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PRP4 (*RNA4*) from *Saccharomyces cerevisiae*: Its Gene Product Is Associated with the U4/U6 Small Nuclear Ribonucleoprotein Particle

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The *PRP4* (*RNA4*) gene product is involved in nuclear mRNA processing in yeast cells; we have previously cloned the gene by complementation of a temperature-sensitive mutation. Sequence and transcript analyses of the cloned gene predicted the gene product to be a 52-kilodalton protein, which was confirmed with antibodies raised against the *PRP4* gene product. These antibodies inhibited precursor mRNA splicing in vitro, demonstrating a direct role of *PRP4* in splicing. Immunoprecipitations with the antibodies indicated that the PRP4 protein is associated with the U4/U6 small nuclear ribonucleoprotein particle.

Nuclear mRNA processing occurs in the spliceosome in a two-step reaction involving a lariat intermediate (for reviews, see references 19 and 43). The spliceosome is a large complex, 60S in HeLa cells (18, 49) and 40S in the yeast *Saccharomyces cerevisiae* (4, 13), that consists of both proteins and small nuclear RNA (snRNA). The snRNAs are found in small nuclear ribonucleoprotein particles (snRNPs) that contain a unique snRNA species as well as proteins. Formation of the spliceosome involves the ordered assembly of at least four snRNPs as well as other factors on the precursor mRNA (11, 26, 47).

The snRNA species of the spliceosome have been identified in both HeLa and yeast cells; they are U1, U2, U4, U5, and U6 (the yeast snRNAs are also known as snR19, LSR1/snR20, snR14, snR7, and snR6, respectively [19a]). Biochemical and genetic analyses have shown that U1 is involved in recognition of the 5′ splice site (27, 55, 57, 65) and also of the branchpoint region (52) and that U2 interacts with the branchpoint region (2, 44); U1 and then U2 are the first snRNPs to assemble on the precursor. Subsequently, U4, U5, and U6 enter the spliceosome. The U5 snRNP probably interacts with the 3′ splice site (9, 17, 61). No specific function has yet been found for U4 and U6. U4 and U6 form a very stable complex with each other (5, 22, 51) and have also been found to interact with the U5 snRNP (11, 26, 37); it is possible that these three snRNPs enter the spliceosome as a single complex. Since U4 is not found in the functional spliceosome, it must be released before splicing occurs (11, 28, 48).

Whereas the snRNAs of the snRNPs have been well characterized, much less is known about the proteins. In HeLa cells, snRNPs have been analyzed biochemically. All snRNPs contain a set of seven core proteins, ranging in size from 9 to 29 kilodaltons (kDa): B, B′, D, D′, E, F, and G. In addition to these, unique proteins are found in U1 and U2: A (34 kDa), C (22 kDa), and 70K (70 kDa) in U1 and A′ (33 kDa) and B″ (29 kDa) in U2 (38). The roles of the proteins in the snRNPs have not yet been determined.

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In *S. cerevisiae*, identification of the protein components of snRNPs has been approached genetically. A group of 10 genes, *PRP2* to *-11* (*RNA2* to *-11*) (20, 21), were suspected to be involved in mRNA processing because cells with temperature-sensitive mutations in these genes accumulated precursor mRNA at the nonpermissive temperature (29, 62). According to a recent consensus, it has been agreed to rename the RNA genes *PRP*, for precursor RNA processing. In this paper, we will use the designations *PRP* for the genes and PRP for the gene products. For example, PRP4 is the protein encoded by *PRP4*. Extracts prepared from temperature-sensitive *prp2*, *prp3*, *prp4*, *prp5*, *prp7*, *prp8*, and *prp11* mutants were also found to be temperature sensitive for processing in vitro (39), indicating a direct role for these gene products in mRNA processing. Recently, additional mutants that accumulate precursor mRNA or processing intermediates have been isolated (*prp17* to *-27*; U. Vishayraghavan and J. Abelson, personal communication). Several of the *PRP* genes have been cloned and analyzed (25, 31, 34, 58). The *PRP8* gene product is a 260-kDa protein that is part of the U5 snRNP (37). The *PRP11* gene product is a 32-kDa protein that is found in the functional spliceosome (10). The *PRP2* gene product is a 100-kDa protein (33) that functions in splicing after formation of the 40S spliceosome complex (35). PRP2, PRP3, and PRP11 have been localized in the nucleus (10, 32). Genetic analysis has identified *PRP16* (14) on the basis of suppression of a mutation in the branchpoint sequence of the precursor mRNA, which suggests that the *PRP16* gene product may be part of the U2 snRNP or a U2 accessory factor.

Here we report our studies of *PRP4*. We have analyzed the gene and its expression in yeast cells. The *PRP4* gene product is an essential 52-kDa protein that is part of the U4/U6 snRNP.

MATERIALS AND METHODS

Strains and plasmids. *S. cerevisiae* LP112 (**a**/α *can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 ade2-1/ade2-1*) is an isogenic diploid (kindly provided by R. Rothstein). W303-1A (*MAT***a**) is a haploid of LP112. LL20 (*MAT*α *his3-11 leu2-3,112 can1*) and YR04-304 (MATα *ade1 his3-11,15 his7 leu2-3,112 lys2*

tyr1 rna4-1) have been described elsewhere (58). Splicing extracts were prepared from EJ101 (36).

Plasmid pYF423 is the original *PRP4* clone (58) that carries *PRP4* as a 2.4-kilobase (kb) *Hin*dIII fragment on pYF91, a 2μm-based replicating plasmid with *LEU2* as selectable marker (59). pYF400 contains *PRP4* sequences from the *Bal*I site at nucleotide 278 (see Fig. 1 for nucleotide numbers) to the *Hin*dIII site at nucleotide 2427; this is carried in a high-copy-number leu-2-d vector that is similar to pJDB207 (3). pYS65 contains *PRP4* sequences from the *Bgl*II site at position 282 to the *Hin*dIII site at 2427 in the vector pint2, a centromere plasmid with *URA3* as selectable marker (46). pYS76 contains *PRP4* sequences from the *Spe*I site at position 789 to the *Hin*dIII site (position 2427) inserted behind the *GAL10* promoter in FGP81 (unpublished data), an integrating *URA3* plasmid. pHK412 and pHK413 are *Escherichia coli* expression vectors (kindly provided by D. Harbrecht, Molecular Genetics Inc., Minnetonka, Minn.); they contain the *lac* promoter-operator driving a *cro-lacI-lacZ* fusion. In pHK412, the fusion is in frame relative to *lacZ*; in pHK413, it is out of frame. pYS43 is an insertion of *PRP4* sequences (nucleotides 938 to 2181) in pHK413 that restores the reading frame of *lacZ*.

Yeast media were essentially as described previously (41). LP112 and W303-1A were grown at 30°C; YR04-304 (temperature sensitive) and LL20 were grown at 23°C.

Plasmids were propagated in *E. coli* JF1754 (58) except for *lacZ* fusion plasmids, which were propagated in JS1060 (F′ *lacI*^{q}; from D. Harbrecht). M13 clones were propagated in *E. coli* JM101 (64).

DNA analysis. All DNA manipulations were performed essentially as described by Maniatis et al. (40). All sequences were obtained by the dideoxy method of Sanger et al. (53). Overlapping restriction fragments from both strands were subcloned into M13mp10 and M13mp11. All reactions were primed with the universal sequencing primer (New England BioLabs, Inc.). The DNA sequence was searched against GenBank version 57.

Transcript analysis. Total yeast RNA was isolated and precipitated with 2 M LiCl to eliminate DNA contamination as described previously (58). RNA was electrophoresed on agarose-formaldehyde gels (40), blotted to nylon membrane, and processed as specified by the manufacturer. Double-stranded probes were prepared by the random-primer labeling procedure (15), and single-stranded probes were prepared by primer elongation on an M13 template (6).

Mapping of transcription start sites. S1 mapping was done essentially as described by Maniatis et al. (40). Total yeast RNA (100 μg) was hybridized to 0.5 μg of 5′-end-labeled DNA fragment for at least 3 h at 43°C. The hybrids were treated with nuclease S1 for 30 min at 37°C (150 U; Boehringer-Mannheim Biochemicals) and loaded on nondenaturing polyacrylamide gels along with size markers and parallel reaction mixtures without added RNA. For mapping of the *PRP4* start site, the labeled ends were at *Fnu*4HI (position 1439), *Taq*I (1213), *Acc*I (1119), *Bgl*II (958), and *Hpa*II (848). The unlabeled end was in all cases *Cfo*I at position 346. For mapping the *ORF2* start site, the labeled 5′ end was *Bgl*II at position 282, and the unlabeled end was *Hpa*II at position 848.

For primer extension analysis of *PRP4* mRNA, a synthetic 20-nucleotide primer (5′-TTATGTTGCAAATCTA CCGG-3′) complementary to sequences flanking nucleotide 845 was 5′ end labeled with [γ-^{32}P]ATP and used to prime cDNA synthesis from total RNA from LL20 as described previously (16). The same oligonucleotide was used to prime a DNA sequencing ladder from M13mp11 DNA carrying the *Hgi*A1 fragment from nucleotides 132 to 1167. The cDNA and sequencing products were electrophoresed on a denaturing polyacrylamide gel.

Yeast transformations. All transformations of LP112 and W303-1A were done by the lithium acetate method (24). Transformations of YR04-304 and LL20 were performed by the spheroplast method (23). For introduction of plasmids, 1 to 2 μg of DNA was used; for gene replacements on the chromosome (null mutations), 2 to 5 μg of purified fragment was used.

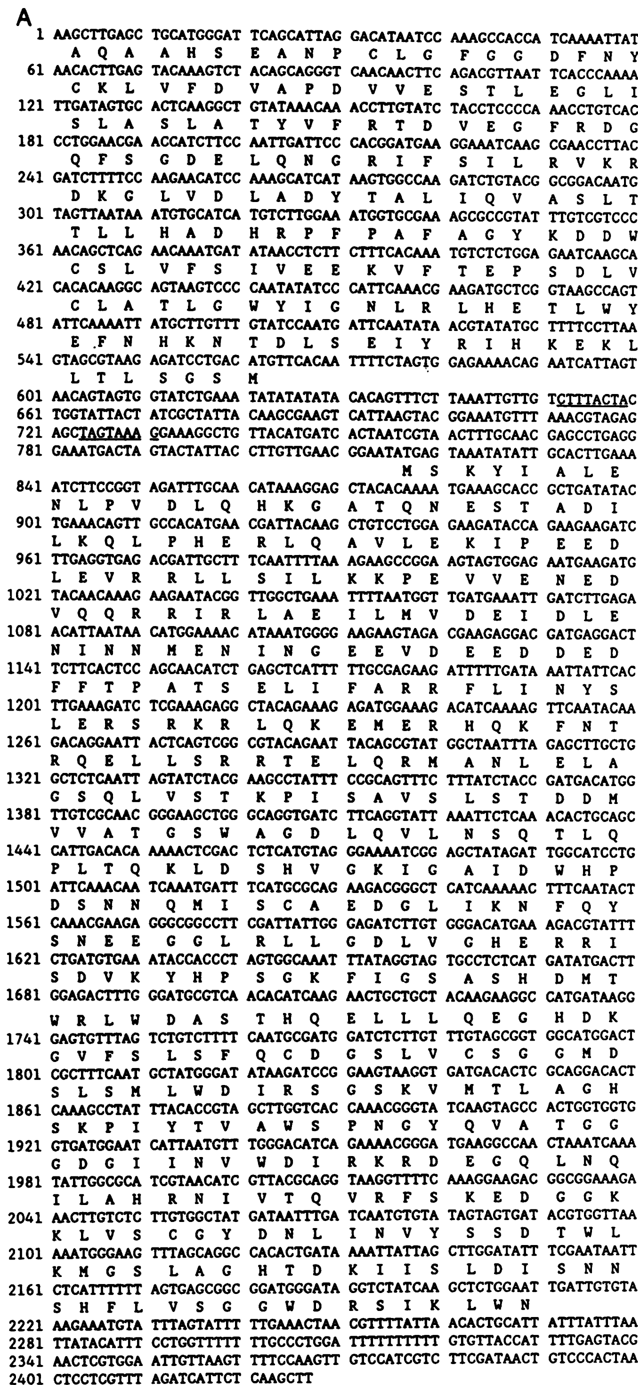
To construct the *prp4* null mutant, LP112 was transformed with the *Sst*I-*Hin*dIII fragment (nucleotides 1167 to 2427) with *HIS3* (isolated on a 1.2-kb *Bam*HI fragment from pYF92 [59]) inserted at the *Bcl*I site at position 2070. To construct the *orf2* null mutant, LP112 was transformed with the *Hin*dIII-*Dra*I fragment (nucleotides 1 to 712 with *LEU2* (isolated on a 3-kb *Bgl*II fragment from pJZ1 [54]) inserted at the *Bgl*II site at position 282. Transformants were analyzed by Southern blots for correct replacements of the chromosomal regions. Tetrad dissections always involved at least 10 tetrads from two independent transformants.

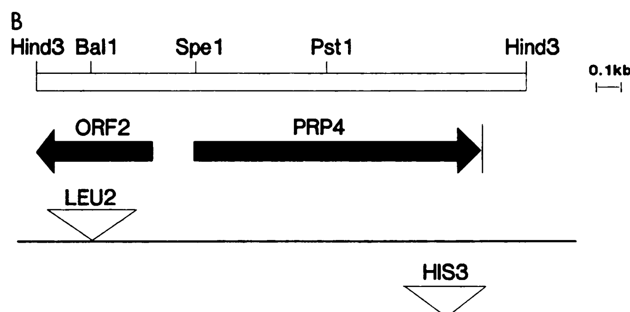
PRP4 antibodies. *PRP4-lacZ* fusions were constructed by cloning random *PRP4* fragments generated by *Bal* 31 digestion into the *Sma*I site of pHK413. Lac$^+$ colonies were isolated; the presence of *PRP4* inserts was confirmed by restriction enzyme analysis, and the induction of fusion proteins was visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of crude *E. coli* extracts. The fusion junctions of two clones were determined by sequencing the fusion junctions; one fusion, pYS43, containing 1,245 base pairs of *PRP4* coding sequence, was chosen for further work.

Antigen for immunization was prepared by isopropyl-β-D-thiogalactopyranoside (IPTG) induction of pYS43 for 45 min, followed by preparative gel electrophoresis. The proteins were visualized by brief staining of the gel with 0.2% Coomassie brilliant blue R in water, followed by destaining in water, and the 165-kDa fusion protein was cut from the gel and electroeluted. (Staining in water rather than methanol prevented precipitation of the protein in the gel and increased recovery at least twofold.) The final yield of fusion protein was about 2 mg/liter of induced *E. coli*.

Two rabbits were injected subcutaneously with 0.3 mg of fusion protein in Freund complete adjuvant and boosted with the same amount in incomplete adjuvant at 1-month intervals. Immunoglobulin G (IgG)-IgA fractions were purified from preimmune and immune sera as described previously (12). Briefly, antibodies were precipitated from total serum with ammonium sulfate, desalted, and dialyzed against 50 mM Tris (pH 8.6)–0.15 M NaCl. Antibodies were affinity purified by eluting antibodies from PRP4 protein bound to nitrocellulose by incubating the filters in 0.2 M glycine hydrochloride (pH 2.8), followed by neutralization with NaOH. Antibodies were concentrated in 50 mM Tris (pH 8)–0.15M NaCl, using Centricon filtration tubes (Amicon Corp.). Purity and titer of purified antibodies were tested on Western blots (immunoblots).

Protein blots. Crude yeast protein extracts were prepared as described by Miller et al. (42). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting were done essentially as described by Burnette (7) except that detection of bands was by incubation with secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG; Promega Biotec), followed by incubation with alkaline





phosphatase dye substrates nitroblue tetrazolium and bromochloroindolyl phosphate.

Inhibition of in vitro splicing. Splicing extracts were prepared as described by Lin et al. (36). Substrate precursor mRNA rp51 was transcribed in vitro with SP6 polymerase from plasmid pHZ18 (62) cut with *Eco*RI and RNA was purified as described previously (36). Splicing assay conditions were as specified by Lin et al. (36). Splicing reaction mixtures were incubated for 20 min at 23°C after addition of substrate. For inhibition studies, splicing extracts (10 μl) were preincubated with 1 μl of antibodies (IgG-IgA fractions at 8 mg/ml) for 1 to 20 min at 23°C before addition of substrate. Splicing products were analyzed on 5% denaturing polyacrylamide gels.

Immunoprecipitations. Precipitations were performed essentially as described by Lossky et al. (37). Whole antibodies were used at 3 or 6 μl per reaction and incubated with 10 or 20 μl of splicing extracts. Proteins labeled in vivo were prepared as described elsewhere (37). Bound RNA was either end labeled with pCp as described previously (37) and electrophoresed on a 6% denaturing polyacrylamide gel or electroblotted to HybondN (Amersham Corp.) membrane after electrophoresis and probed with oligonucleotides complementary to U1 (5'-CACGCCTTCCGCGCCGT-3'), U2 (5'-CTACACTTGATCTAAGCCAAAAGGC-3'), U4 (5'-ATATGCGTATTTCCCGTGCT-3'), U5 (5'-GTTGACCTCCCTCCGCC-3'), and U6 (5'-TGCTGATCATCTCTGTATTG-3') (19a). The antigen fractions for the competition experiments were prepared from *E. coli* JS1060 transformed with pYS43 (*PRP4-lacZ*) or pHK412 (*lacZ*). Cells grown in LB medium were induced with IPTG for 45 min, harvested, suspended in 15% sucrose–50 mM Tris hydrochloride (pH 8)–50 mM EDTA with 1 mg of lysozyme per ml, incubated for 30 min, lysed with 0.2% Triton X-100, and centrifuged at 20,000 × *g* for 10 min. This supernatant is designated fraction 1. The pellet was suspended in 2 M urea, sonicated, and centrifuged as described above. The supernatant is referred to as fraction 2. The pellet was suspended in 6 M urea, sonicated, and centrifuged as described above. The supernatant and pellet obtained after dialysis against phosphate-buffered saline (10 mM $NaPO_4$ [pH 7.4], 150 mM NaCl) are designated fractions 3 and 4, respectively. Whole-cell extracts and fractions were analyzed on sodium dodecyl sulfate-polyacrylamide gels and Western blots. PRP4–β-galactosidase fusion protein was found predominantly in fraction 3 and in smaller amounts in fraction 2; the β-galactosidase itself was predominantly in fraction 2.

RESULTS

***PRP4* is on chromosome 16.** The *PRP4* gene has been cloned by complementation of a temperature-sensitive *prp4* mutant (58); it is contained on a 2.4-kb *Hin*dIII fragment. This fragment was used to probe a blot of an orthogonal-field

FIG. 1. (A) Nucleotide sequence of *PRP4* and *ORF2*. Numbering starts at the *Hin*dIII site that is the cloning site 5' to *PRP4*. The *PRP4* reading frame starts at position 816. The *ORF2* reading frame starts at position 562 on the complementary strand and reads toward the left. A conserved sequence upstream of *PRP4* and *ORF2* is underlined. The amino acid sequence is written in one-letter code below the middle nucleotide of codons (for *ORF2* of the complementary strand). (B) Summary of the organization of *PRP4* and *ORF2*. Transcripts and their directions are indicated by arrows. Positions of the insertions of *LEU2* and *HIS3* used to create the null alleles are indicated below the transcripts.

agarose gel on which intact chromosomes from two different yeast strains had been separated (kindly provided by M. Olson [8]). The probe hybridized specifically to chromosome 16 of both strains (data not shown).

Nucleotide sequence. Overlapping restriction fragments of the 2.4-kb *Hin*dIII fragment were subcloned into bacteriophages M13mp10 and M13mp11, and both strands were sequenced by the dideoxy method. The region that complements the *prp4* temperature-sensitive allele contains an open reading frame (ORF) of 465 amino acids (Fig. 1A). The 2.4-kb *Hin*dIII fragment contains part of a second ORF (*ORF2*) on the strand opposite *PRP4* and reading in the opposite direction. The nucleotide sequence is shown in Fig. 1A. The *PRP4* ORF starts at position 816 and terminates at position 2210; *ORF2* starts at position 562. The intergenic region contains several potential TATA boxes. The only similarity between the *PRP4* sequence and others in sequence data bases is to the beta subunit of transducin (60). *ORF2* was not found to have any significant similarity to other known DNA or protein sequences. The gene organization of the *PRP4-ORF2* region is summarized in Fig. 1B.

Transcript analysis. Soltyk et al. (58) found that the 2.4-kb *Hin*dIII fragment hybridized to two transcripts, a 1.6-kb RNA that hybridized to sequences to the right end of the clone (Fig. 1B) and a 1.3-kb RNA that hybridized to sequences from the left end. To determine the orientations of the two transcripts, RNA blots were probed with complementary single-stranded probes. The 1.4-kb *Hin*dIII-*Pst*I fragment, which hybridized to both the 1.3- and 1.6-kb transcripts, was cloned in bacteriophages M13mp8 and M13mp9, and single-stranded DNA probes were prepared. The orientation of the inserts was such that the M13mp8 construction would detect transcripts going from left to right (Fig. 1B) while the M13mp9 construct would hybridize to transcripts in the opposite direction. The M13mp8 probe hybridized to the 1.6-kb transcript (*PRP4*; Fig. 2, lanes a to d), and the M13mp9-derived probe hybridized to the 1.3-kb transcript (*ORF2*; lanes e and f). The two transcripts are therefore divergent, and their polarity is in agreement with the orientation of the ORFs.

The 5′ end of the *PRP4* mRNA (the 1.6-kb transcript) was localized by a series of nuclease S1 mapping experiments (see Materials and Methods). The region examined for transcript start sites extended from positions 346 to 1439. In all cases, the 5′ end of the mRNA was found to be near position 770 (data not shown).

The 5′ end of the *PRP4* transcript was determined precisely by primer extension analysis. A 20-nucleotide-long primer complementary to the RNA sequence spanning nucleotides 846 to 865 was used to prime cDNA synthesis from total RNA. The cDNA extension products were electrophoresed together with the DNA sequencing ladder primed with the same oligonucleotide. The major transcription start was the A at position 778, with minor starts at nucleotides 763, 771, and 772 (Fig. 2B).

The 5′ end of the 1.3-kb RNA (*ORF2*) was mapped to a site near position 580 by nuclease S1 analysis (data not shown).

Both *PRP4* and *ORF2* are essential genes. To determine whether *PRP4* and *ORF2* code for essential functions, diploid strains were constructed in which one of the homologous chromosomes had either *LEU2* integrated within *ORF2* (at the *Bgl*II site; position 282) or *HIS3* integrated within *PRP4* (at the *Bcl*I site; position 2070) (Fig. 1B). The chromosomal configurations were confirmed by DNA blot analysis (not shown). The strains were sporulated, and

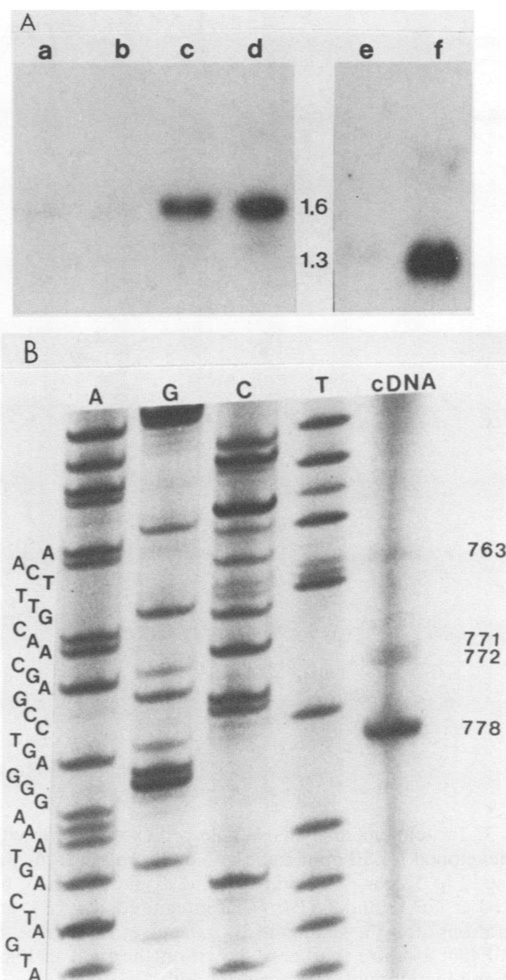


FIG. 2. (A) RNA blot analysis of total RNA from strain LL20 (lanes a, b, and e) and from LL20 transformed with pYF423 (*PRP4*, 2 μm; lanes c, d, and f). Sizes (in kilobases) are shown in the margin. Lanes a to d were probed with single-stranded DNA prepared from M13mp8 carrying the 1.5-kb *Pst*I-*Hin*dIII fragment; lanes e to f were probed with DNA from a similar construct in M13mp9. (B) Primer extension analysis of *PRP4* mRNA. Lanes: cDNA, primer extension products of total RNA from strain LL20 primed with an oligonucleotide complementary to *PRP4* mRNA; A, G, C, and T, DNA sequence obtained by using the same primer on M13 carrying the 1-kb *Hgi*A1 fragment. Positions of primer extension products relative to the DNA sequence are indicated. Numbers on the right refer to the nucleotide sequence shown in Fig. 1A.

tetrads were dissected. In both the *ORF2*/*ORF2*::*LEU2* and *PRP4*/*PRP4*::*HIS3* strains, only half of the spores were viable, and these were invariably Leu$^-$ and His$^-$, respectively. These observations indicate that *PRP4* and *ORF2* are essential genes. To ensure that the lethality of the null alleles was indeed due to *PRP4* or *ORF2*, we attempted to rescue the null phenotype by providing *PRP4* in *trans* from a plasmid (pYS65; *PRP4*, *URA3*). The *PRP4*::*HIS3* allele could be rescued by pYS65, as determined by the recovery of His$^+$ Ura$^+$ haploids, but the *ORF2*::*LEU2* allele was not rescued by this plasmid. We therefore conclude that *ORF2* is a separate essential gene. We also found *PRP4* to be essential during active proliferative growth by removing the nutritional selection for a rescuing plasmid (pYF423; *PRP4* *LEU2*) in wild-type and *PRP4*::*HIS3* haploids. Whereas the

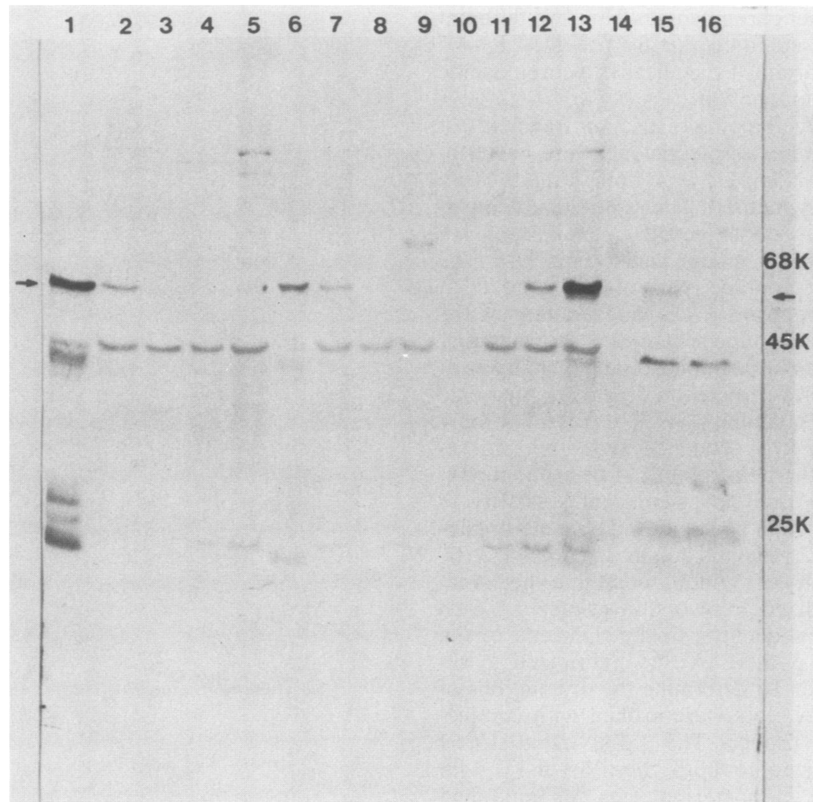


FIG. 3. Protein blot analysis. Whole-cell extracts or in vitro translation reactions were probed with a 100-fold dilution of serum from rabbit F and developed for 10 min. Lanes: 1, total protein from strain W303 with pYS76 (*pGAl10-PRP4 URA3*) integrated at *URA3*, grown in 2% galactose; 2 to 5, same strain grown in 2% glucose for 3, 6, 9, and 12 generations after the shift from 2% galactose; 6, W303 with pYS76 integrated at *URA3* and *PRP4*::*HIS3* integrated at *PRP4*, grown in 2% galactose; 7 to 10, same strain grown in 2% glucose for 3, 6, 9, and 12 generations after the shift from 2% galactose; 11, total protein from strain LL20; 12, LL20 transformed with pYF423; 13, LL20 transformed with pYF400; 14, size markers (shown on the right in kilodaltons); 15, in vitro translation reaction with SP6-*PRP4* mRNA; 16, in vitro translation reaction with no transcript added.

wild type (His^-) rapidly lost pYF423 during growth in yeast extract-peptone-dextrose medium (2% loss per generation), the *PRP4*::*HIS3* strain never lost it; i.e., His^+ Leu^- clones were never recovered.

Antibodies to PRP4: identification of the gene product. A *PRP4-lacZ* gene fusion, pYS43, was constructed in the *E. coli* expression vector pHK413 as described in Materials and Methods. The fusion gene contained all of the *PRP4* coding sequence except for 41 amino acids at the N terminus and 10 amino acids at the C terminus, as determined by DNA sequencing of the fusion junctions. Upon induction with IPTG in *E. coli*, a 165-kDa fusion protein was produced. The fusion protein was isolated from a preparative gel and was used to immunize two rabbits, F and K.

Crude antiserum was used to probe protein blots containing various whole-cell extracts and in vitro-translated *PRP4* gene product. The antibodies faintly detected a 52-kDa protein in untransformed cells (Fig. 3, lane 11); this band was more intense in a strain carrying *PRP4* on a 2μm-based plasmid (lane 12) and very intense in a strain carrying *PRP4* on a high-copy plasmid (lane 13) or in strains expressing the *PRP4* gene from the strongly inducible *GAL10* promoter, *pGAL* (lanes 1 and 6). Upon shift of the *pGAL-PRP4* strains to glucose medium, the band corresponding to the *PRP4* gene product gradually became fainter as transcription from *pGAL* was repressed (lanes 2 to 5 and 7 to 10). A band of the same mobility (52 kDa) was present as an in vitro translation product of *PRP4* mRNA (lane 15) but absent when no *PRP4* mRNA was added (lane 16). The 52-kDa band was detected by the immune sera from both rabbits but not by either preimmune serum (not shown). We conclude that the 52-kDa protein corresponds to the *PRP4* gene product, in agreement with the size predicted from the DNA sequence.

Antibodies to the *PRP4* gene product inhibit mRNA splicing in vitro. PRP4 is essential for mRNA processing in vitro, since a splicing extract from a temperature-sensitive *prp4* mutant displays temperature sensitivity and fails to make spliceosomes (39), suggesting that PRP4 is stably or transiently associated with the spliceosome or one of its components. Antibodies to PRP4 might therefore be expected to inhibit splicing in vitro.

To test this possibility, the IgG fractions from whole preimmune and immune sera (see Materials and Methods) were isolated and added to in vitro splicing reactions. (Addition of crude serum caused complete degradation of the substrate RNA, necessitating the use of IgG fractions.) Preincubation of the splicing extract with immune antibodies abolished splicing (Fig. 4, lane 2). Upon dilution of the antibodies, splicing activity gradually ceased to be inhibited (lanes 3 and 4). Antibodies from preimmune serum had no effect (lane 5). To show that the inhibition was due specifically to PRP4 antibodies and not to some other component in the immune sera, PRP4 antibodies were affinity purified, i.e., eluted from the 52-kDa PRP4 protein on preparative blots

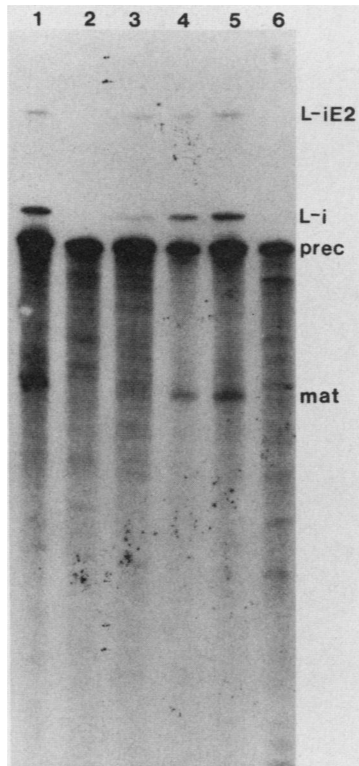


FIG. 4. In vitro splicing inhibition. Substrate precursor RNA (rp51) was incubated with splicing extract for 20 min at 23°C. The splicing extract was preincubated for 5 min at 23°C with no antibodies (lane 1), immune antibodies from rabbit K (final concentration, 0.8 mg/ml of IgG) (lane 2), antibodies diluted fourfold (lane 3), antibodies diluted eightfold (lane 4), preimmune antibodies to the same final concentration as immune antibodies (lane 5), and antibodies affinity purified to PRP4 protein (lane 6). Positions of splicing products are indicated (L-iE2, lariat intron-exon 2; L-i, lariat intron; prec, precursor mRNA; mat, mature mRNA).

(see Materials and Methods), and tested for inhibition of splicing in vitro. The affinity-purified PRP4 antibodies inhibited mRNA splicing (Fig. 4, lane 6).

Formation of the spliceosome involves the ordered formation of at least four snRNPs and several other factors on the precursor mRNA; several of these steps are ATP dependent. To determine at which point the splicing reaction is inhibited by PRP4 antibodies and whether the inhibition is ATP dependent, ATP and antibodies were added at various times before and after addition of substrate RNA (Fig. 5). The results showed that (i) splicing was dependent on the addition of ATP (Fig. 5, lane 12); (ii) the antibodies inhibited splicing if added before the substrate, independent of the presence of added ATP (lanes 1 to 3); (iii) antibodies added at the same time or later than substrate RNA and ATP did not inhibit splicing (lane 4, 5, and 7 to 9); and (iv) antibodies added at the same time as substrate RNA inhibited splicing if ATP was added later (lane 6). Presumably, formation of the spliceosome occurred faster than that of the antibody complex(es), although once the antibody complexes had formed, they inhibited splicing efficiently even when ATP was added later. An experiment in which antibodies were added at shorter time intervals showed that complete inhibition of splicing occurred if the antibodies were present 2 min or more before precursor substrate, but if preincubation was for 1 min, a small amount of splicing could take place (not shown).

Identification of the PRP4 snRNP(s). To determine whether PRP4 is associated with any of the snRNAs known to be involved in splicing, U1, U2, U4, U5, and U6, immunoprecipitation experiments were performed. IgG from whole antiserum was bound to protein A-Sepharose beads and subsequently incubated with splicing extracts (see Materials and Methods). To show that the antibodies react with PRP4 under these conditions, in vivo-labeled proteins were first precipitated from total-cell extracts and visualized by autoradiography after gel electrophoresis. A 52-kDa protein was precipitated by immune but not by preimmune antibodies (not shown).

Coprecipitated snRNAs were then visualized by end labeling total bound RNA with RNA ligase and pCp or by probing for specific snRNAs (Fig. 6). As a positive control, anti-trimethyl cap antibodies (generously provided by R. Lührmann) were used; these antibodies precipitate several snRNAs, including U1, U2, U4, and U5. U6, which does not have a trimethyl cap, is precipitated through its association with U4. As a negative control, especially for the sensitive pCp labeling, no antibodies were used. The result of probing for U1, U2, U4, U5, and U6 is shown in Fig. 6A. Preimmune serum did not precipitate any of these snRNAs above background level (compare lanes 3 and 5). However, immune serum precipitated U4, U6, and both forms of U5 (U5-long and U5-short [45]) but no U1 or U2 above background (lane 2). A similar result was obtained by pCp end labeling (Fig. 6B and C). Preimmune serum did not precipitate any RNA species above background. However, in addition to background bands, the precipitate from immune serum contained RNA species migrating with the mobility of U4 (160 nucleotides) and the two forms of U5 (179 and 214 nucleotides) (Fig. 6B, lane 7). Yeast U6 labels very inefficiently with pCp (37), but upon long exposure a band migrating like U6 (112 nucleotides) could be seen in the immunoprecipitate (Fig. 6C, lane 6). No other RNA species were precipitated specifically by immune serum.

We wanted to determine whether the precipitation was due specifically to antibodies to PRP4 rather than to other antibodies present in the rabbit serum, e.g., anti-β-galactosidase. To this end, the antibodies were incubated with increasing amounts of antigen before incubation with splicing extract. The rationale was that excess antigen would bind antibodies and thereby prevent them from subsequent interaction with the yeast splicing extract. Added antigens would therefore compete for precipitation of the snRNPs, presumably in a concentration-dependent manner. As antigens, fractionated extracts of *E. coli* that had been induced for PRP4–β-galactosidase or for β-galactosidase itself were used (see Materials and Methods). In extracts in which *PRP4-lacZ* was induced, the fusion protein was preferentially recovered in fraction 3 of the extract, although a small amount was present in fraction 2. In extracts in which only *lacZ* was induced, β-galactosidase was preferentially recovered in fraction 2. In addition to these two proteins, the extracts contained a number of *E. coli* proteins that were common to both. To ensure that any observed competition was due to PRP4–β-galactosidase, different concentrations of both fractions 2 and 3 from both extracts (PRP4–β-galactosidase and β-galactosidase alone) were used in the competition experiments.

Extract fractions containing induced β-galactosidase showed no inhibition of precipitation of U4 and U6, even at the highest protein concentrations (Fig. 7, lanes 9 to 14). In

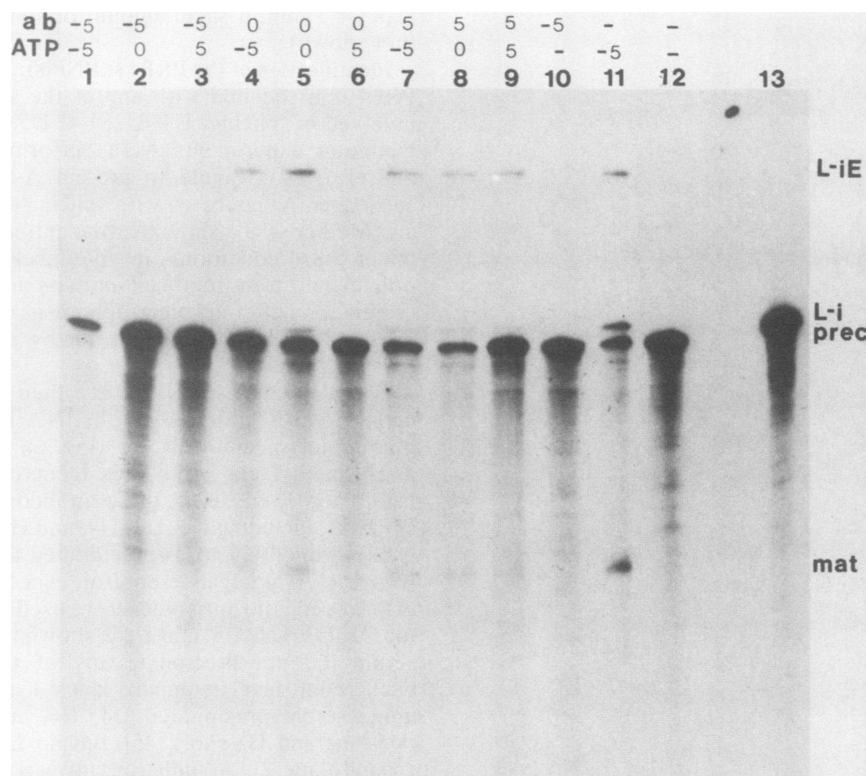


FIG. 5. Effect of ATP on inhibition of splicing in vitro. Substrate RNA (rp51) was added to splicing extracts at time zero, and the reaction was stopped after 20 min at 23°C. ATP and antibodies (ab) were added at −5 , 0, or +5 min, as indicated at the top. Lanes: 1 to 3, and 10, antibodies from rabbit K added at −5 min; 4 to 6, antibodies added at 0 mm; 7 to 9, antibodies added at +5 min; 1, 4, 7, and 11, ATP added at −5 min; 2, 5, and 8, ATP added at 0 min; 3, 6, and 9, ATP added at +5 min; 12, no ATP added; 13, substrate RNA. Splicing products are as indicated in the legend to Fig. 5.

contrast, fractions with PRP4–β-galactosidase competed efficiently and with the expected pattern: fraction 3 inhibited completely when undiluted and at a 10-fold dilution (lanes 3 and 4) and, to a lesser extent, at a 100-fold dilution (lane 5). Fraction 2 inhibited when undiluted (lane 6) but not at a 10- or 100-fold dilution (lanes 7 and 8). In similar experiments, precipitation of U5 was found also to be inhibited, with the same pattern as found for U4 and U6 (not shown). We therefore conclude that the precipitation of U4, U5, and U6 was due specifically to the PRP4 antibodies.

The observation that antibodies to PRP4 precipitated three snRNAs, U4, U5, and U6, could mean that PRP4 is present in all three snRNPs or that PRP4 is present in only one or two and the other(s) precipitates through association. U4 and U6 are known to form a very stable complex, both in HeLa cells (51) and in yeast cells (5). Also, U4/U6 and U5 form a dynamic complex; in extracts competent in RNA splicing, U4/U6 can associate with U5 upon incubation with ATP (26, 37). Precipitation of U4, U5, and U6 by anti-PRP4 was not greatly affected by addition of ATP (Fig. 8A). This finding could indicate that PRP4 is associated with both U4/U6 and U5 or that this extract contained preformed U4/U5/U6 complex. Indeed, precipitation of U5 was variable and very extract dependent, whereas U4 and U6 consistently precipitated quite efficiently. Figure 8B shows precipitation of U4, U5, and U6 from four different extracts. The total amounts of U4, U5, and U6 were comparable in all extracts (lanes 1 to 4), but the amount of U5 precipitated by anti-PRP4 antibodies relative to the amounts of U4 and U6 was variable (lanes 5 to 8); in one extract (lane 7), very little U5 was precipitated. This result indicated that the PRP4 protein was present in the U4/U6 snRNP.

To determine whether we could further establish that PRP4 was present independently in the U4/U6 snRNP and that precipitation of U4/U6 was not due primarily to association with U5, serial immunoprecipitations with anti-PRP4 and a U5-specific antibody (anti-PRP8) were performed. PRP8 has been shown to be part of the U5 snRNP (37), and anti-PRP8 antibodies efficiently precipitate U5 in the presence of low levels of ATP. U4/U6 is precipitated by anti-PRP8 through association with U5 at higher ATP levels. In the serial precipitation experiments, extracts were first precipitated with anti-PRP4 or anti-PRP8 antibody, the precipitate was recovered, and the supernatant was incubated with the other antiserum. By first using anti-PRP8, we hoped preferentially to remove U5 from the extract. A subsequent precipitation with anti-PRP4 should then show whether precipitation of U4 and U6 is dependent on the presence of U5. Conversely, a first precipitation with anti-PRP4, followed by anti-PRP8, would indicate how efficiently U5 is precipitated.

The result of such an experiment (Fig. 8C) showed the following. (i) Anti-PRP4 precipitated U4, U6, and U5 (lane 2). (ii) Anti-PRP8 precipitated U5 and a trace of U4 and U6 (lane 4). (The amount of U4/U6 precipitated by anti-PRP8 without addition of ATP is extract dependent [37].) (iii) When the residual extract from the anti-PRP4 precipitation was reprecipitated with anti-PRP8, a very strong U5 signal was seen in addition to some U4 and U6 signal (lane 3). (Note that the amount of U4 and U6 precipitated without

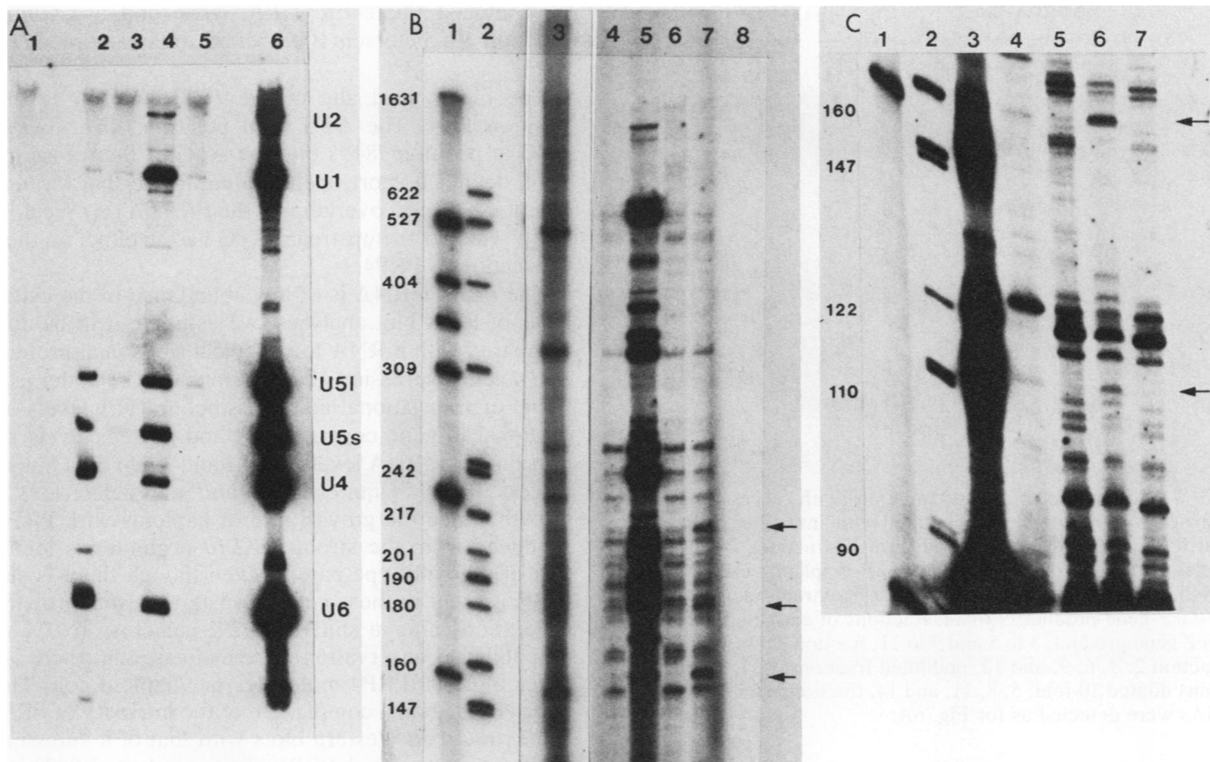


FIG. 6. Immunoprecipitations with antibodies from rabbit K. Splicing extract (10 μl) was incubated with preimmune serum, immune serum, anti-trimethyl cap antibodies, or no antibodies, and precipitated RNAs were recovered. (A) Immunoprecipitated RNAs were fractionated on a 6% denaturing acrylamide gel, and snRNAs U1, U2, U4, U5, and U6 were visualized by probing an RNA blot with specific oligonucleotides. Lanes: 1, calf thymus carrier DNA; 2, immune antibodies; 3, preimmune antibodies; 4, anti-trimethyl cap antibodies; 5, no antibodies; 6, total RNA in 10 μl of extract. Positions of U1, U2, U4, U5-long and U5-short, and U6 are indicated. The signal from the carrier DNA is due mainly to hybridization to the U2 probe. The band between U4 and U6 is an endogenous U4 degradation product. (B and C) Immunoprecipitated RNAs were end labeled with pCp and RNA ligase and fractionated on a 6% denaturing acrylamide gel with size markers (shown in nucleotides on the left). (B) Lanes: 1, pBR322 digested with *Hin*fI; 2, pBR322 digested with *Hpa*II; 3, total RNA; 4, no antibodies; 5, anti-trimethyl cap antibodies; 6, preimmune serum; 7, immune serum; 8, calf thymus carrier DNA. Arrows indicate U5-long, U5-short, and U4. (C) Lanes: 1, pBR322 digested with *Hin*fI; 2, pBR322 digested with *Hpa*II; 3, total RNA; 4, anti-trimethyl cap antibodies; 5, preimmune serum; 6, immune serum; 7, no antibodies. Arrows indicate U4 and U6.

ATP added was increased relative to that in the first precipitation [compare lanes 3 and 4]. Perhaps the prolonged incubation during the serial precipitation steps promoted association of U4/U6 with U5.) Anti-PRP4 clearly precipitated U5 much less efficiently than did anti-PRP8. (iv) When the extract partially depleted of U5 by anti-PRP8 was reprecipitated with anti-PRP4, little or essentially no U5 was detected, although U4 and U6 could still be seen (lane 5). These results suggest that PRP4 must be part of the U4/U6 complex.

DISCUSSION

We have analyzed the structure and function of the *S. cerevisiae PRP4* gene and its gene product. The *PRP4* gene encodes a 465-amino-acid, 52-kDa protein, as determined from the following observations. (i) *PRP4* was cloned by complementation of a temperature-sensitive *prp4* mutant on a 2.4-kb *Hin*dIII fragment; the region that complements the mutation contains an ORF of 465 amino acids, predicting a 52-kDa protein product. (ii) This ORF is transcribed into a 1.6-kb RNA that is enriched in poly(A)$^+$ RNA, and the 5′ end of this mRNA is 38 bases upstream of the ATG of the 465-amino-acid ORF. (iii) The ORF codes for an essential function, since insertion of *HIS3* in the ORF is lethal in haploids. (iv) The ORF codes for a 52-kDa protein, since antibodies raised against the protein encoded by the ORF fused to β-galactosidase recognize a 52-kDa protein in yeast cells.

The only similarity that we have detected between the *PRP4* sequence and others in sequence data bases is to the beta subunit of transducin ($G_{t\beta}$), a G protein involved in signal transduction in the retina (60). The biological significance, if any, of this sequence similarity is not clear at present and will be discussed elsewhere. The sequence CTAGTAAAG, 93 bases upstream of the ATG of *PRP4*, is also found at a similar position upstream of *PRP3*, and closely related sequences are found at similar positions upstream of *PRP11* and *ORF2* (an essential gene upstream of *PRP4*). We have found that these boxes are important for transcription of both *PRP4* and *ORF2* (unpublished observations) and are currently investigating this observation in more detail.

ORF2 was identified through sequence analysis, transcript mapping, and genetic analysis. It is an essential gene and shares regulatory sequences with *PRP4*, suggesting that it might be involved in RNA processing, since genes that are found head-to-head often have related functions (1). We

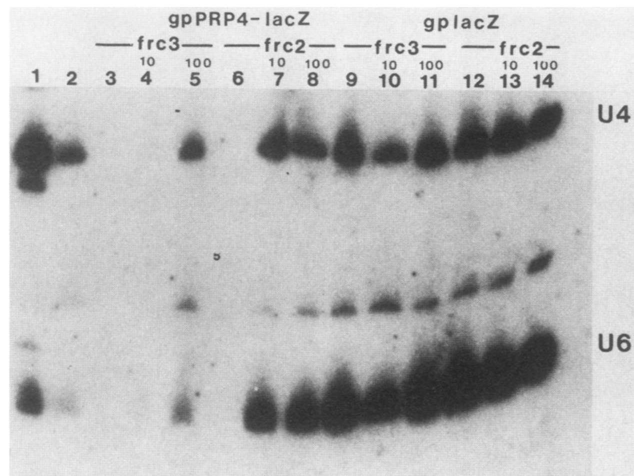


FIG. 7. Competition for precipitation with excess antigens. Splicing extract (10 μl) was precipitated with antibodies from rabbit K that had been preincubated with protein fractions from *E. coli* extracts. Lanes: 1, total RNA from 4 μl of splicing extract; 2, no competing antigen added; 3 to 8, fractions of extracts induced for the *PRP4-lacZ* gene product; 9 to 14, fractions of extracts induced for the *lacZ* gene product; 3 to 5 and 9 to 11, fraction 3; 6 to 8 and 12 to 14, fraction 2; 3, 6, 9, and 12, undiluted fractions; 4, 7, 10, and 13, fractions diluted 10-fold; 5, 8, 11, and 14, fractions diluted 100-fold. snRNAs were detected as for Fig. 6A.

have cloned the entire *ORF2* gene and are studying its function (S. Petersen Bjørn et al., manuscript in preparation).

The major start site of the *PRP4* mRNA is the A at position 1744. The ATG is at position 816; however, the ATG at position 784 is the first ATG of the transcript. This ATG defines a short open reading frame that terminates at position 817, i.e., overlapping the *PRP4* ATG. We do not yet know whether the upstream ATG has an effect on the rate of translation of PRP4.

The *PRP4* mRNA is of low abundance in the cell; on the basis of RNA blot analyses, we estimate it to be about 5% compared with mRNA for a typical ribosomal protein gene. *PRP4* gene expression does not appear to be under transcriptional or translational control, since mRNA levels are proportional to gene copy number and protein levels are proportional to mRNA levels (Fig. 2 and 3 and data not shown). Excess PRP4 is quite stable and not deleterious to cell growth, since the growth rate of haploids with PRP4 overproduced from the strong *GAL10* promoter is identical to that of the wild-type parent, even though there is sufficient PRP4 present in these cells to sustain normal growth for 12 generations after a shift from 2% galactose to 2% glucose (unpublished observation). We estimate that there is of the order of 1,000 PRP4 molecules per haploid cell. This estimate is based on a comparison of the intensity of PRP4 from cell extracts on Western blots with that of a known amount of in vitro-translated PRP4. This number, 1,000, is of the same order of magnitude as that inferred from relative

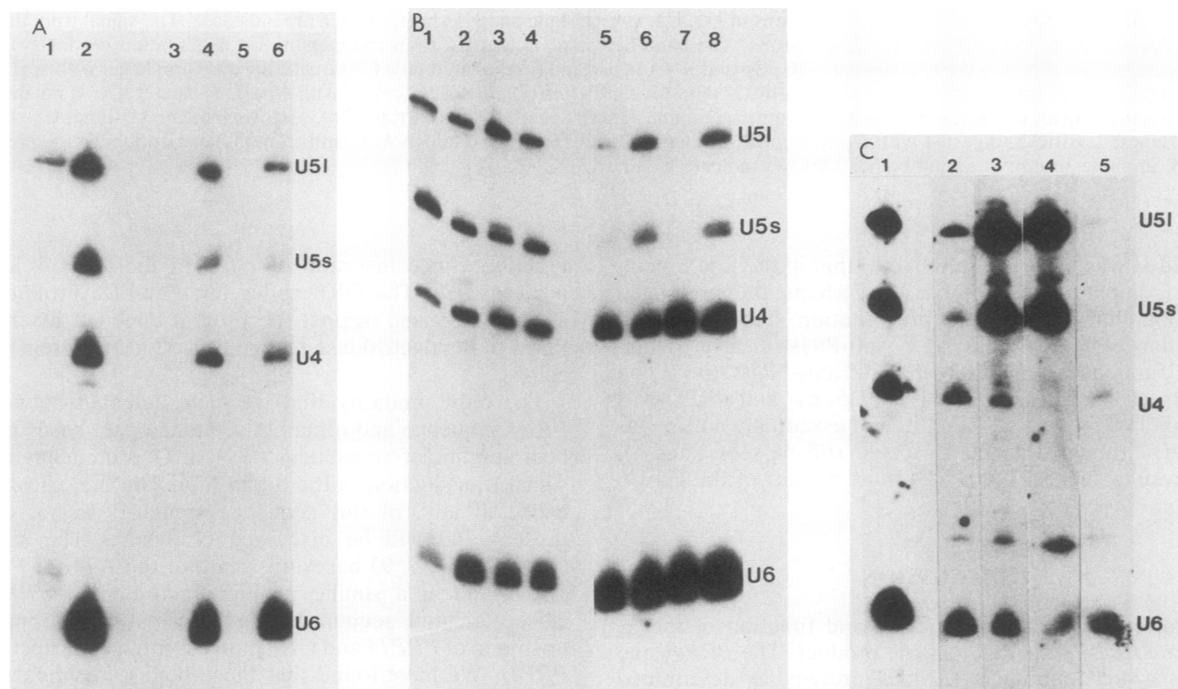


FIG. 8. Differential precipitation of U4, U5, and U6. Splicing extract (10 μl) was precipitated with antibodies, and U4, U5, and U6 were detected as for Fig. 6A. (A) Effect of ATP. Lanes: 1, size marker; 2, total RNA in 4 μl of extract; 3 and 5, precipitation with preimmune antibodies from rabbit K; 4 and 6, precipitation with immune antibodies from rabbit K; 3 and 4, no ATP added; 5 and 6, ATP added to a final concentration of 2 mM. (B) Extract-dependent precipitation. Lanes: 1 to 4, total RNA in 4 μl of four different splicing extracts; 5 to 8, precipitations with anti-PRP4 of the four extracts in the same order. Extracts in lanes 1 and 5 are from strain YR04-304; the others are from strain EJ101. (C) Serial precipitation with anti-PRP4 and anti-PRP8 antibodies. Lanes: 1, total RNA in 4 μl of splicing extract; 2, precipitation with anti-PRP4; 3, supernatant from lane 2 reprecipitated with anti-PRP8.4 (37); 4, precipitation with anti-PRP8.4; 5, reprecipitation of the supernatant from lane 4 with anti-PRP4.

mRNA abundances and correlates well with the estimated amount of snRNAs (50, 63).

Antibodies to PRP4 inhibit mRNA splicing in vitro. The inhibition occurs before any detectable splicing intermediates or products have formed. This finding is in agreement with the in vivo phenotype of the *prp4* temperature-sensitive mutant, which accumulates precursor mRNA at the restrictive temperature (29, 39). Also, spliceosome formation in extracts of the *prp4* temperature-sensitive mutant is blocked before formation of the 40S spliceosome (35). Inhibition is not dependent on exogenously added ATP. However, our data could indicate that the step affected by the antibodies is just before an ATP-dependent step in spliceosome formation, since no inhibition occurs if ATP, substrate, and antibodies are added at the same time, but if ATP is added later than antibodies, no splicing takes place.

In immunoprecipitation experiments, PRP4 antibodies precipitated U4, U5, and U6 snRNAs. The relative amounts of U4 and U6 recovered were fairly constant, whereas the amount of U5 relative to the amounts of the other two varied somewhat more from experiment to experiment. That the precipitation was due specifically to the PRP4 antibodies is indicated by the fact that preimmune sera did not precipitate these snRNAs, whereas both immune sera did; furthermore, precipitation could be inhibited by excess PRP4–β-galactosidase but not by β-galactosidase itself.

The fact that PRP4 antibodies precipitate three snRNAs, U4, U5, and U6, could mean that (i) PRP4 is stably associated with all three; (ii) PRP4 is associated with just one or two, and the other(s) precipitates through association; or (iii) an epitope in PRP4 is conserved in other snRNPs. Conversely, if an snRNA is not precipitated, it could be because the antigen is not present or merely because the epitopes are not accessible. Our data suggest that PRP4 is associated primarily with U4/U6. First, U5 recovery is more variable than that of U4 and U6 and is extract dependent. If PRP4 were primarily in U5, and U4/U6 were precipitated by association with U5, one would expect the opposite pattern. Second, when the extract has been partially depleted of U5 (by anti-PRP8 serum), a detectable amount of U4 and U6 is precipitated by anti-PRP4, but little if any U5 can be seen. This finding indicates that PRP4 must be present in U4/U6, although we cannot exclude the possibility that it is also in U5. Direct proof must await biochemical fractionation of individual snRNPs or demonstration of interactions genetically.

At the moment, we cannot say whether PRP4 is stably associated with both U4 and U6 or with only one of them. If PRP4 is stably associated with only one of these snRNAs, it is not surprising that the other would be precipitated very efficiently, since U4 and U6 are stably base paired with a dissociation temperature of 53°C (5). In the spliceosome, however, the two snRNAs dissociate, since only U6, not U4, is present in the functional spliceosome (48). In addition, in yeast cells there appears to be an excess of U6 relative to U4 (11, 56). The following observations could indicate that PRP4 is primarily associated with U4: (i) anti-PRP4 does not precipitate spliceosomes (data not shown), which could indicate that PRP4 is associated with U4, since U4 is not found in the functional spliceosome; and (ii) anti-PRP4 serum and anti-trimethyl cap serum precipitate similar relative amounts of U4 and U6 (Fig. 6A). Since anti-trimethyl cap serum precipitates U6 only through its association with U4 (U6 itself does not have a trimethyl cap; reference 38 and data not shown), anti-PRP4 serum may not precipitate free U6. However, on both points we cannot exclude the possibility that the inability of anti-PRP4 serum to precipitate spliceosomes or free U6 could be due to unavailability of PRP4 epitopes in spliceosome-bound and free U6.

Finally, PRP4 might be only transiently part of the U4/U6 complex. U4 and U6 presumably enter the spliceosome as a complex, but before splicing occurs they separate and U4 leaves the spliceosome. It is possible that reassociation of the two snRNAs is aided (for example, by PRP4). We think that this possibility is less likely because Lustig et al. (39) found that heat-inactivated splicing extracts from a *prp4* temperature-sensitive strain could not be complemented in vitro; one reason for this could be that PRP4 is tightly bound or buried and nonexchangeable. If PRP4 were transiently part of U4/U6, one would expect efficient complementation in vitro.

We do not see an effect of ATP on anti-PRP4 precipitation of U4/U6 and U5. Lossky et al. (37) found that coprecipitation of U4/U6 with U5 by using anti-PRP8 was dependent on incubation of extract with at least 0.1 mM ATP. In some of our extracts, anti-PRP8 precipitates some U4/U6 independently of exogenously added ATP. It is therefore possible that endogenous levels of ATP in the extracts are above the threshold needed to support the interaction between U4/U6 and U5, although splicing itself is dependent on the addition of ATP. The inhibition of splicing in vitro by the anti-PRP4 antibodies may therefore not be due primarily to interference with formation of U4/U6/U5 complex but could be due to the antibodies interfering with a subsequent step.

What is the function of the proteins in splicing? It is possible that their primary role is structural and that the actual RNA scission and ligation reactions are essentially autocatalytic or aided by snRNAs (43). However, all of the identified splicing factors appear to be essential and may be needed to recognize introns and to juxtapose the exons in the appropriate way for splicing to occur, as well as for keeping the splicing intermediates in the proper conformation for final exon ligation. Identification of one protein of an snRNP is a first step toward an analysis of its composition, structure, and function. In yeast cells, two snRNP proteins have now been identified, PRP8 in the U5 snRNP and PRP4 in the U4/U6 snRNP, and a link between PRP4 and another protein, PRP3, has already been established genetically (30). PRP4 may be U4/U6 specific. If further studies prove it to be, PRP4 will be the first protein identified that is unique to the U4/U6 snRNP in any organism studied thus far and so may help in determining the role in splicing of this snRNP.

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