Mutational analysis of the PRP4 protein of *Saccharomyces cerevisiae* suggests domain structure and snRNP interactions

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ABSTRACT

The PRP4 protein of *Saccharomyces cerevisiae* is an essential part of the U4/U6 snRNP, a component of the mRNA splicing apparatus. As an approach to the determination of structure–function relationships in the PRP4 protein, we have isolated more than fifty new alleles of the PRP4 gene through random and site-directed mutagenesis, and have analyzed the phenotypes of many of them. Twelve of the fourteen single-point mutations that give rise to temperature-sensitive (ts) or null phenotypes are located in the portion of the PRP4 gene that corresponds to the β-transducin-like region of the protein; the remaining two are located in the central portion of the gene, one of them in an arginine–lysine-rich region. Nine additional deletion or deletion/insertion mutations were isolated at both the amino- and carboxy-termini. These data show that the amino-terminal region (108 amino acids) of PRP4 is non-essential, while the carboxy-terminal region is essential up to the penultimate amino acid. A deletion of one entire β-transducin-like repeat (the third of five) resulted in a null phenotype. All ts mutants show a first-step defect in the splicing of U3 snRNA primary transcript *in vivo* at the non-permissive temperature. The effects on prp4 mutant growth of increased copy-number of mutant prp4 genes themselves, and of genes for other components of the U4/U6 snRNP (PRP3 and U6 snRNA) have also been studied. We suggest that the PRP4 protein has at least three domains: a non-essential amino-terminal segment of at least 108 amino acids, a central basic region of about 140 residues that is relatively refractile to mutation and might be involved in RNA interaction, and an essential carboxy-terminal region of about 210 residues with the five repeat-regions that are similar to β-transducins, which might be involved in protein–protein interaction. A model of interactions of snRNP components suggested by these results is presented.

INTRODUCTION

The spliceosome, a ribonucleoprotein structure which is required for pre-messenger RNA (pre-mRNA) splicing in all eukaryotes, is composed of the pre-mRNA, five species of small nuclear ribonucleoprotein particle (snRNP) and associated factors (1–7). The snRNPs consist of five different species of small nuclear RNA (snRNA; reviewed in 8) and a large number of proteins (reviewed in 1, 7, 9–12), some of whose functions are beginning to be understood.

Knowledge of the protein components of the spliceosome has come primarily from biochemical studies in HeLa cells and genetic studies in the budding yeast, *Saccharomyces cerevisiae*. The former have revealed the so-called core or Sm proteins that are common to four of the five snRNPs (U1, U2, U4 and U5; reviewed in 4). These proteins, along with the trimethylguanosine cap that is found on the 5′-terminus of most snRNAs, have been implicated in the transport of assembled snRNPs from cytoplasm to nucleus (13, 14). Proteins that are specific for the U1, U2, and U5 snRNPs, as well as for the U4/U6 di-snRNP, have also been found in HeLa cells (1, 9–12, 15). In particular, the U1 70K, A, and U2 B proteins of HeLa have been studied both biochemically and structurally; these proteins have a sequence that has been implicated in the binding of some proteins to RNA (16, 17). Homologues of the U1 70K (18) and A (19) proteins have been found in yeast.

Genetic studies in yeast have identified at least thirty PRP (precursor RNA processing) (6, 7) genes, many of which are temperature-sensitive (ts) both for cell growth and mRNA splicing. Their participation in splicing has been studied both *in vivo* and *in vitro*. Some of these gene products are involved in specific steps of the splicing process: PRP9 (20), PRP21 (21, 22) and PRP5 (23) act in spliceosome assembly; PRP2 acts on assembled spliceosomes in the presence of ATP and other factors in the first step of the splicing reaction (24); PRP19 also acts early in the spliceosome assembly process (22); PRP16 (25) and PRP18 (26) are required in the second step, and PRP22 (27) in release of the spliced mRNA from the spliceosome. PRP17/SLU4 and SLU7 appear to be involved in 3′-splice-site selection (28,
A yeast de-branching activity is associated with DBR/PRP26 (30); presumably this is a late step, following or concomitant with the splicing reactions.

Some of the PRP proteins have structural motifs that suggest their function. For example, six splicing-related proteins in yeast, PRP2, 5, 16, 22, 28 and SPP1 have amino acid motifs that are associated with proteins which have RNA-dependent ATPase activity and/or ATP-dependent RNA-helicase activity (23, 27, 31–34), suggesting that conformational changes in RNA are crucial events in most steps of mRNA splicing. To date, PRP2 and PRP16 have been shown biochemically to possess RNA-dependent ATPase activity (25, 36). PRP4 and PRP17 have a repeated amino acid motif with similarity to β-transducins (4, 7, 37); whose significance is not yet understood. Three gene products have an RNA-binding consensus, PRP24 (38), MUD1 (19) and SNP1 (39); the latter is a yeast homologue of the HeLa U1 70K protein. At least two of the PRP gene products, PRP11 (40) and PRP19 (41), have been shown to be associated with the spliceosome, but not yet with a particular snRNP. Several other PRP proteins are known to be associated with a specific snRNP: PRP8 with U5 (42), PRP3 (J.Woolford, personal communication), PRP4 (43, 44), PRP6 (20) and PRP24 (38) with the U4/U6 snRNP, and SNP1 (39) and MUD1 (19) with the U1 snRNP.

The PRP4 gene product is the first protein identified as a component of the U4/U6 snRNP (43, 44). Using a combination of RNase H digestion and immunoprecipitation it was shown that this protein is found in association with the 5’-portion of U4 snRNA, although association with U6 snRNA was not ruled out completely (45). Deletion of the 5’-stem-loop of the U4 snRNA abolished association of the PRP4 protein with the U4/U6 snRNP (46). Neither of these studies demonstrate the direct binding of PRP4 to U4 snRNA. The PRP4 gene product is a 52 kDa protein of 465 amino acid residues (43, 44). Its primary amino acid sequence indicates a structure of five repeats that are similar to repeats in the β-subunit of bovine transducin, as well as the yeast STE4, CDC4 gene products, and other β-subunits of the G-protein family (37). The function of the β-transducin-like sequences is not known. A second structural feature of the PRP4 protein is an acidic stretch in the amino-terminal portion, located between amino acids 99–108 and consisting of a stretch of nine Glu and Asp residues with only one non-charged amino acid in it. A third, central, region of the PRP4 protein is basic.

The role in mRNA splicing of the PRP4 protein, or for that matter of the U4 snRNP, is not yet understood. As a step in defining that role, we report here the results of a genetic dissection of the PRP4 gene product using random and site-directed mutagenesis. We have isolated more than fifty prp4 mutations, of which thirty-seven have been sequenced and partially characterized. These studies indicate that the amino-terminal 108 residues, including the acidic stretch, are non-essential for PRP4 function; that the β-transducin-like segment is essential, although with a surprising degree of tolerance for replacement of residues in the most conserved amino acids of this motif; and that the central segment of PRP4 is also important for function, since mutations in this region cause severe impairment of growth. Cells carrying one of several ts alleles of prp4 show a significantly decreased level of U6 snRNA at the non-permissive temperature, suggesting a role for either PRP4 or the U4 snRNP in assembling or stabilizing the U6 snRNP.

MATERIALS AND METHODS

Yeast strains, plasmids and media

LP112 (Mata ale can1-100/can1-100 his3-11.15/ his3-11.15 leu2-3,112/leu2-3,112 trpl-1/ trpl-1 ura3-1/ura3-1 ade2-1/ ade2-1) is an isogenic diploid strain provided by R.Rothstein. W303-1A (Mata) is a haploid form of strain LP112. The null allele of prp4 (prp4::HIS3 strain YF1582) was constructed as described previously (44). prp4-1 was integrated into the chromosome of strain W303-1A as follows. An integrative, URA3 plasmid (pFL34, given to us by F.Lacroute) was targeted to the chromosome at the PRP4 locus. 5-flouro-orotic acid (5-FOA)-resistant derivatives were isolated and were screened for temperature-sensitive (ts) phenotype. The ts phenotype was verified as being due to a mutation at the prp4 locus by introducing a PRP4-containing plasmid and observing that the phenotype reverted to wild-type; this strain is called YF1634. The prp3-1 mutant strain (Mata adel leu2 his7 prp3-l) was given to us by J.Woolford, and the prp6-l mutant strain (Mata adel lys2 ura3-52 prp6-1) by M.Rosbash.

The following plasmids were used. pBC ks+ (Stratagene, Inc.), YEP13 (LEU2-2-µm), pRS314 (TRP1-CENARS), pRS315 or pSB32 (LEU2-CENARS), pRS316 (URA3-CENARS), all from P.Hieter (47); YEP24 (URA3-2-µm, from D.Botstein), pJAY100 (TRP1-pGAL1-CEN6-ARS) (based on pFL39; derived from F.Lacroute and J.Archambault), pEMBLYE4X (2-µm-URA3-pGAL10). The following plasmids were constructed for this study: pYX112 has the PRP3 gene driven by pGAL10 on pJAY100 (J.Archambault). pYX172 has the U6 snRNA gene under the control of its normal promoter and carried on YEP13. pYX117 has the U6 snRNA gene under the control of its normal promoter and carried on YEP24; the U6 snRNA gene was isolated from the yeast genome by using the polymerase chain reaction (PCR) with two U6 snRNA-gene-specific oligonucleotide primers. pYF1378 has the U4 snRNA gene under the control of its normal promoter and carried on YEP13; pYX185 is the same, but is carried on YEP24. pYW4 was constructed by inserting the PRP4 coding region into pEMBLYE4X between the Xbal and and HindIII sites; a sequence (GGCCATCATCAT- CATCACTCATCCTACAGACCGGCGCATATC-GAAGGGTCGTCATATGCCAGGGATCC) encoding ten histidine residues was inserted immediately downstream of the first ATG codon of PRP4.

Yeast growth media were described previously (48). Growth temperatures are indicated in the text. Plasmids were propagated in E.coli JF1754 (44) or JM101. M13 clones were propagated in E.coli JM101. Single-stranded DNA templates for site-directed mutagenesis were isolated from E.coli strain CJ236 or RZ1032. The prp4-l mutant strain (ts 339; 49) was obtained from the Yeast Genetic Stock Center, Berkeley, CA. Total chromosomal DNA was digested with HindIII, fractionated on a 5—20% sucrose gradient, and DNA in the range of 1.5—4.0 kilobases (kb) was pooled and concentrated by precipitation. These DNA fragments were cloned into the HindIII site of pUC18. This library was introduced into E.coli and the colonies were probed with oligonucleotides specific for the PRP4 gene in order to select the correct plasmid construct. Mutants prp4-2 and 4-3 were obtained from L.Hartwell. The mutant gene sequences were amplified directly from the genome of the original strains by using PCR (50) and oligonucleotide primers that span codons 75—80.
and a region 210 bp downstream of the stop codon; two independent PCR products from each mutation were sequenced.

The various prp4 mutant genes were placed under the control of the GAL10 promoter (pGAL10) by ligating a 1.63 kb Scal–HindIII fragment from the appropriate mutant-bearing plasmid (see below) into HindII–HindIII sites downstream of pGAL10 in pJAY100.

Yeast transformation was carried out as described (51). All DNA manipulations were done according to conventional procedures (50).

PRP4 antibody production and use
For production of antibody against PRP protein, the PRP4 coding sequence was cloned into the BamHI site of pAR3038, which contains a T7 promoter (52). The resulting plasmid, pKSR4 was introduced into E. coli BL21(DE3). Two liters of transformed cells were grown at 37°C and induced (at OD_600 = 0.5) with 1 mM IPTG for 3 h at the same temperature. The cells were harvested by centrifugation at 6,000×g for 10 min and resuspended in 200 ml of 100 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl, pH 8.0. Following addition of lysozyme to 1 mg/ml and phenylmethylsulfonyl fluoride to 1 mM, the cell suspension was incubated at room temperature for 20 min. The cells were collected again by centrifugation and the membrane disrupted by resuspension in the same buffer plus 1% Triton X-100 and 1% Tween-20, then freezing and thawing three times in dry ice–ethanol and 37°C water baths. The cellular DNA and RNA were digested by adding MgCl2 to 8 mM, DNase I to 10 μg/ml and RNase A to 1 μg/ml. The inclusion bodies were collected at room temperature for 10 min and were resuspended in the same buffer containing 25% sucrose, 1% Triton X-100 and 1% Tween-20. The insoluble PRP4 protein was collected by centrifugation again and further purified by using a Prep-cell. Two rabbits were injected subcutaneously with 100 μg of the purified PRP4 protein and were boosted four times each with the same amount of protein at one-month intervals.

The crude antiserum from both rabbits were found to be highly specific when used for immunoprecipitation. In order to decrease the background for protein blots, and since the specific antibodies against PRP4 are expected to be in excess compared to the levels of the non-specific antibodies, total yeast proteins were used to absorb an antibody solution. This was done by immobilizing 5 mg of yeast proteins on a polyvinylidene difluoride (PVDF) membrane (5×10 cm) and incubating the membrane with 50 μl of antiserum in 10 ml of 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20 (TBST) at 4°C for 1 h after blocking the membrane with 5% Carnation instant milk powder in TBST at room temperature for 1 h. The treated antibody solution was saved and used for immunoblotting following an additional 100-fold dilution.

Isolation of random and site-directed PRP4 mutations
Random mutagenesis was carried out as follows. Plasmid pYF423 (LEU2-2-μm-PRP4; 53) was treated with N-methyl-N-nitrosoguanidine as described (53). DNA was prepared in purified form for introduction into strain YF1582, which has a null mutation of prp4 (PRP4::HIS3) as the sole chromosomal copy, and the normal PRP4 function supplied on a URA3-PRP4-CEN-ARS plasmid (pYF1043). Following transformation of this strain with a library of mutagenized PYF423 DNA and growth for many generations on Leu−His−Ura drop-out medium, mutant cells were diluted and grown overnight at 23°C on Leu−His drop-out medium. These were spread on solid medium, grown at 23°C and then were patched on solid Leu−His drop-out medium containing 1 mg/ml 5-FOA, which selects for cells that have lost the PRP4-URA3 maintenance plasmid. Surviving colonies were replica-patched onto 5-FOA-solid medium and were grown at different temperatures; those with temperature-sensitive (ts) or no-growth (null) phenotypes were retained. Plasmids were recovered from these strains by conventional methods, passed through E. coli, and were re-introduced into yeast strain YF1582 (PRP4::HIS3) in order to confirm the plasmid-related phenotype. Those plasmids that survived this test were retained for further analysis.

Site-directed mutations were constructed by conventional methods (50), using PRP4 carried on bacteriophage M13. All mutations were verified by sequence analysis. Mutant genes were then transferred to plasmid pRS315 or pSB32 (LEU2-CEN-ARS) to be introduced into yeast for further analysis.

RNA blots and primer-extension analysis
RNA blots were done with total cellular RNA as described previously (44). Primer-extension analysis was done with 5 μg of total yeast RNA per sample. This was hybridized to 2 ng of γ-32P-ATP-labelled, U3 snRNA-specific and U2 snRNA-specific (as loading control) oligonucleotide in a buffer consisting of 50 mM Tris–HCl, pH 8.0, 40 mM NaCl and 0.5 mM EDTA in a total of 15 μl (54). Hybridization was carried out at 65°C for 5 min, then 52°C for 30 min. Following this 2.5 units of AMV reverse transcriptase (BRL) in a buffer consisting of 0.7 mM dNTPs, 30 mM MgCl2, 3 mM dithiothreitol and 0.6 μg/μl actinomycin D in a total of 50 μl was added and the mixture was incubated for 1 h at 42°C. Following phenol extraction the reaction products were applied to a 6% denaturing polyacrylamide gel.

Yeast protein preparation and immunoblot analysis
Wild-type and mutant PRP4 strains were grown in selective medium at 23°C. The cultures were shifted to 37°C (at OD_600 = 0.3–0.4) for 2 h. Total yeast proteins from 100 ml cultures were prepared as described (55) and were concentrated by precipitation with ammonium sulfate at 350 mg/ml. Protein concentration was determined by using the Bradford assay. Protein blot and the immuno-detection with ECL (Amersham) were done according to the protocol provided by Amersham.

RESULTS
Localization of the prp4-1, prp4-2 and prp4-3 temperature-sensitive mutations
A set of ten (subsequently nine) ts complementation groups was isolated many years ago by random mutagenesis of yeast strain A364A, followed by screening for the inability to accumulate RNA at the non-permissive temperature (49). The fundamental defect in many members of this class of mutants proved subsequently to lie in mRNA splicing (56). PRP4 is among this set of genes (49, 56). It is a component of the U4 snRNP (44, 46) and has a region of intriguing similarity to β-transducin proteins (37). Our aim was to establish functionally essential
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(1) Deletion of the first 9 amino acids and insertion of 2 amino acids (Met-Gly) resulting from the addition of an Ncol site.
(2) Deletion of amino acids 99 to 108 and insertion of 2 amino acids (Gly and Ser in between) in order to get an BamHI site (GGATCC).
(3) Deletion of the first 108 amino acids and insertion of 4 amino acids (Met-Ala-Gly-Ser) resulting from flushing the BamHI site and addition of an Ncol linker to prp4-18.

si refers to slow growth.
chromosome did not contain the first 80 codons; this was done in order to deal with a fragment of practical size for the PCR-mediated recovery, and because the first 108 amino acids are not essential for PRP4 function (see below). The positions of prp4-1, -2 and -3 are shown in Fig. 1a and Table 1—they lie in the region of the gene that corresponds to the β-transducin-like region of the protein. prp4-2 and prp4-3 proved to be identical.

Isolation of site-directed and random point mutations

We obtained additional mutations in the β-transducin-like region by site-directed mutagenesis, targeting three of the most conserved residues (H306, D328 and W334; 37) and changing each of them to four different amino acids. Fig. 1b shows in more detail the amino acid sequence of the five repeats of this region of the PRP4 protein; the consensus amino acids and the locations of changes due to the PRP4 site-directed mutations in the region are indicated. Surprisingly, eight of twelve amino acid changes in these highly conserved residues produced no detectable growth phenotype. The only noticeable changes were due to D328R or D328K (ts or slow growth; prp4-10, -11), W334L (slow growth; prp4-15), and W334P (no-growth; prp4-14).

In addition, we obtained nine single-point mutations by using random mutagenesis (see Materials and Methods). Fig. 1a shows the distribution of these mutations on the PRP4 protein. Six of those that displayed a ts or null phenotype are located in the portion of the gene that corresponds to the β-transducin-like or central region of the protein, indicating the importance of these two regions. Three of the mutations that showed a wild-type phenotype were obtained by separating double mutations that were obtained through random mutagenesis. It is striking that none of the mutations fell in the region of the gene that encodes the amino-terminal region of the protein, suggesting that all or part of this region might not be essential for PRP4 function (see also below).

Isolation of prp4 deletion mutations

The results described above suggested that the amino-terminal segment of PRP4 might be non-essential, in contrast to the carboxy-terminal portion. We obtained further evidence on this point by constructing deletion mutations. Fig. 2 and Table 1 show the distribution of these and the phenotypes conferred by them.

Deletion of the amino-terminal 108 amino acids (prp4-34) or subsets of them (prp4-32, -33) had no effect on cell growth at any temperature. In particular, precise deletion of the stretch of acidic amino acids that lies between residues 99 and 108 (prp4-33) yielded normal PRP4 function (data not shown). However, a deletion that reached to residue 142 (prp4-35) was lethal for growth, indicating that at least part of the region of PRP4 that lies between residues 108 and 142 is essential for function. In contrast to the amino-terminus, the carboxy-terminus of PRP4 was much more sensitive to deletion. Loss of as little as one residue from the PRP4 gene product (prp4-38) led to slow growth at an elevated temperature; removal of two amino acids (prp4-21) yielded ts growth at 35°C. (Note that the prp4-21 mutation, due to a translation stop-codon, results in a deletion of the terminal two residues of the protein.) Removal of nine amino acids (prp4-37) led to temperature sensitivity at 30°C. prp4-30 is a double mutation (Table 1). It contains a stop codon (W457STOP), which yields essentially the same mutation as prp4-37, and an additional amino acid change (A238T). Separation of the two mutations in prp4-30 showed that A238T did not affect growth at any temperature tested. Yet, when this 'wild-type' mutation is combined with the W457STOP ts mutation, the double mutant showed a null phenotype. Another pair of mutations, prp4-21 (W464STOP) and prp4-19 (W464STOP and D240N), showed a similar phenomenon—while prp4-21 displayed a ts phenotype at 35°C, the double mutant (prp4-19) showed a null phenotype but a strain carrying only D240N (prp4-20) was wild-type. These results suggest that the amino-terminal and carboxy-terminal regions of the β-transducin-like domain might interact—the former can tolerate some amino acid changes, but not in the context of changes to the latter.

The β-transducin-like region of PRP4 extends to the carboxy-terminus of the protein (37). The mutations discussed so far lie in four of the five repeat-regions (excepting the third). In order to test whether the third of these repeats is also essential for function, it was deleted precisely from the PRP4 gene product (prp4-36). This destroyed PRP4 function, suggesting that each repeat contains at least one essential residue. This result also implies that other repeat regions cannot substitute for the absence of the third repeat, or that a minimum of five repeats is necessary.

Splicing defects in PRP4 mutants in vivo

The defect in prp4-1 mutant strains has been attributed directly to a defect in mRNA splicing (56). We determined whether the conditional-lethal mutations that were isolated in the present study were unable to splice RNA in vivo. Following a period of exponential growth at room temperature, cells were shifted to non-permissive conditions (37°C) for 3 h. Total RNA was extracted and was analyzed by primer-extension for the presence of unspliced precursors of U3 snRNA, which contains an mRNA-type of intron (55). As can be seen in Fig. 3A, in addition to prp4-1 (lanes 3, 4), prp4-11 (lanes 5, 6), -21 (lanes 7, 8) and -37 (lanes 9, 10) showed a U3 snRNA splicing defect at the non-permissive temperature, accumulating both species of U3 primary transcript. Fig. 3B shows the quantitation of the ratios of the total pre-U3 snRNAs to the mature U3 snRNAs. In addition, three null mutations that showed a ts phenotype when they were transferred to a 2-μM-based plasmid (prp4-23, -25 and -26; see Table 1) also were unable to splice U3 snRNA to completion at the non-permissive temperature (unpublished results). Thus, all ts mutants that were isolated in this study showed a splicing defect in vivo at 37°C.

Over-expression of some PRP4 mutant gene products suppresses their own mutant phenotype

It has been demonstrated that over-production of the gene products of at least one conditionally-lethal mutant mRNA splicing gene (prp11-1; 57) can reverse the mutant phenotype. This might be explained by assuming that the mutational defect is in the rate of assembly or stability, due to weak binding of the mutated protein to a complex structure, which can be overcome by enhancing the intracellular concentration of gene product, thus driving assembly. We tested a selection of prp4 mutations, some null and some conditional-lethal, for this effect by increasing gene copy-number by placing them on a high-copy (2-μM) plasmid, or in some cases by transcribing them from the highly inducible GAL10 promoter (pGAL10). Previously we have shown that the cellular level of PRP4 gene product is enhanced when the wild-type gene is present on a 2-μM plasmid (44). This was confirmed by immuno-blotting for both mutant and wild-
Figure 1. Distribution of PRP4 point-mutational changes and phenotypes conferred by them. Point mutations were obtained by random and site-directed mutagenesis (see text); the phenotypes were determined by a plasmid shuffle method as described in Materials and Methods. (a) The upper section indicates the mutations that gave rise to ts or slow-growth phenotypes. The middle section represents the distribution of point mutations that conferred a null phenotype. The lower section shows the distribution of point mutations that gave rise to a wild-type phenotype. All point mutations obtained either by site-directed or random mutagenesis are described in Materials and Methods, (a). The upper section indicates the mutations that showed a ts or slow-growth phenotype when on a 2-μm-based plasmid. Mutations labeled * showed a null phenotype when placed on a CEN-ARS plasmid, but became ts when expressed under the control of pGAL10. Italized numbers refer to pprp4 alleles. (b). The locations of mutations in the β-transducin-like region of the PRP4 protein. All point mutations obtained either by site-directed or random mutagenesis are underlined to show their locations in the five repeat-regions. Three conserved amino acids (H306, D328 and W334) were targeted and each of them was changed to four different amino acids, as indicated in the lower part of the figure. Amino acid changes indicated with * are null or ts; all others are wild-type. Underlined amino acids are mutated in this study.

Figure 2. PRP4 mutations that give rise to protein deletions, and phenotypes conferred by them. pprp4-32, 33, 34, 35, 36, 37 and 38 were constructed by site-directed mutagenesis. pprp4-39 and 40 were constructed by XbaI linker-insertion mutagenesis. pprp4-21 was obtained by random mutagenesis, which resulted in a change of codon 464 to a stop codon.

Table 1: Phenotypes of prp4-11, -23, -25, -26, -31, -32, -34, -35 and -39 mutant alleles.

<table>
<thead>
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<th>Allele</th>
<th>Phenotype</th>
<th>ts at 30°C</th>
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<td>prp4-40</td>
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Type PRP4 protein. As shown in Fig. 4, PRP4 levels were higher when the alleles were expressed from 2-μm plasmids (Fig. 4A, lanes 2, 4, 6 and Fig. 4B, lanes 3, 5, 7) than when they were expressed from CEN-ARS plasmids (Fig. 4A, lanes 1, 3, 5 and Fig. 4B, lanes 2, 4, 6) regardless of whether they were wild-type or mutant alleles. The only exception is prp4-11 protein, which, although over-expressed when the gene is carried on a 2-μm plasmid, appears to be more unstable than its siblings.

As is shown in Table 1, one of the original ts mutations, prp4-11, when present on a 2-μm-based plasmid, yielded normal growth at both 30°C and 37°C. A similar result was obtained for prp4-19 and prp4-27. A rather more pronounced effect was obtained with other mutants, such as prp4-19, -21, -23, -25, -26 and -30. These changes resulted in either a non-functional phenotype or temperature-sensitivity at 30°C when the mutant gene was carried on a CEN-ARS plasmid, but a ts phenotype when carried on a 2-μm plasmid. The latter class of plasmids has a higher copy-number than the former, which probably also results in a higher production of gene product. Increased gene product was obtained independently by transcribing pprp4-23, -25, -26 and -29 from pGAL10; these phenotypes were essentially the same as when these alleles were present on a 2-μm-based plasmid. prp4-17, when over-produced from pGAL10 changed from a null phenotype to a ts phenotype, although when the allele was present on a 2-μm plasmid, its phenotype was null (Table 1).
These results suggest that the defect in the mutant *prp4* gene products that we tested lies in their ability to bind to the U4 or other components of the U4/U6 snRNP; increasing the intracellular protein concentration drives the defective gene product towards association with the snRNP, reversing the conditional-lethal phenotype.

**Over-expression of PRP3 suppresses the temperature-sensitive phenotype of *prp4-1* and *prp4-11**

We sought to determine whether reversal of the ts or null phenotype of any of the *prp4* mutants could be obtained by over-production of any other component that might be expected to interact with the *PRP4* gene product. A good candidate is *PRP3*, since elevated copy-number of this gene has been shown to suppress the ts phenotype due to the *prp4-1*, -2 and -3 alleles (58). Furthermore, anti-PRP3 antibody can precipitate the U4/U6

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**Figure 3. In vivo splicing of U3 snRNA.** (A). Cells carrying *prp4-1, 11, 21* or 24 were grown at room temperature to $A_{600} = 0.8-1.0$. The cultures were divided in two parts and were incubated continuously at room temperature or 37°C for a further 3 h. Total RNA was isolated and 5 μg of RNA was used for primer-extension analysis as described in Materials and Methods. RT indicates room temperature. (B). The primer-extension products were quantitated by using a Phosphorimager (Molecular Dynamics). The ratios of total pre-U3 to mature U3 snRNAs from wild-type and mutant *prp4* strains are presented.

**Figure 4. Intracellular content of PRP4 protein in wild-type and mutant *prp4* stains.** Preparation of yeast proteins and immuno-blotting were done as described in Materials and Methods. Protein blots in Fig. 4A and Fig. 4B were done according to the same protocol except that a re-used primary antibody solution used in Fig. 4B gave a lower background so that the film was allowed to be exposed longer in ECL detection. 50 μg total yeast proteins were used in each lane, but because of a higher level of His-tagged PRP4 proteins expressed from pYW4, only 2 μg total yeast proteins were used in lane 7 of Fig. 4A and lane 8 of Fig. 4B. Molecular weight standards are indicated on the right side of the figure. Closed arrowheads mark the location of PRP4 and open arrowheads point to a non-specific band that can be used as a loading control. Note that the gene product of *prp4-37* should be 1 kDa smaller and the His-tagged PRP4 protein should be 2 kDa larger than the wild-type PRP4 protein. The poor resolution in lanes 4, 6 and 7 of Fig. 4B was caused by a high salt concentration in the samples loaded; because in these samples the protein concentration was lower, a larger volume had to be loaded in order to apply the same amount of protein to each lane. Abbreviations: CEN-ARS, a *PRP4* allele carried on a CEN-ARS plasmid; 2-μm, a *PRP4* allele carried on a 2-μm plasmid; WT, wild-type *PRP4* on chromosome.
Figure 5. Suppression by over-expression of the PRP3 gene of the phenotypes due to some PRP4 mutations. pJAY100 (control plasmid) and pYX112 (pGAL10-PRP3-TRP1-CEN-ARS) were introduced into strains carrying prp4-l (sectors 1a and 1b) or prp4-l1 (sectors 1a and 11b). The prp4-l strain carries the mutant gene integrated into the chromosome in a strain W303-la background. The prp4-l1 strain carries the mutant gene on pRS315 (LEU2-CEN-ARS) and an interrupted PRP4 gene on the chromosome. Transformants were grown on His-Leu-Trp drop-out solid medium with 2% galactose as the sole carbon source; they were then streaked on to the same medium and were grown at different temperatures. Sectors labelled 1a and 11a contain cells that were transformed with the control plasmid (pJAY100); sectors labelled 1b and 11b contain cells that were transformed with pYX112 (pGAL10-PRP3-TRP1-CEN-ARS). Duplicate experiments are shown. RT denotes room temperature.

Figure 6. Suppression by over-production of U6 snRNA of the phenotypes due to three prp4 mutations. Strains carrying prp4-l, 11 or 24 were transformed with control plasmid YEP24 (a) or pYX117 YEP24 carrying the U6 snRNA gene (b). Transformants were grown on His-Leu-Ura drop-out solid medium and were then streaked on the same medium for growth at different temperatures.

snRNP (J. Woolford, personal communication), indicating that PRP3 is one of its components. A CEN-ARS plasmid that carried PRP3 under the control of the pGAL10 (pYX112; see Materials and Methods) was introduced into host strain YF1582 (PRP4::HIS3), which also harboured a plasmid that carried various prp4 ts alleles. Individual colonies of these strains were streaked on solid, selective growth medium containing either 2% glucose (repression of PRP3) or 2% galactose (induction of PRP3) and were incubated at 23°C, 30°C or 37°C. For experiments with non-functional prp4 mutants, pYX112 (pGAL-PRP3) was introduced into host strains that carried both wild-type and mutant PRP4 genes on different plasmids, the wild-type being on a URA3 plasmid; the transformants were tested on galactose-5-FOA agar, which selected for cells that express PRP3 and have lost the PRP4-URA3 plasmid.

As is shown in Fig. 5, prp4-1 (S320F) and prp4-11 (D328R) were suppressed by over-production of the PRP3 gene product (see sectors 4-1b and 4-11b in the figure), in agreement with earlier findings (58); prp4-2/prp4-3 (K272E) could also be suppressed under these conditions (data not shown). However, none of the other twelve mutations that gave a ts or null phenotype on a CEN-ARS plasmid could be suppressed in this way. It is noteworthy that pGAL-PRP3 could not suppress any null alleles of the PRP4 gene, regardless of their location in the gene. This suggests strongly that PRP3 does not substitute for PRP4, but rather compensates for defective PRP4 by interacting with it. A second observation of importance is that of all the prp4 alleles tested, the only ones that can be suppressed by increased PRP3 protein lie in the ß-transducin-like repeat region of the protein. This is compatible with the suggestion that the PRP3 and PRP4 proteins interact with one another, and that certain alterations in the PRP4 protein (particularly in the region of amino acids 272 and 320-328) render it unfavourable for binding to PRP3. Enhancing the intracellular concentration of PRP3 by transcription of that gene from pGAL10 might drive it to association with certain PRP4 proteins that are partially debilitated in function. Many of the other mutationally altered PRP4 proteins are either incapable of associating with PRP3 under any circumstances, even when they are over-produced, or if they can associate they are non-functional for other reasons.

Over-expression of U6 snRNA partially relieves the phenotype of some prp4 mutants

U4 snRNA and U6 snRNA were tested for over-production-reversal of the phenotypes due to prp4 mutants. The U4 snRNA gene when carried on a 2-μm plasmid failed to reverse the ts phenotypes due to prp4-1, -11, 21 and 24; a pGAL10-driven U4 snRNA also did not suppress the prp4-l ts phenotype. Subsequent experiments (data not shown) indicated that the intracellular content of U4 snRNA was not increased under these conditions, perhaps due to rapid degradation of over-produced, free snRNA. Consequently, this line of investigation was not pursued further.
Allele: PRP4 prp4-lla prp4-llb
Temp: RT 37 RT 37 RT 37

Figure 7. RNA blot-analysis of snRNAs present in cells with and without the U6 snRNA gene carried on YEP24. Wild-type PRP4 and the 4-21 strains were transformed with control plasmids (designated a; lanes 1, 2, 3, and 4) and pYX117 (YEP24 carrying the U6 snRNA gene; designated b; lanes 5 and 6). Cultures were grown at room temperature to $A_{600} = 0.8$. The cultures were then divided into two and incubated at room temperature or 37°C for a further 3 h. RNA blot-analysis was done as described in Materials and Methods. Oligonucleotides specific for U4a, U5a, and U6a (34) were used as probes for snRNA U4, U5 and U6, respectively. RT designates room temperature.

In contrast, additional U6 snRNA gene copy-number (carried on a 2-μm plasmid) partially reversed the ts phenotypes due to prp4-ll and -21 (Fig. 6, sectors 4-1 lb and 4-2 lb) and also prp4-l (data not shown), but not of any others. In these strains the relative intracellular amount of U6 snRNA was noticeably reduced at the non-permissive temperature and addition of a plasmid that carried the 2-μm-U6 plasmid reversed this; results for prp4-l are shown in Fig. 7. This suggests that some mutant PRP4 proteins lead to destabilization of U6 snRNA, perhaps by rendering the U6 snRNA susceptible to RNase degradation; overproduction of U6 snRNA might partially reverse this effect by enhancing snRNP assembly.

DISCUSSION

We have isolated and characterized point and deletion mutations in the Saccharomyces cerevisiae PRP4 gene, which is part of the U4/U6 snRNP (43, 44), an essential component of the mRNA splicing apparatus. Analysis of some of these (including three of the original isolates; 49), consisting of nine ts and slow-growing mutations, six null and eleven wild-type alleles, suggests the following conclusions about the PRP4 protein.

First, the PRP4 protein consists of three major domains. Domain I, the amino-terminal 108 residues, is entirely unnecessary for the function of the protein under any growth conditions that we have used. No random mutations were recovered in this region, and its outright deletion has no growth phenotype. In particular, a stretch of ten amino acids (residues 99—108), whose acidic nature might have suggested a function, is non-essential.

Domain II, the central portion of the protein from residue 109 to 252, which shows no similarity to any other known protein, is essential for function. We have isolated two null phenotype point mutations in this region: E151K (prp4-17) and G193E (prp4-22). Neither was suppressible by over-expression of any other U4/U6 snRNP component that we tested, including U6 snRNA and the mutant proteins themselves, although E151K can be partially self-suppressed if over-produced from pGAL10. Deletion of the protein from its amino-terminus up to residue 142 resulted in a null phenotype, confirming the importance of domain II.

Domain II has a region that is relatively rich in arginine and lysine residues (amino acids 135—156). Might this region have a function? If one assumes an α-helical structure for this region, then four arginine residues can be seen to be aligned on one face of the helix, and two lysine and one arginine residues lie on a face immediately adjacent to it (unpublished observations). The available data show that PRP4 is associated primarily with the 5′-stem-loop of U4 snRNA (45, 46), although no evidence yet shows that PRP4 binds RNA directly. However, if indeed PRP4 does bind to U4 snRNA, either singly or cooperatively with another protein, then perhaps it is through the arginine—lysine-rich region noted above. The binding of Tat protein to Tar RNA in HIV is reminiscent of this mechanism of RNA—protein
interaction (59).

In support of this suggestion, we note that the phenotype of at least one conditional-lethal mutation in the 5'-stem-loop of U4 snRNA can be suppressed by over-production of PRP4 (Hu et al., submitted), suggesting interaction of PRP4 and U4 snRNA.

Domain III is the β-transducin-like region of the protein, consisting of amino acids 252–465. About thirty mutations are located in this domain. Mutations occur as frequently in non-conserved as in conserved residues of the β-transducin-like motif. Even the most highly conserved residues show surprising plasticity; this is probably not due to redundancy of function, since deletion of one complete repeat is lethal. One of the most highly conserved residues in the repeat region, D328 (37), an acidic amino acid, can tolerate a substitution of either another acidic amino acid, E, or a neutral one, G, but not a positively charged one, K or R, indicating the importance of charge at this site. W334, which lies on a highly conserved site that was studied in some detail, can be restored by supplying additional copies of the U4 snRNA gene product was (45). Apparently in some mutant prp4 strains the defective PRP4 protein results in destabilization of the U4/U6 complex, perhaps exposing the U6 snRNA to intracellular nuclease attack, as is suggested in Fig. 8. Overproduction of U6 snRNA might simply enhance the U6 snRNA pool, driving U4/U6 interaction, thus protecting U6 snRNA.

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