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PRP19: A Novel Spliceosomal Component

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We have isolated the gene of a splicing factor, PRP19, by complementation of the temperature-sensitive growth defect of the *prp19* mutant of *Saccharomyces cerevisiae*. The gene encodes a protein of 502 amino acid residues of molecular weight 56,500, with no homology to sequences in the data base. Unlike other PRP proteins or mammalian splicing factors, the sequence of PRP19 has no discernible motif. Immunoprecipitation studies showed that PRP19 is associated with the spliceosome during the splicing reaction. Although the exact function of PRP19 remains unknown, PRP19 appears to be distinct from the other PRP proteins or other spliceosomal components.

Removal of the introns from nuclear precursor mRNA proceeds via two transesterification reactions. The first step involves cleavage at the 5′ splice site and the formation of a lariat intron-3′ exon intermediate. In the second step, the 3′ splice site is cleaved, and the two exons are joined (for reviews, see references 21 and 37). The splicing reaction takes place on a large complex called the spliceosome (4, 13, 20), which comprises the precursor mRNA, the small nuclear ribonucleoprotein particles (snRNPs) (for reviews, see references 36 and 46), and other protein components (for reviews, see references 23 and 44). The reaction requires five U-class snRNAs and an as yet undetermined number of protein factors, some of which are integral components of snRNPs.

Identification of proteins essential for pre-mRNA splicing has been greatly facilitated in the yeast *Saccharomyces cerevisiae* by the isolation of temperature-sensitive mutants that affect pre-mRNA splicing (for reviews, see references 50 and 52). The first group of protein factors were identified by characterization of a set of temperature-sensitive mutations (*rna2* to *rna11*) isolated in 1967 by Hartwell (24) as mutations affecting RNA metabolism. Several of these mutations have been shown to affect pre-mRNA splicing (for reviews, see references 39 and 54). Henceforth, all of the *rna* mutants have been renamed *prp* (precursor RNA processing) mutants. At least 30 *PRP* genes have now been identified (for a review, see reference 39). Some of them were isolated in screens of new collections of temperature-sensitive mutants for those defective in pre-mRNA splicing. Others have been isolated as suppressors of mutations either in *PRP* or snRNA genes or in an intron (5, 28, 43). These *prp* mutants can be classified into three categories. One class of the mutants accumulate pre-mRNA at the nonpermissive temperature, suggesting that the corresponding gene product is involved in assembly of the spliceosome or the first step of the cleavage-ligation reaction. The other two accumulate splicing intermediates and the lariat intron product, respectively.

Most of the *PRP* genes have been cloned and sequenced. Many of them show striking homology between each other (for a review, see reference 39). Four interesting and identifiable motifs have appeared in genes that have been sequenced. These include the zinc finger motif, ATP-dependent RNA helicase, the RNA binding motif, and the motif for the β subunit of G protein (for a review, see reference 39). Although possible functions of PRP proteins are suggested by their associated sequence, only PRP2 and PRP16 have been demonstrated to possess RNA-dependent nucleoside triphosphatase and RNA-dependent ATPase activities, respectively (29, 42).

Several of the *PRP* gene products have been characterized in vitro. PRP4 and PRP6 are part of the U4/U6 snRNP (1–3), and PRP8 is part of U5 snRNP (35). PRP8 and PRP11 are integral components of the spliceosome (6, 53). PRP2 is required for the first cleavage-ligation reaction and is dispensable for assembly of a functional 40S splicing complex (33). PRP16 is required only for the second cleavage-ligation reaction (42). Both PRP2 and PRP16 appear to be associated with the spliceosome only transiently during the splicing reaction (30, 42). PRP22 is required for release of the mature message from the spliceosome (11).

PRP19 is required for the first cleavage-ligation of the splicing reaction, since the *prp19* mutant accumulates precursor mRNA at the nonpermissive temperature. To study the function of PRP19, we have isolated the *PRP19* gene and determined its sequence. No discernible motif is associated with the sequence. No homology was found to sequences in the data base. By immunoprecipitation studies, we also showed that PRP19 is tightly associated with the spliceosome during the splicing reaction.

MATERIALS AND METHODS

Strains and microbiological techniques. The wild-type *S. cerevisiae* strains used in this study were EJ101 (*MATα his1 prb1-1122 prc1-126*), SS330 (*MATa ade2-101 his3Δ200 ura3-52 tyr1 suc2 GAL*$^+$), and SEY6210 (*MATα his3Δ200 leu2-3 leu 2-112 ura3-52 trp1-Δ901 lys2-801 suc2-Δ9 GAL*$^+$ *mel*). The *prp19* strain (*MATa ade2-101 his3Δ200 ura3-52 lys2-801 ts*) was from the collection of the temperature-sensitive mutants described in Vijayraghavan et al. (51). The yeast strains were propagated by standard methods on media as described by Sherman et al. (45). Temperature-sensitive strains were maintained at 23°C, and 37°C was used as the nonpermissive temperature. Yeast transformation was performed by the lithium acetate procedure of Ito et al. (27). Transformants were selected on omission media at 23°C, and colonies were then replica plated to prewarmed YPD plates

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and incubated at 37°C to test for temperature resistance. The *Escherichia coli* strain used for cloning and DNA amplification was MC1061 [F⁻ *araD139* Δ(*araABIOC-leu*)*7679* Δ(*lac*) *X74 galU galK rpsL hsr hsm*⁺].

Isolation of the *PRP19* gene. The *PRP19* gene was isolated by complementation of the temperature-sensitive growth defect of the *prp19* mutant strain (38) with a YCp50-based *Sau*3A genomic library obtained from M. Rose and P. Novick. Subsequent subclonings were done with the yeast shuttle vector pPHYC18 (26), kindly provided by P. Herman and S. Emr. A derivative of the yeast integrative plasmid YIp5, in which the unique *Sal*I site was deleted, was used to subclone the complementing DNA fragment for directed integration. DNA sequencing was carried out using the dideoxy method (41) with the Sequenase kit (U.S. Biochemical). Fragments to be sequenced were cloned into Bluescript plasmids (Stratagene) and sequenced by the deletion method (25).

Preparation of antibodies. PRP19 was expressed in *E. coli* for raising antibodies. The *Pst*I-*Bam*HI fragment of the *PRP19* gene was subcloned into the M13mp19 vector, and oligonucleotide-directed mutagenesis was performed to introduce *Nco*I sites at the first or second ATG of the open reading frame and 49 bases downstream of the stop codon. The oligonucleotides used are ATAGCACAAACCATGG TTCCCTAGT and AAGTTTTCGACCATGGTAGCATCC CA for the first and second ATGs, respectively, and TTTT TACTGGCCATGGTACTACTATT for the 3′ end. The *Nco*I fragment was then inserted into the *Nco*I site of plasmid vector PAR3039-1 to place the *PRP19* gene under T7 promoter control (47). The recombinant plasmid was transformed into *E. coli* BL21(DE3) (47) for expression of PRP19. Cell extracts prepared from the PRP19-expressing strain were fractionated by chromatography on a phosphocellulose column. Fractions containing PRP19 were further fractionated on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel. The PRP19 protein was excised from the gel and used to inject rabbits for raising antibodies. To affinity purify the antibody, the PRP19 protein isolated from *E. coli* was coupled to Sepharose according to the method provided by Pharmacia. Thirty milligrams of PRP19 produced from *E. coli* was incubated with 3.5 ml of swollen CNBr-activated Sepharose. Approximately 85% of the protein was coupled to Sepharose. Twelve milliliters of anti-PRP19 antisera was loaded on the PRP19-coupled Sepharose column and recycled three times. After extensive washing of the unbound materials with phosphate-buffered saline (PBS), the antibody was eluted with 25 ml of 50 mM glycine-HCl (pH 2.3) containing 0.15 M NaCl. The eluted fraction was concentrated by precipitation with 60% saturated $(NH_4)_2SO_4$ and dissolved in 0.5 ml of PBS. The final concentration of the antibody was 4.5 mg/ml. The anti-(m_3G)cap antibody was a generous gift of Reinhard Lührmann.

Southern blot and Western immunoblot analyses. Small-scale preparation of yeast genomic DNA was carried out as described by Sherman et al. (45). Southern blots were performed as suggested by Dupont, NEN Research Products. Hybridizations were carried out according to Cheng and Abelson (8), and the probe was prepared by the random primer method (12). Western blot analysis was performed according to Towbin et al. (49), and the anti-PRP19 antiserum was used at a dilution of 1:1,000.

Construction of gene replacement. The *prp19*::*LEU2* allele was created by deleting the internal *Xba*I fragments from the clone and replacing it with a 2-kb DNA fragment containing the *LEU2* gene. The resulting plasmid was digested with *Sph*I and *Hin*dIII prior to transformation into a diploid strain. After selection for Leu⁺ transformants, DNA was isolated from individual transformants and restricted with *Hin*dIII. The digested DNA was fractionated on a 1% agarose gel and then transferred to a GeneScreen membrane for Southern blot analysis, using the 2-kb *Eco*RI fragment of the *PRP19* gene or the 2-kb *LEU2* fragment as a probe.

Preparation of splicing extracts and substrates. Yeast whole cell extracts were prepared according to Cheng et al. (9). Actin precursors were synthesized in vitro by using SP6 RNA polymerase according to Cheng and Abelson (8).

Splicing assay and immunoprecipitation. Splicing assays were done according to Lin et al. (34), using uncapped actin pre-mRNA as the substrate. Immunoprecipitation was carried out as described by Cheng and Abelson (7) except that 6 μg of the affinity-purified anti-PRP19 antibody or 0.8 μl of the anti-(m_3G)cap antiserum was used per mg of protein A-Sepharose. Twenty microliters of the splicing reaction mixture was incubated with 10 μl of the antibody-coupled protein A-Sepharose (approximately 2.5 mg) for precipitation of the spliceosome.

RESULTS AND DISCUSSION

Isolation of the *PRP19* gene. The *PRP19* gene was isolated on the basis of its ability to complement the temperature-sensitive mutation. A *prp19* strain was transformed with a YCp50-based yeast genomic library, and transformants were replica plated to YPD plates to select for temperature-resistant colonies. Yeast plasmids were recovered from four of these colonies and propagated in *E. coli*. Retransformation of the *prp19* mutant with these plasmids confirmed that the presence of the cloned plasmid DNA confers temperature resistance. Preliminary restriction mapping of these plasmids indicated that they are related. Two of them were analyzed in more detail and named pPRP19A and pPRP19B (Fig. 1). pPRP19A carries a yeast genomic DNA fragment of 11 kb, which contains the 9-kb fragment carried by pPRP19B. Subcloning of various restriction fragments from these two plasmids indicated that the *Cla*I-*Bam*HI fragment of subclone pSCC12 confers temperature resistance and that the *Sal*I site interrupts the complementing sequence (Fig. 1). The insert of pSCC12 was further restriction mapped and subcloned. The results indicated that the *Eco*RV-*Bam*HI fragment carried by plasmid pSCC12-7 complements the temperature-sensitive phenotype.

Genetic mapping by integrative transformation. To prove that the complementing clone obtained corresponds to the *PRP19* gene instead of an extragenic suppressor capable of relieving the *prp* phenotype, integration of the cloned complementing genomic DNA was performed. The complementing DNA fragment from pSCC12-7 was subcloned into a derivative of yeast integrative plasmid YIp5, in which the unique *Sal*I site was deleted. Directed integration was accomplished by transformation of the resulting plasmid linearized at the unique *Sal*I site in the genomic fragment into the wild-type strain SS330 (Fig. 2A). Southern blot analysis was used to confirm correct integration of the cloned DNA fragment. Genomic DNA isolated from the integrated *URA* strain and the untransformed strain was digested with restriction enzyme *Hpa*I or *Bgl*II and subjected to Southern blot analysis using the 2-kb *Eco*RI fragment, the subcloned fragment in pSCC12-1 (Fig. 1), as a probe. As expected, the blot detected single, larger restriction fragments from the integrated strain than from the untransformed strain, since integration brings in no *Hpa*I or *Bgl*II site (Fig. 2B). This

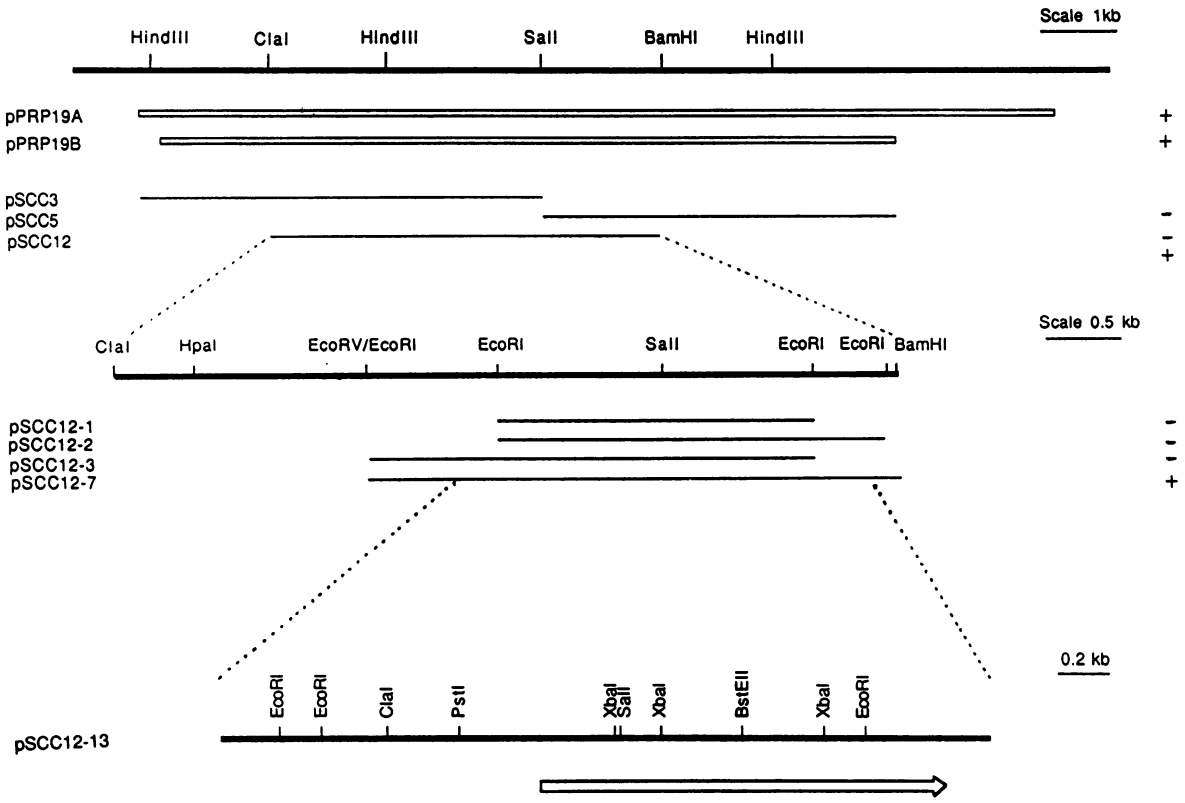

FIG. 1. In vivo complementation analysis with *PRP19*. pPRP19A and pPRP19B are two positive clones isolated from a YCp50-based *Sau*3A genomic library that complement the temperature-sensitive growth defect of *prp19*. The deduced restriction map is shown. pSCC3, pSCC5, and pSCC12 are subclones of restriction fragments from these two clones. pSCC12-1, pSCC12-2, pSCC12-3, and pSCC12-7 are subclones of fragments from pSCC12. pSCC12-13 is generated from deletion clones and contains the minimum sequence required for complementation. The complete sequence of this fragment was determined. The restriction map of pSCC12-13 is deduced from the sequence. Symbols: +, ability to complement the temperature-sensitive growth defect of *prp19*; −, inability to relieve temperature sensitivity.

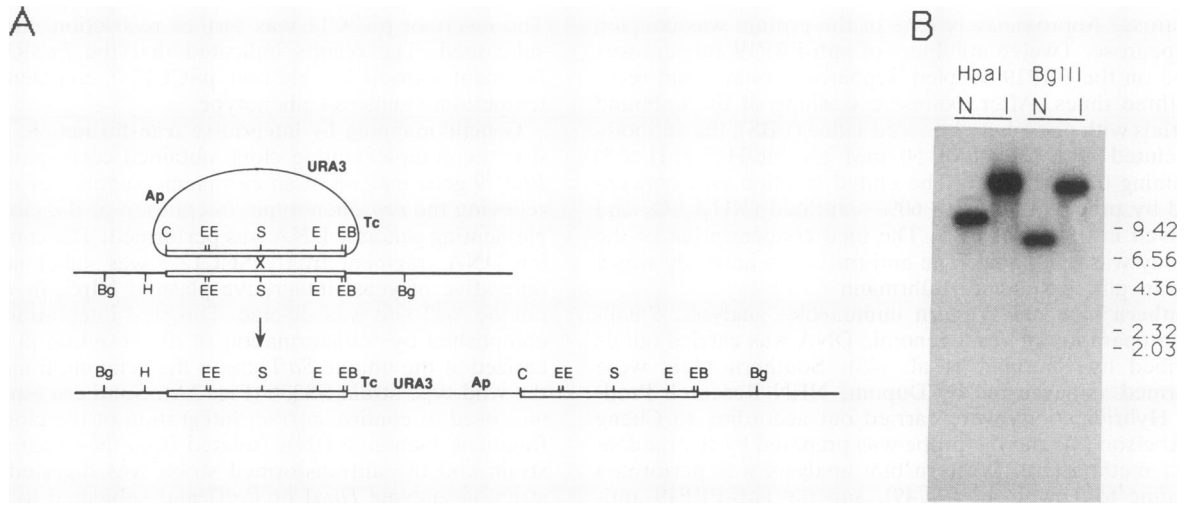

FIG. 2. Integration of the cloned DNA fragment. (A) An integrative plasmid containing the DNA fragment which complements the temperature-sensitive growth defect of *prp19* was linearized at the unique *Sal*I site and used to transform yeast strain SS330. Restriction sites: C, *Cla*I; E, *Eco*RI; S, *Sal*I; B, *Bam*HI, Bg, *Bgl*II; H, *Hpa*I. (B) DNAs from the integrated and untransformed strains were isolated and digested with *Hpa*I or *Bgl*II for Southern blot analysis, using the 2-kb *Eco*RI fragment as a probe. N, DNA with integration; I, DNA without integration. Sizes are indicated in kilobases.

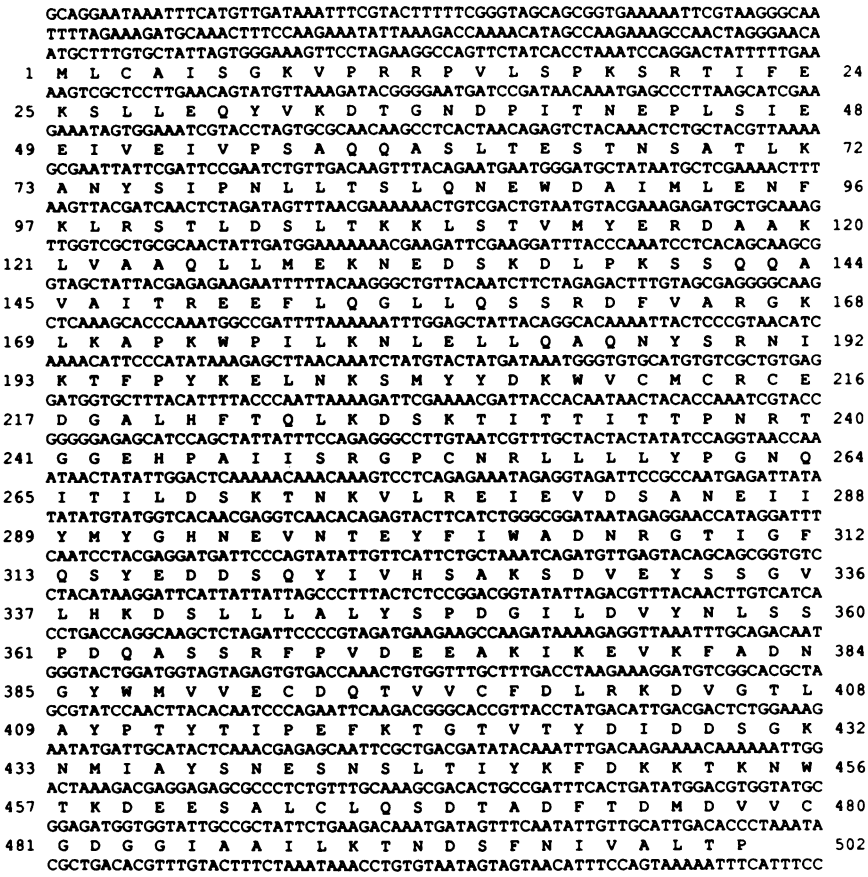

FIG. 3. Nucleotide and protein sequences of *PRP19*. The gene encodes a protein of 502 amino acid residues. No identifiable motif is found in the protein sequence. Neither the nucleotide nor the protein sequence has homology to existing sequences in the data base.

analysis also suggests that *PRP19* is a single-copy gene in yeast cells, since only one restriction fragment was detected in each digestion with *Hpa*I or *Bgl*II and with other enzymes which do cut within the integrated fragment (data not shown). The faint band present in the sample of *Hpa*I-digested nonintegrated DNA was due to incomplete digestion in the reaction, since it did not appear in repeated experiments.

The integrated *URA* strains were crossed to the temperature-sensitive *ura prp19* strain, and diploids were sporulated for tetrad analysis. Of 15 four-spored tetrads, all yielded two temperature-sensitive and two ts^+ spores, with cosegregations of the ts^+ and *URA* phenotype and of the temperature-sensitive and *ura* phenotype in all tetrads. This analysis shows that the cloned DNA was integrated at the homologous chromosomal *PRP19* locus.

Sequence determination and analysis. The cloned DNA in plasmid pSCC12-7 was subcloned into the Bluescript plasmid, and two sets of deletion clones, one from each end of the fragment, were generated by the exonuclease III-S1 deletion method as instructed by Stratagene. Several deletion clones were subcloned to the yeast shuttle plasmid pPHYC18 for the complementation assay. A total 2.8-kb complementing DNA sequence (pSCC12-13 in Fig. 1) was determined by the dideoxy-chain termination method (41). A more detailed restriction map of this DNA fragment was generated from the sequence and is shown in Fig. 1. Analysis of the sequence reveals a unique open reading frame of 502 amino acid residues, the relative position of which is shown in Fig. 1. The nucleotide sequence and the translated protein sequence of this open reading frame are shown in Fig. 3.

Functional complementation of the *prp19* mutation requires sequences at least 800 bases upstream of the open reading frame, since deletion of 350 bases further from the 5′ end to the second *Eco*RI site of pSCC12-13 (Fig. 1) abolished the complementation activity. This finding indicates that there might be upstream activation sites 800 to 1,150 bases upstream of the open reading frame required for transcription activation of the *PRP19* gene. This is not unusual, since upstream activation sites are normally found between 1,400 and 100 nucleotides upstream of the TATA box in yeast genes (for a review, see reference 22).

The first AUG in the open reading frame is the initiation codon used. Examination of the sequence in the open reading frame revealed that the second AUG is 273 bases downstream from the first AUG. To determine whether the first AUG of the open reading frame is the likely initiation codon used, the transcription start site of the *PRP19* gene was mapped by primer extension. The results indicated multiple transcription start sites, all beginning within 8 to 39 bases upstream of the first AUG of the open reading frame (data not shown). To prove that the first AUG is used as the initiation codon, *PRP19* was fused to the bacteriophage T7 promoter, using the first or the second AUG as the initiation codon to produce PRP19 polypeptides of two different lengths. Comparison of the length of PRP19 produced in

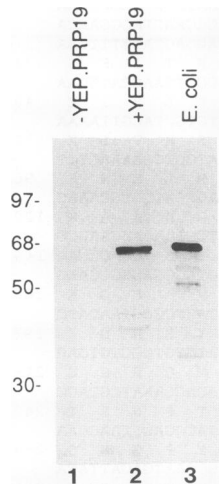

FIG. 4. Western blot analysis to confirm that the first AUG of the open reading frame is the initiation codon. Lanes: 1, wild-type yeast extract; 2, yeast extract from the PRP19-overproducing strain; 3, *E. coli* extract. Sizes are indicated in kilodaltons.

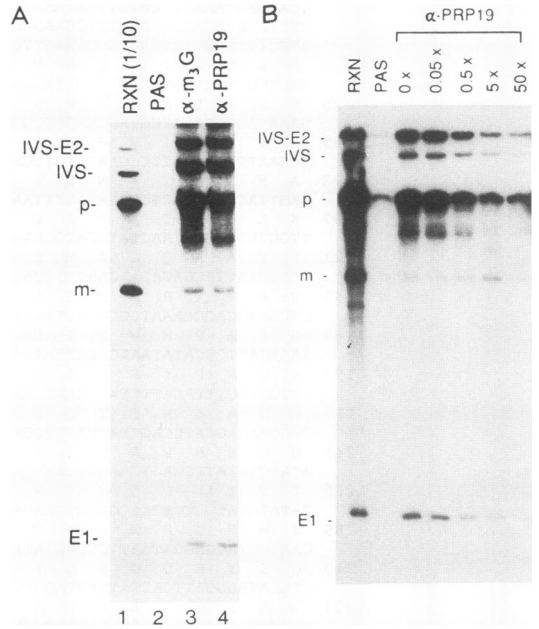

FIG. 5. Immunoprecipitation of the splicing reaction mixture. (A) The splicing reaction was carried out under standard conditions for 20 min. Each 20 μl of the reaction mixture was subjected to immunoprecipitation with anti-(m_3G)cap antibody (lane 3), anti-PRP19 antibody (lane 4), or protein A-Sepharose alone (PAS; lane 2). Lane 1 contained 2 μl of the splicing reaction mixture without immunoprecipitation. (B) Precipitation of the spliceosome by anti-PRP19 antibody was inhibited by preincubation of the antibody with PRP19 isolated from *E. coli*. 0×, 0.05×, 0.5×, 5×, and 50× represent no addition and 0.05-, 0.5-, 5-, and 50-fold molar ratio of PRP19 to the antibody, respectively. IVS, intervening sequence; p, precursor; m, mature message.

yeast cells with that of the recombinant proteins, as can be monitored by antibodies, will clearly demonstrate whether the first AUG is the initiation codon used in yeast. Cell extracts prepared from the PRP19 expressed *E. coli* strains were fractionated on a phosphocellulose column, and the PRP19-containing fractions were further fractionated by SDS-polyacrylamide gel electrophoresis (PAGE). The PRP19 protein was isolated from the gel and used to inject rabbits for raising antibodies.

Immunoblot analysis of the yeast splicing extracts showed that anti-PRP19 antibody detected no protein at 1.2 mg of total proteins in the extract (Fig. 4, lane 1). We then subcloned the *PRP19* gene into the yeast multicopy plasmid YEp24, and the recombinant plasmid, YEP.PRP19, was transformed into strain SS330 for overproduction of PRP19. Extracts prepared from such a strain carrying plasmid YEP-.PRP19 were subjected to immunoblot analysis. At 1.2 mg of total proteins, a protein of 65 kDa was detected (Fig. 4, lane 2). Although the size of the protein detected is different from that predicted from the sequence (56.5 kDa), it is the same as that of the protein produced in *E. coli*, using the first AUG as the initiation codon (Fig. 4, lane 3). The recombinant protein with the T7 promoter fused to the second AUG as the initiation codon migrated normally as a 46-kDa protein, as predicted from the sequence (data not shown). We therefore conclude that the first AUG of the open reading frame is the initiation codon for PRP19 and that the sequence in the N-terminal region of the protein is responsible for its anomalous mobility on SDS-PAGE.

***PRP19* is essential.** To test whether *PRP19* is an essential gene, we made a null allele in which the two *Xba*I fragments in the coding region of the *PRP19* gene (Fig. 1) are deleted and replaced with a 2-kb DNA fragment containing the *LEU2* gene. A diploid strain was transformed with a DNA fragment containing the disrupted *PRP19* gene, and Leu^+ transformants were selected. Insertion at the *PRP19* locus was confirmed by Southern analysis (data not shown). Tetrads from this strain yielded only two viable spores, and none of these were Leu^+. We therefore conclude that *PRP19* is an essential gene.

PRP19 is a spliceosomal component. Anti-PRP19 antibodies were affinity purified on a PRP19-coupled Sepharose column, and association of PRP19 with the spliceosome was examined by immunoprecipitation of the spliceosome with anti-PRP19 antibody. The splicing reaction was carried out under standard conditions, and the reaction mixtures were subjected to immunoprecipitation with anti-PRP19 antibody. After removal of the unreacted material, RNA species bound to the antibody were recovered and analyzed by gel electrophoresis. As shown in Fig. 5A, the splicing intermediates were quantitatively precipitated by the antibody (compare the amounts of the intermediates in lanes 1 and 4). Only 1/10 equivalent of the reaction mixture for immunoprecipitation was loaded in lane 1 as the total reaction mixture. More than 40% of the precursor, but only less than 2% of the mature message, was precipitated. Precipitation of splicing intermediates, the excised intervening sequence, and the precursor, but not the mature message, is a diagnosis for precipitating the spliceosome, since the mature message is not associated with the spliceosome (7). When such experiments were carried out with protein A-Sepharose alone (lane 2) or the unrelated antibody 12CA5 (see fig. 5, lane 4, in the accompanying report [47a]), no RNA was precipitated. Immunoprecipitation of the same reaction mixture with anti-(m_3G)cap antibody precipitated approximately the same amounts of the precursor RNA and the splicing intermediates (lane 3). Preincubation of the antibody with increasing amounts of PRP19 isolated from *E. coli* decreased the amounts of the RNA precipitated (Fig. 5B), whereas preincubation with bovine serum albumin at the level equivalent

to the highest PRP19 concentration used (50×) had no effect on the efficiency of immunoprecipitation (data not shown), indicating that such precipitation of splicing complexes is specific for PRP19. These results indicate that, like some of the U-class snRNAs, PRP19 is associated with the spliceosome during the splicing reaction.

PRP19 is a novel splicing factor. At least four other yeast PRP proteins have been demonstrated to be associated with the spliceosome during the splicing reaction. Sedimentation analysis of the spliceosome formed in *prp11* extracts complemented with the in vitro-synthesized ^{35}S-labeled PRP11 protein shows that PRP11 cofractionates with the spliceosome on glycerol gradients (6). PRP2 is shown to be associated with the spliceosome during the splicing reaction by immunoprecipitation studies (30). PRP16, required only for the second step of the cleavage-ligation reaction, appears to interact with the spliceosome only transiently (42). PRP8, as an integral component of U5 snRNP, has been shown to be associated with the spliceosome during the splicing reaction (53). PRP4 and PRP6 are integral components of the U4/U6 snRNP (1–3). They are also likely to be associated with the spliceosome during the splicing reaction through association with snRNPs, although they have not been demonstrated to be so. PRP19 is a new member of yeast splicing factors that are associated with the spliceosome during the splicing reaction.

Among all other *PRP* genes sequenced, four identifiable motifs have been found. These include the zinc finger motif, ATP-dependent RNA helicase, the RNA binding motif, and the motif for the β subunit of G protein (for a review, see reference 39). Several splicing factors have been identified in the mammalian system, including the intron-binding protein (18, 48), U2AF (40, 55), heterogeneous nuclear RNP C protein (10), SF2/ASF (31), SC-35 (14), and the polypyrimidine tract-binding protein (16). Among them, genes encoding U2AF, SF2/ASF, SC-35 and the polypyrimidine tract-binding protein have been cloned and sequenced, and the RNA binding motif is found in all of these genes (15, 17, 19, 32, 56). U2AF, SF2/ASF, and SC-35 also contain an arginine/serine-rich motif shared by the U1 snRNP 70-kDa protein and the *Drosophila* splicing regulators *transformer*, *transformer-2*, *suppressor-of-stable*, and *suppressor-of-white-apricot* (15, 17, 32, 56). In contrast, we did not find any discernible motif in the PRP19 sequence. A data base search also detected no homology in the nucleotide or protein sequence. Although its function is not known, PRP19 represents a novel splicing factor and a novel spliceosomal component.

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