grown at 23°C; (lanes 3–5) RNA extracted from an *rrp4-1* strain after temperature shift to 37°C for the times indicated; (lane 6) RNA extracted from an *rrp2-1* strain grown at 18°C; and (lane 7) RNA extracted from an *rrp2-1* strain grown at 25°C. RNA extracted from 0.1 OD_{600} unit of cells was loaded in each lane. The migration positions of the 7S(L), 7S(S), 5.8S(L), and 5.8S(S) rRNAS are indicated on the *left*; major extended 5.8S rRNA species that accumulate in the *rrp2-1* and *rrp4-1* strains are indicated as cartoons on the *right*. Panel A is a composite of two exposure times (relative duration, 20:1) of the same Northern blot to show both the extended 5.8S rRNA species and the levels of the mature 5.8S rRNAs. A schematic representation of ITS1, the 5.8S-coding region, and ITS2 is shown; processing sites are denoted in uppercase letters, positions of sites complementary to the probes used for Northern hybridizations (lowercase letters) are indicated.
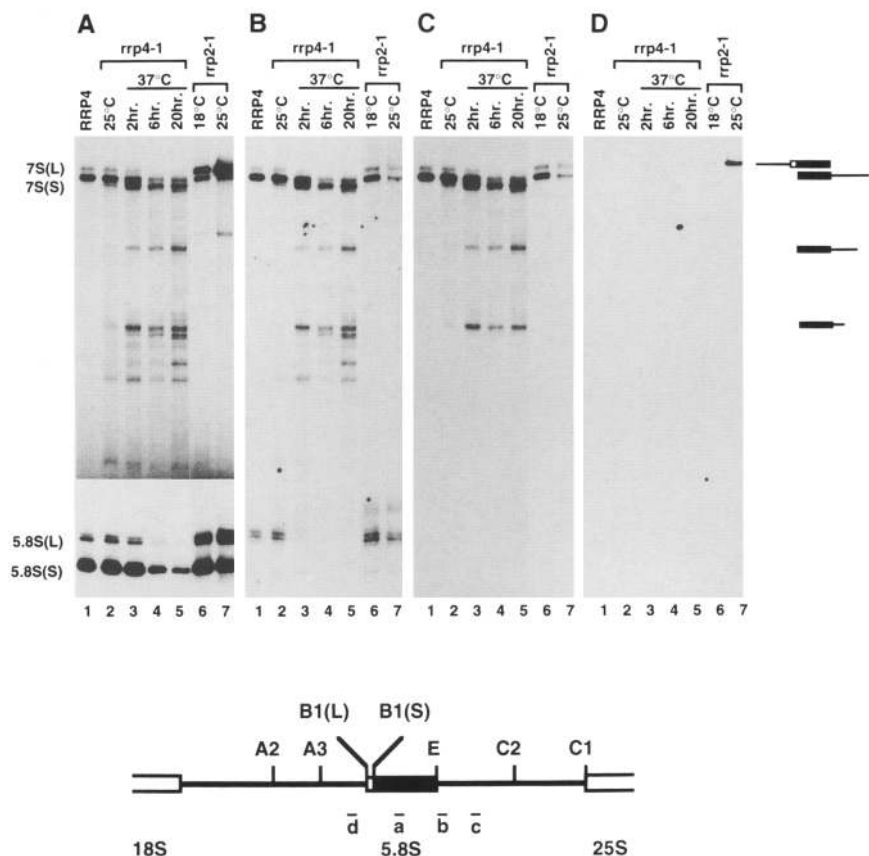
and methods) the 7S(S) and 7S(L) pre-rRNAs are well resolved; the 3′ end of 7S pre-rRNA is reported to be homogeneous (Klootwijk and Planta 1989), and the observed difference in length between these two species can be fully accounted for by the heterogeneity at their 5′ ends. In addition to these RNAs, a number of fragments of intermediate lengths are detected with probe a in the *rrp4-1* strain grown at the permissive temperature (Fig. 2A, lane 2); no species are detected in the *rrp4-1* mutant that are either shorter than the mature 5.8S rRNAs or longer than the 7S pre-rRNAs. The fragments of intermediate length accumulate five- to tenfold within 2 hr after shift to the nonpermissive temperature (Fig. 2A, lane 3), at which point the levels of 5.8S rRNAs and 7S pre-rRNAs are not grossly affected and no inhibition of growth was observed. No additional rRNA processing defect could be detected by Northern analyses or primer extension experiments at this time point (data not shown). With longer incubation at 37°C the levels of these additional fragments increase further, whereas the levels of 5.8S(S) and 5.8S(L) rRNAs decrease (Fig. 2A, lanes 4,5). Depletion of all pre-rRNA processing intermediates and mature rRNA species was observed in the *rrp4-1* strain after 6 hr of incubation at the nonpermissive temperature (data not shown). Because these general effects are only observed at late time points after growth inhibition, it is unclear whether they are direct effects of the *rrp4-1* mutation.

Probe b is partially complementary to both the 3′ end of the 5.8S rRNA and the 5′ end of the ITS2 spacer but hybridizes specifically to 5.8S rRNA species with 3′ extensions. All of the extended species detected with the probe against the mature 5.8S rRNA sequence are also detected by hybridization with probe b, (Fig. 2B, lanes 2–5), thereby demonstrating that they contain ITS2 sequences. In addition, Northern blot analysis with this probe reveals a distinct set of 5.8S rRNA species with short 3′ extensions in the wild type, *RRP4* strain, and in the *rrp2-1* strain (Fig. 2B, lanes 1,6,7); these RNAs are also observed in the *rrp4-1* strain grown at the permissive temperature (Fig. 2B, lane 2) and are depleted upon incubation at 37°C (Fig. 2B, lanes 3–5). Hybridization with probe c, which is complementary to a site ~50 nucleotides farther 3′ in ITS2, detects only a subgroup of the extended 5.8S rRNA species (Fig. 2C, lanes 2–5), the lengths of which are sufficient to span the sequence between sites B1 and the site complementary to probe c. In contrast, hybridization with probe d, which is complementary to a site in ITS1 proximal to the 5′ end of 5.8S rRNA, fails to detect any of the extended species in the *rrp4-1* strain and reveals only the 5.8S rRNA 5′ extended to site A2 (Fig. 2D, lane 7) which accumulates in *rrp2-1* strains grown at 25°C (Shuai and Warner 1991; Lygerou et al. 1994).

The Northern blot analyses shown in Figure 2 demonstrate that the 5.8S rRNA species that are accumulated in the *rrp4-1* strain contain sequences of the ITS2 spacer. Were the 3′-extended 5.8S rRNA species to be generated by an inaccurate endonucleolytic activity, which in wild-type *RRP4* strains cleaves at site C2, then it would

be predicted that the resulting 5′ termini could be detected in RNA from the *rrp4-1* strain by primer extension using primers complementary to sites 3′ of this processing site. The analysis shown in Figure 3 shows that no such primer extension stops are detected. The stop detected between sites E and C2 in Figure 3 is also present in RNA from the *RRP4* strain and is not accumulated in the *rrp4-1* strain during incubation at the nonpermissive temperature and therefore, it is probably a structural stop. Consistent with the Northern data (see Fig. 2), no stops are observed in the 5.8S rRNA-coding region and no 5′-extended 5.8S rRNA species are detected; the same result was obtained with probes c and f (see Fig. 1A; data not shown). No defect in processing at
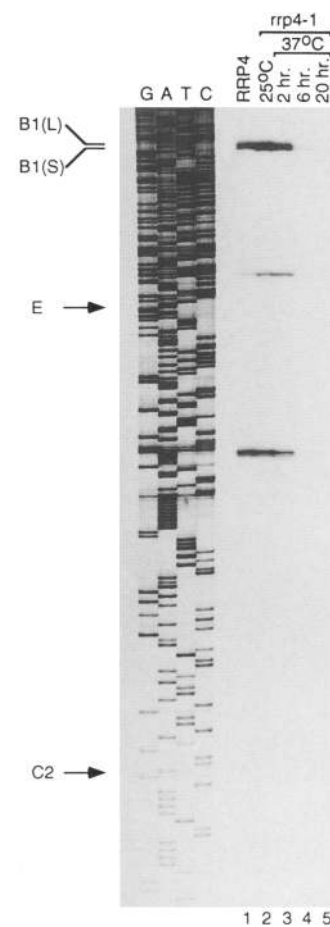

Figure 3. Primer extension analysis of the 7S pre-rRNA region in *RRP4* and *rrp4-1* strains. (Lane *1*) RNA extracted from a wild-type, *RRP4* strain after growth at 37°C; (lane *2*) RNA extracted from an *rrp4-1* strain after growth at 25°C; (lanes *3–5*) RNA extracted from an *rrp4-1* strain after growth at 37°C for the times indicated. Primer extension was performed using oligonucleotide e, which hybridizes to ITS2 at a site 3′ to processing site C2 (see Fig. 1). Sequencing reactions on the rDNA repeat using the same primer are also shown. The positions of processing sites corresponding to the 5′ ends of 5.8S rRNA [sites B1(S) and B1(L)] and the 3′ ends of 5.8S rRNA (site E) and 7S pre-rRNA (site C2) are indicated.

B1(S) or B1(L) was observed in the *rrp4-1* strain (Fig. 3; data not shown).

Taken together, the Northern analyses and primer extension data show that the additional fragments detected in the *rrp4-1* strain are 5.8S rRNA species that are processed correctly at their 5' ends but that contain 3' extensions of variable length up to, but not beyond, the 3' end of the 7S pre-rRNAs. The biochemical phenotype observed in the *rrp4-1* strain is consistent with a defective 3' → 5' exonuclease activity that, in wild-type strains, processes the 7S pre-rRNA to 5.8S rRNA. Notably, the existence of 5.8S rRNA species with short 3' extensions in *RRP4* strains and in the *rrp4-1* strain grown at the permissive temperature (see Fig. 2) is characteristic of previously studied maturation processes involving 3' → 5' exonuclease activities (see Discussion section).
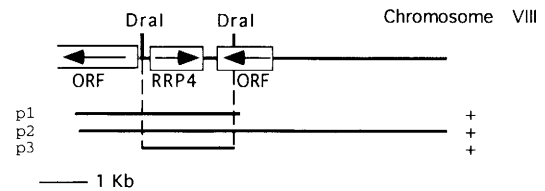
Mutations in the previously characterized 5' → 3' exonucleases Rat1p and Xrn1p have pleiotropic effects on RNA metabolism. To investigate whether *rrp4-1* mutants are defective in the maturation or processing of RNAs other than 5.8S rRNA, Northern blots were hybridized with probes specific for tRNA$_3^{Leu}$, 5S rRNA, a number of snoRNAs (snR190, U14, U3, snR10, snR30, and RNase MRP RNA), RNase P RNA, and the excised rRNA spacer fragment extending from A0 to A1 (see Fig. 1). In no case was any clear alteration observed in the *rrp4-1* mutant strain (data not shown). An excised rRNA spacer fragment extending from site C1 to C2 was not detected in *rrp4-1* or *RRP4* strains.

RRP4 encodes a 39-kD protein that is evolutionarily conserved

To determine whether the phenotype observed in the *rrp4-1* strain is attributable to a single mutation, the original mutant isolate was backcrossed to the wild-type strain BSY360 (see Materials and methods) and the meiotic progeny were screened for ts-lethal growth and defective 7S pre-rRNA processing. The temperature sensitivity of the *rrp4-1* strain was found to be recessive to *RRP4* in the heterozygous diploid. The ts-lethal growth phenotype and the 7S pre-rRNA processing phenotype, analyzed by Northern hybridization, cosegregated 2:2 in the progeny of four tetrads analyzed, indicating that the rRNA processing defect of *rrp4-1* strains is the result of a single, recessive ts-lethal mutation.

The *RRP4* gene was cloned from a wild-type yeast genomic DNA library by complementation of the ts-lethal growth phenotype of two outcrossed *rrp4-1* strains. Thirteen complementing plasmids were recovered in total, all of which were shown to overlap by restriction digestion analyses. Sequence determination of the ends of the inserts of two plasmids (plasmids p1 and p2 in Fig. 4A) and comparison of the sequences obtained with the European Molecular Biology Laboratory (EMBL) data base mapped the inserts to chromosome VIII (Johnston et al. 1994). The sequence common to both complementing plasmids contains a single complete open reading frame (ORF) (Fig. 4A). This ORF was subcloned from plasmid

Figure 4. Chromosomal locus of the *RRP4* gene and alignment of Rrp4p with homologous proteins from humans and *S. pombe*. (*A*) Schematic representation of the *RRP4* locus. The direction of transcription of the *RRP4* gene and the adjacent ORFs are indicated. The extent of plasmid inserts either isolated from a genomic library (p1 and p2) or constructed as a subclone thereof (p3) that complement the temperature sensitivity of *rrp4-1* strains are shown. Complementation is indicated by +. (*B*) Alignment of the deduced protein sequence of Rrp4p (S.c.) with homologous proteins from *S. pombe* (S.p.) and humans (H.s.). Identities are indicated by asterisks, similarities are indicated by dots. The sequence of the human homologue is extended from that given in data base entry U07561, based on sequence analysis of a cDNA clone from HeLa cells (data not shown). The Leu$_{136}$ residue, which is mutated in *rrp4-1* strains, is boxed.

p1 and the resulting construct (plasmid p3 in Fig. 4A) was shown to complement the ts-lethal growth phenotype of both *rrp4-1* strains.

The *RRP4* gene encodes a weakly acidic protein (pI = 6.5) of 39.4-kD predicted molecular mass. Comparison of the DNA and protein sequences with the EMBL and Swissprot data bases revealed homologs of as yet uncharacterized function in humans and *Schizosaccharomyces pombe* (43% and 52% identity throughout the

entire length of the protein, respectively). An alignment of the Rrp4p sequence with the homologous proteins is shown in Figure 4B. No motifs or repeated sequences could be detected in the Rrp4p protein sequence. The completely conserved amino acid sequences MRGHGTY and HTRSLKYGKL could not be identified in other entries in the Swissprot protein sequence data base and their functional significance, if any, is therefore unclear.

The RRP4 gene is essential for viability in yeast

A null allele of *RRP4* was constructed by replacing the entire *RRP4*-coding region with the *HIS3* marker (see Fig. 5A). The *rrp4Δ::HIS3* construct was used to transform both the wild-type diploid strain RS453 and a heterozygous *RRP4/rrp4-1* diploid strain (see Materials and methods). Correct integration of the null allele was confirmed by Southern blot analysis (Fig. 5B). Transformation of the heterozygous *RRP4/rrp4-1* diploid strain yields correctly integrated His⁺ strains that are either ts-lethal (*rrp4-1/rrp4Δ::HIS3*) or non-ts (*RRP4/rrp4-1Δ::HIS3*) (Fig. 5B, lanes 3,4, respectively), thereby demonstrating that the cloned *RRP4* gene is the same genetic locus as the *rrp4-1* mutation.

To analyze the phenotype of the *rrp4Δ* null allele, tetrad analyses were performed on a *RRP4/rrp4Δ::HIS3* diploid strain derived from RS453. Each of the 22 tetrads dissected yielded either one or two viable spores and none of the progeny carried the *HIS3* marker, strongly indicating that the *rrp4Δ* null allele is lethal. Consistent with this result, His⁺ progeny could be recovered by tetrad dissection of the same strain after transformation with a plasmid carrying a functional *RRP4* allele (see Materials and methods). The plasmid also contains the *URA3* marker and all the His⁺ progeny were also Ura⁺. His⁺, Ura⁺ progeny were nonviable on medium containing 5-fluoro-orotic acid (5-FOA), which stringently counterselects against the plasmid-encoded *URA3* marker, demonstrating that Rrp4p is essential for cell viability.

A single missense mutation results in defective 7S pre-rRNA processing and a ts-lethal growth phenotype in rrp4-1 strains

The ts mutation in *rrp4-1* strains was determined by sequence analysis, using PCR-amplified DNA prepared from an outcrossed, ts-lethal *rrp4-1* strain and from the wild-type strain used for mutagenesis. A single missense mutation at nucleotide +407 (taking the first base of the initiation codon as +1) was observed in each of three transformants containing *rrp4-1* DNA when compared to the wild-type controls (data not shown). Sequence analysis of the *rrp4-1* allele isolated by gap repair (see Materials and methods) confirmed the identification of the ts lesion. The point mutation is a T → C transition, resulting in a proline residue in place of a conserved leucine residue at amino acid 136 of Rrp4p (see Fig. 4B).

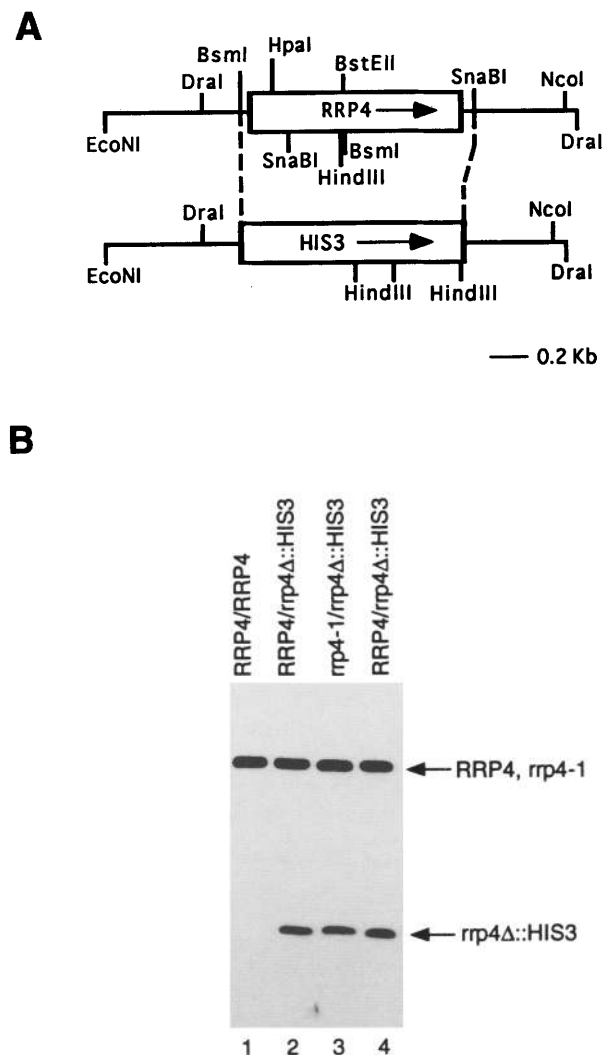To analyze the *rrp4-1* phenotype in a nonmutagenized

Figure 5. Construction of an *rrp4-Δ* null allele. (*A*) Partial restriction maps of the *RRP4* gene and the *rrp4Δ::HIS3* construct. The directions of transcription of the *RRP4* and *HIS3* genes are indicated by arrows. (*B*) Southern blot analysis of *rrp4Δ::HIS3* strains. Total yeast DNA was digested with *HindIII* and *NcoI*; hybridization was performed with a random primed probe generated from a fragment extending from the 3′ end of the *RRP4* gene to the downstream *DraI* site. (Lane *1*) DNA from the wild-type diploid strain RS453; (lane *2*) DNA from an integrative transformant of RS453; and (lanes *3,4*) DNA from a ts and a non-ts integrative transformant of a heterozygous *RRP4/rrp4-1* strain, respectively.

strain, the *rrp4-1* allele was integrated in place of the *RRP4* gene in the wild-type haploid strain BWG1-7A (see Materials and methods). Replacement of the *RRP4* gene with the *rrp4-1* allele imparted both a ts-lethal growth phenotype and a defective 7S pre-rRNA processing phenotype indistinguishable from those of the original mutant isolate (data not shown). These results confirm that the gene cloned by complementation is indeed allelic with the ts mutation. Moreover, both the growth pheno-

type and the rRNA processing phenotype are attributable to the $Leu_{136} \rightarrow Pro$ mutation in Rrp4p.

Immunoprecipitated prot.A–Rrp4p exhibits a $3' \rightarrow 5'$ exonuclease activity in vitro

From the in vivo analysis of *rrp4-1* mutants we concluded that these strains are deficient in a $3' \rightarrow 5'$ exonuclease activity. Therefore, we assayed Rrp4p for $3' \rightarrow 5'$ exonuclease activity in vitro. To purify Rrp4p from yeast under nondenaturing conditions, a *prot.A–RRP4* fusion gene was constructed that encodes two tandem IgG-binding domains of protein A from *Staphylococcus aureus* fused in-frame at the amino terminus of Rrp4p. This construct was shown to complement the *rrp4Δ::HIS3* null allele (see Materials and methods), demonstrating that the *prot.A–RRP4* fusion gene encodes a functional protein. The epitope-tagged prot.A–Rrp4p protein was enriched selectively from cell lysates by immunoprecipitation with IgG agarose. To test for exonuclease activity in vitro, assays were performed on immunoprecipitates of whole cell lysates from an *rrp4Δ::HIS3* strain expressing the prot.A–Rrp4p fusion protein. As a negative control, assays were performed in parallel on immunoprecipitates of lysates from an isogenic *rrp4Δ::HIS3* strain expressing the nontagged, wild-type Rrp4p, which is not expected to be immunoprecipitated.

When a 5'-labeled RNA substrate is incubated with immunoprecipitated prot.A–Rrp4p, RNAs shortened by an integral number of nucleotides are detected (Fig. 6A).

The lengths of these degradation products decrease progressively with increasing amounts of immunoprecipitated lysate (Fig. 6A, lanes 2–6), consistent with a $3' \rightarrow 5'$ exonuclease activity. Incubation of immunoprecipitated prot.A–Rrp4p with 3'-labeled RNA carrying a 3'-hydroxyl group leads to the complete loss of substrate without any detectable shortened fragments (Fig. 6B), consistent with quantitative release of the 3' terminal nucleotide (cf. Fig. 6A, lane 6). No degradation is observed when 3'-labeled RNA carrying a 3' terminal phosphate group is used as substrate (Fig. 6C). Low levels of fragments shortened by one or two nucleotides are observed in the non-tagged Rrp4p control (Fig. 6, A, lanes 7–11, and B, lane 3) or with an additional control strain expressing a prot.A–Nsp1p fusion protein (data not shown). The yields of these fragments are, however, independent of the quantity of lysate immunoprecipitated.

A distributive or processive mode of hydrolysis can be inferred by the pattern of fragments observed upon gel electrophoresis. A distributive activity (i.e., one that dissociates from the substrate after each nucleolytic cleavage) simultaneously decreases the length of the whole population of substrate molecules. In contrast, a processive activity hydrolyzes the bound RNA to completion, leaving the unbound portion of substrate intact. The detection of populations of 5'-labeled RNA fragments of decreasing length with increasing amounts of immunoprecipitated prot.A–Rrp4p (Fig. 6A, lanes 4–6) is indicative of a distributive exonuclease activity. The in vitro exonuclease activity is inhibited at monovalent cation concentrations >200 mM or by preincubation with

Figure 6. Assays of immunoprecipitates from *prot.A–RRP4* and *RRP4* strains for exoribonuclease activity. Immunoprecipitates were assayed for exoribonuclease activity using RNA substrates labeled with ^{32}P either at the 5' or 3' terminus; the position of the label and the presence or absence of terminal phosphate groups in each substrate are indicated schematically under the corresponding panel. (*A*) Assay with 5' ^{32}P-labeled RNA substrate. (Lane *1*) Input RNA; (lanes *2–6*) reaction products from immunoprecipitates of increasing amounts of lysate (containing 8, 16, 40, 80, and 160 μg of total protein) from a strain expressing the prot.A–Rrp4p fusion protein; and (lanes *7–11*) reaction products from immunoprecipitates of equivalent amounts of lysate from a strain expressing Rrp4p. (*B,C*) Assays with 3' ^{32}P-labeled RNA substrates. Immunoprecipitations were performed on lysate aliquots containing 160 μg total protein. (*B*) RNA lacking a phosphate group at its 3' terminus. (*C*) RNA with a phosphate group at its 3' terminus. (Lane *1*) Mock-treated substrate; (lane *2*) reaction products from immunoprecipitate of lysate from a strain expressing the prot.A–Rrp4p fusion protein; and (lane *3*) reaction products from immunoprecipitate of lysate from a strain expressing wild-type Rrp4p.
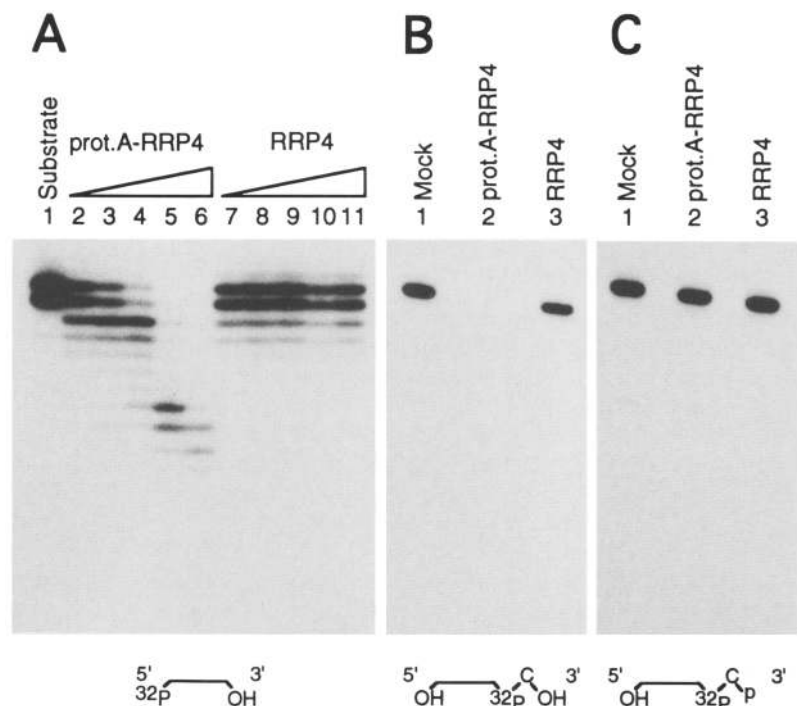
EDTA or EGTA, has a pH optimum close to 7, does not require the addition of NTPs or inorganic phosphate, and is not inhibited by RNasin, an inhibitor of RNase A-like activities (data not shown).

To determine the nature of the released product, assays were performed with an RNA substrate transcribed in the presence of $[\alpha\text{-}^{32}P]UTP$. To generate nucleoside monophosphate (NMP) standards with either a 3' or 5' phosphate group the substrate was subjected to complete alkaline hydrolysis or digestion with nuclease P1, respectively. Reaction mixtures and NMP standards were resolved in parallel by chromatography on polyethyleneimine (PEI)–cellulose thin layer plates.

Conversion of substrate transcribed in the presence of $[\alpha\text{-}^{32}P]UTP$ to nucleoside 3' monophosphates by alkaline hydrolysis results in the release of all four 3' NMPs as ^{32}P-labeled species (Fig. 7, lane 1). In the analysis shown, Ap is resolved into two species, presumably corresponding to adenosine 3' monophosphate and the 2',3' cyclic phosphate intermediate. In contrast, digestion of the substrate to nucleoside 5' monophosphates with nuclease P1 generates a single ^{32}P-labeled product, uracil 5'-monophosphate (pU) (Fig. 7, lane 2). Incubation of the substrate with immunoprecipitated prot.A–Rrp4p releases only pU, as shown by comigration of the product with that of nuclease P1 digestion (Fig. 7, lane 3); nucleoside 3' monophosphates are not observed. Only a small amount of the total radioactivity is released as pU upon

incubation with immunoprecipitated prot.A–Rrp4p, as the majority of ^{32}P radioactivity remains in partially digested products (cf. Fig. 6A) which are not resolved chromatographically in this system.

From the above data we conclude that Rrp4p is associated with an exonuclease activity in vitro that requires RNA with a 3' terminal hydroxyl group and hydrolyzes $3' \rightarrow 5'$, releasing nucleoside 5' monophosphates.

Discussion

We have characterized a mutation in a novel gene, designated *RRP4*. In yeast strains carrying the ts-lethal *rrp4-1* allele, 5.8S rRNA species accumulate that are processed correctly at their 5' termini but are processed incompletely at their 3' termini. The extended 5.8S rRNA species are heterogeneous, with 3' termini extending up to, but not beyond, the 3' end of the 7S pre-rRNA, the immediate precursor to 5.8S rRNA (see Fig. 1). We attribute the biochemical phenotype of *rrp4-1* strains to a defect in a $3' \rightarrow 5'$ exonuclease activity that processes 7S pre-rRNA to mature 5.8S rRNA.

Several lines of evidence support this conclusion. (1) Immunoprecipitated prot.A–Rrp4p exhibits an in vitro activity that correlates with the biochemical defect observed in strains mutant for Rrp4p; the epitope-tagged protein is associated with an exoribonuclease activity that degrades RNA exclusively in the $3' \rightarrow 5'$ direction. (2) Northern analyses of total RNA from both *RRP4* strains and from *rrp4-1* strains grown at permissive temperature reveal 5.8S rRNA species with short 8- to 10-nucleotide 3' extensions. Relatively stable intermediates have been reported in previously characterized processing reactions involving $3' \rightarrow 5'$ exonucleases in tRNA and snoRNA maturation (Reilly and RajBhandary 1986; Kiss and Filipowicz 1995) and in mRNA deadenylation (Lowell et al. 1992). In each case, processing occurs in two steps that are kinetically distinguishable; initial rapid $3' \rightarrow 5'$ exonuclease processing yields a metastable product with a short 3' extension, which is then removed by a slower trimming activity to yield the mature product. It is unclear whether a single exonuclease carries out both steps in 5.8S rRNA maturation, or whether an additional activity is responsible for the second step. (3) Primer extension experiments using primers complementary to sequences within ITS2 or 25S rRNA do not detect any processing sites within the 7S pre-rRNA region in *rrp4-1* or *RRP4* strains, which might have been expected had the extended 5.8S rRNA species been generated by endonucleolytic cleavages. (4) The accumulation of 3' extended forms of 5.8S rRNA in *rrp4-1* strains is comparable to the ladder of 5' extended 5.8S rRNA species observed in strains mutant for the $5' \rightarrow 3'$ exoribonucleases Xrn1p and Rat1p (Henry et al. 1994).

Previous genetic analyses in yeast have identified a number of factors that are essential for specific steps in the processing of the 5' ETS and ITS1 regions of the pre-rRNA (for review, see Venema and Tollervey 1995;
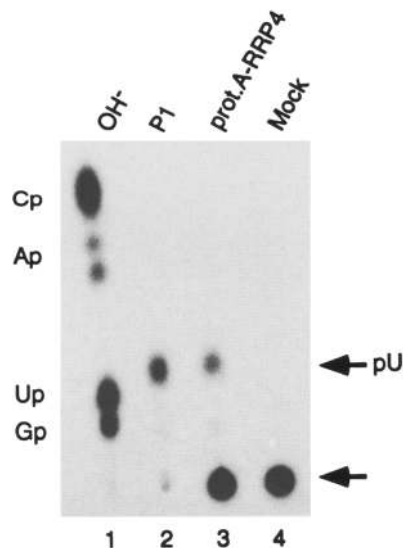

Figure 7. Analysis of the product released by the Rrp4p-associated exonuclease activity. Thin-layer chromatographic analysis of reaction products; (Lane *1*) Alkaline hydrolysate of RNA substrate; (lane *2*) RNA treated with nuclease P1; (lane *3*) RNA incubated with immunoprecipitate from a strain expressing the prot.A–Rrp4p fusion protein; and (lane *4*) mock-treated RNA substrate. The sample application points are indicated by a small arrow, the direction of chromatography was from bottom to top. Migration positions of ^{32}P-labeled 5' and 3' monophosphate nucleosides are indicated; the Rrp4p-associated exonuclease product pU is indicated with an arrow.

summarized in Fig. 1), many of which are components of small nucleolar ribonucleoproteins (snoRNPs). The U3, U14, and snR30 snoRNPs are required for early processing events at sites A0, A1, and A2, which generate the 20S and 27SA$_2$ pre-rRNA intermediates. The precise functional roles of these components in the processing reactions are, however, not clear. In contrast, direct functional roles have been assigned to components required for the 5′ end formation of 5.8S(S) rRNA. Endonucleolytic cleavage of the 27SA$_2$ pre-rRNA by RNase MRP at site A3 (Shuai and Warner 1991; Lindahl et al. 1992; Schmitt and Clayton 1993; Chu et al. 1994; Lygerou et al. 1994) provides the entry site for the 5′ → 3′ exonucleases Xrn1p and Rat1p (Henry et al. 1994). These exonucleases subsequently remove the remaining ITS1 sequence, generating the mature 5′ end of the 5.8S(S) rRNA.

Both the 5′ and 3′ termini of the predominant form of the yeast 5.8S rRNA are therefore generated by exonucleolytic processing mechanisms. This is in contrast to the synthesis of 18S rRNA, which is apparently generated by endonucleolytic cleavages at both termini (Stevens et al. 1991; Venema et al. 1995). The 3′ maturation of 5.8S rRNA in vivo is rapid and presumably accomplished by a processive exonuclease activity. In contrast, the 3′ → 5′ exonuclease activity observed in vitro with immunoprecipitated prot.A–Rrp4p is distributive. This discrepancy may be attributable to the assay conditions used but is most likely due to cooperativity in vivo with other factors that confer processivity and are lost during the purification of Rrp4p.

3′ → 5′ Exonuclease activities have been identified previously from a range of eukaryotes, including human (Kwan 1977; Åström et al. 1992; Caruccio and Ross 1994), mouse (Lazarus and Sporn 1967; Eichler and Eales 1985), rat (Kumagai et al. 1979), Aspergillus (Ito et al. 1994), and yeast (Sachs and Deardorff 1992; Min et al. 1993). One such activity was isolated from the nucleoli of Ehrlich ascites tumor cells (Eichler and Eales 1985) and has properties comparable with the Rrp4p-associated exonuclease. However, none of the genes encoding these 3′ → 5′ exonuclease activities has been identified. It is therefore unclear whether any of these activities are attributable to Rrp4p homologs. From yeast, a poly(A) nuclease activity has been characterized (Lowell et al. 1992; Sachs and Deardorff 1992), the genetic locus of which is not certain. The other yeast 3′ → 5′ exonuclease reported was isolated from mitochondria (Min et al. 1993) and is clearly distinct from the Rrp4p-associated exonuclease activity, as it exhibits an absolute requirement for NTPs.

The identification of highly conserved homologs of Rrp4p in S. pombe and humans (see Fig. 4) strongly suggests that the mechanism of 5.8S rRNA 3′ end formation has been conserved throughout eukaryotes. Rrp4p is the first component identified that is directly demonstrated to be involved in ITS2 processing of pre-rRNA. Biochemical analyses of Rrp4p-associated factors and secondary mutational analyses of rrp4-1 strains should further elucidate this aspect of pre-rRNA processing.

Materials and methods

Strains

The original rrp4-1 mutant isolate (At187; MATa, ura3-52, arg4Δ–EcoRV, leu2-3,112, ade2, rrp4-1, TRP1::GAL–SNR14) was identified from a collection of ts-lethal yeast strains (Lygerou et al. 1994). Backcrosses were performed with the strain BSY360 (MATα, ura3-52, his3-Δ1, leu2-3,112, ade2, ade5, TRP1::GAL–SNR14); the resulting heterozygous rrp4-1/RRP4 diploid was used for genetic linkage analyses. Allele replacement was performed in the strain BWG1-7A (MATa, ura3-52, leu2-3,112, ade1-100, his4-519), kindly provided by L. Guarente (Massachusetts Institute of Technology, Cambridge). Gene disruption was performed in the diploid strain RS453 (MATa/MATα, ade2-1/ade2-1, his3-11/his3-11, leu2-3/leu2-3, trp1-1/trp1-1, ura3-52/ura3-52, can1-100/can1-100); haploid rrp4-Δ::HIS3 strains complemented with plasmid-encoded wild-type or epitope-tagged Rrp4p were derived from RS453. The E. coli strain MC1066 used for cloning has the genotype galU, galK, strAr, hsdR⁻, Δ(lacIPOZYA)X74, trpC9830, leuB6, pyrF74::Tn5(km^r).

Cloning of RRP4

To clone the RRP4 gene, two outcrossed ts-lethal strains from independent tetrads were transformed with a genomic wild-type S. cerevisiae library constructed in pUN100 (CEN, LEU2) (Elledge and Davis 1988). Transformants were selected for growth at 37°C on YPD plates, colony-purified and screened for leucine prototrophy. Plasmids recovered from 13 Leu⁺ colonies were amplified in E. coli. Restriction mapping, end-sequence analyses of the inserts and sequence comparison with the EMBL data base revealed previously determined sequences from chromosome VIII (Johnston et al. 1994) with a common overlap of 3.3 kb. This common sequence contained a single complete ORF of 1.1 kb, which was subcloned into pRS316 (Sikorski and Hieter 1989) as a 1.9-Kb DraI fragment (p3, Fig. 4) and shown to complement the temperature sensitivity of both outcrossed rrp4-1 strains.

To construct a rrp4-Δ null allele, a 2.4-kb EcoNI–NcoI fragment containing the RRP4 gene (see Fig. 5A) was recovered from plasmid p1 (Fig. 4) and blunt-end ligated into the SmaI site of pBluescript II KS(+) (Stratagene). A 1.2-kb BsmI–SnaBI deletion containing the complete RRP4-coding sequence was replaced with a 1.1-kb BamHI fragment from YDpH (Berben et al. 1991) containing the HIS3 gene. In the transformant selected for further analysis, the orientation of the HIS3 gene was the same as that of the deleted RRP4 gene. The entire insert was recovered as a EcoRI–BamHI fragment and used to transform the wild-type diploid RS453 and a heterozygous RRP4/rrp4-1 diploid strain to histidine prototrophy. His⁺ transformants of the heterozygous RRP4/rrp4-1 diploid strain were selected at 23°C and screened subsequently for growth at 37°C. Integration of the HIS3 gene at the cloned locus was confirmed by Southern blot analysis.

Analysis of the rrp4-1 mutation and allele replacement

To identify the mutation in the rrp4-1 strain responsible for the ts phenotype, the RRP4-coding region and promoter were amplified by PCR on genomic DNA isolated from one of the outcrossed ts strains and the parental strain used for mutagenesis. The PCR fragments were cloned into pBluescript II KS(+) (Stratagene) and sequence analysis was performed on three independent transformants obtained from each source. A single missense mutation at nucleotide +407 was observed in all

transformants containing *rrp4-1* DNA and was absent from the wild-type controls.

To verify that the +407 mutation was responsible for the ts phenotype, the *rrp4-1* allele was recovered and used for subsequent gene replacement. To recover the *rrp4-1* allele, a 374-bp *Bst*EII–*Hpa*I fragment (see Fig. 5A) encompassing the +407 site was excised from plasmid p3 and the gapped plasmid was transformed into an *rrp4-1* strain. Fifty-five Ura⁺ transformants were recovered and all exhibited a ts growth phenotype. Plasmids were recovered from five transformants and shown to contain the complete *RRP4* gene by restriction analysis. Two plasmids were sequenced through the *Bst*EII–*Hpa*I region and were shown to be wild-type except for the +407 mutation. The recovered *rrp4-1* allele was cloned into the integration vector pRS406 (Stratagene), which carries the *URA3* marker. The construct was linearized within the *RRP4*-coding region with *Hpa*I and used to transform the wild-type haploid strain BWG1-7A. Ura⁺ transformants were grown overnight in YPD medium and Ura⁻ segregants were selected by plating on 5-FOA medium. Temperature-sensitive Ura⁻ segregants (carrying the *rrp4-1* allele) were identified by replica plating at 23°C and 37°C. The biochemical phenotype of these *rrp4-1* strains were confirmed by Northern blot analysis.

Construction of the prot.A–RRP4 *fusion gene*

To construct a *prot.A–RRP4* fusion, a 395-bp *Nco*I–*Eco*RI fragment containing two IgG-binding domains of *S. aureus* protein A was recovered from plasmid p28NZZtrc (Grandi et al. 1993) and fused in frame by PCR mutagenesis to the *RRP4*-coding sequence immediately before the initiation codon. The *RRP4* promoter region was thereby left intact. The efficacy of the construct was confirmed by sequence analysis.

The *prot.A–RRP4* fusion gene construct in plasmid pRS316 (Sikorski and Hieter 1989) was used to transform a heterozygous *rrp4-Δ/RRP4* diploid strain derived from RS453. A haploid strain carrying the chromosomal *rrp4-Δ* deletion complemented by the *prot.A–RRP4* fusion gene was obtained by subsequent tetrad dissection. An isogenic haploid strain carrying the chromosomal *rrp4-Δ* deletion complemented by a wild-type copy of *RRP4* cloned into pRS415 (Stratagene) was constructed by plasmid shuffling.

Immunoprecipitation of prot.A–Rrp4p *and in vitro exonuclease assays*

Isogenic yeast strains bearing the *rrp4-Δ* allele and complemented with plasmids containing either the *prot.A–RRP4* construct or the wild-type *RRP4* gene were grown in YPD at 30°C to an OD_{600} of 1. One liter of culture was harvested and washed with 50 ml of cold H_2O followed by 50 ml of cold lysis buffer, consisting of 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM $MgCl_2$, and 0.1% NP-40. All subsequent steps were performed at 4°C. The cell pellets were resuspended in 5 ml of lysis buffer, also containing 5 mM vanadyl ribonucleoside complex, 5 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and lysed by vortexing 10 times for 30 sec in the presence of a half-volume of glass beads (0.45–0.5 mm, B. Braun, Melsungen). After centrifugation at 5000 rpm for 10 min in a Megafuge 1.0 centrifuge (Heraeus), the lysates were clarified by centrifugation at 50,000 rpm for 20 min in an SW 55 Ti rotor (Beckman). The aqueous phases were recovered and glycerol was added to a final concentration of 8.7%. Aliquots of 100 μl were frozen in liquid nitrogen and stored at −80°C.

Immunoprecipitations were performed as described previously (Lygerou et al. 1994). In vitro assays were routinely per-

formed directly on the immunoprecipitates with ³²P-labeled RNA substrate in 20 μl volumes of 10 mM Tris-HCl (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$, 10 mM DTT, 100 μg/ml of BSA, 0.8 U/μl of RNasin (Promega). Reaction mixtures were incubated with shaking at 30°C for 30 min, diluted to 100 μl, and extracted with an equal volume of phenol/chloroform. RNA was recovered by ethanol precipitation in the presence of 300 mM NaCl and 20 μg glycogen (Boehringer) and resolved on 12% polyacrylamide sequencing gels. For analysis of the released product, [α-³²P]UTP-labeled substrate was incubated with immunoprecipitates as above. EDTA (pH 8.0) was added to a final concentration of 5 mM, and 2-μl volumes were analyzed by chromatography on PEI–cellulose plates (Mackery and Nagel) (Volckaert and Fiers 1977).

Synthesis of RNA substrates for in vitro assays

RNA substrates were synthesized in vitro by T3 polymerase transcription of pBS(−) (Stratagene) linearized with *Xba*I. Internally labeled RNAs were synthesized in the presence of [α-³²P]UTP. For the synthesis of end-labeled RNAs, transcription reactions were performed with nonlabeled NTPs, the reaction mixture was treated with calf intestine alkaline phosphatase, extracted three times with phenol/chloroform and the RNA was recovered by ethanol precipitation in the presence of 20 μg of glycogen. For 5' ³²P-labeled substrate, ~10% of the RNA was labeled with [γ-³²P]ATP to low specific activity using polynucleotide kinase. The incubation mixture was combined with unlabeled RNA and the full-length transcript was isolated by electrophoresis on a 12% polyacrylamide sequencing gel. The RNA was eluted from the excised gel slice by vortexing overnight at 4°C in the presence of equal volumes of extraction buffer [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.1% SDS] and phenol/chloroform. The RNA was recovered from the aqueous phase by ethanol precipitation in the presence of 20 μg of glycogen. Aliquots of the purified, full-length transcript were then labeled with [γ-³²P]ATP to high specific activity to provide substrate for a given assay. For 3' ³²P-labeled substrate, the RNA was labeled with [5'-³²P]pCp using RNA ligase (Bruce and Uhlenbeck 1978). To obtain 3'-labeled RNA with and without a 3' phosphate group, half of the RNA was treated with alkaline phosphatase. Full-length RNAs were then purified and recovered as described above.

RNA analyses

Isolation of total cellular RNA from yeast, Northern hybridizations, and primer extension analyses were performed as described previously (Tollervey 1987; Tollervey and Mattaj 1987; Beltrame and Tollervey 1992). The oligodeoxyribonucleotides used for Northern hybridization and primer extension are as follows; probe a, TTTCGCTGCGTTCTTCATC; probe b, TGAGAAGGAAATGACGCT; probe c, GGCCAGCAATTTC-AAGTTA; probe d, CCAGTTACGAAAATTCTTG; probe e, GAACATTGTTCGCCTAGA; and probe f, CTCCGCTTATT-GATATGC. To characterize the 3'-extended 5.8S rRNA species in *rrp4-1* strains, DNA sequencing reactions were resolved in parallel with total RNA by migration on 40-cm-long, 1-mm thick 8% polyacrylamide sequencing gels. Electrotransfer and Northern hybridization were as described previously (Tollervey and Mattaj 1987) and the lengths of RNA species detected by hybridization were estimated by comparison with the DNA sequencing ladder.

Miscellaneous

Growth and handling of *S. cerevisiae* were performed using

standard techniques (Sherman 1991). Yeast strains were transformed using a lithium acetate procedure (Gietz et al. 1992). Plasmids were recovered from yeast and transformed into *E. coli* using the method of Robzyk and Kassir (1992). Gap repair and allele replacement were carried out according to Rothstein (1991). Data base searches between query sequences and protein or nucleic acid data bases were made using the FASTA and TFASTA programs of the GCG package (Devereux et al. 1984). The sequence alignment was compiled using the CLUSTAL W program (Thompson et al. 1994) and subsequently hand-edited.

Data base accession numbers

The *RRP4* sequence (YHR069c gene) has been submitted to the EMBL and GenBank data bases as part of the chromosome VIII sequence (Johnston et al. 1994) and has the accession number U00061. The accession numbers of the human and *S. pombe* homologs are U07561 and SPAC2F7, respectively.

Acknowledgments

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