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The *Saccharomyces cerevisiae PRP21* gene product is an integral component of the prespliceosome

(splicing/spliceosome/U2 small nuclear ribonucleoprotein)

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ABSTRACT **In *Saccharomyces cerevisiae*, the *prp21* mutation causes accumulation of unspliced pre-mRNA at the nonpermissive temperature. We have cloned the *PRP21* gene by complementation of its temperature-sensitive phenotype and found it to be the same as *SPP91*, an extragenic suppressor of the *prp9* mutation previously studied *in vivo* by Chapon and Legrain [Chapon, C. & Legrain, P. (1992) *EMBO J.* 11, 3279–3288]. We have analyzed the effects of the *prp21* mutation on splicing *in vitro* and have found that PRP21 is a splicing factor required for prespliceosome assembly. We also have analyzed the interaction of PRP21 with splicing complexes using anti-PRP21 antibodies and found that the RNA components of the prespliceosome—U1 and U2 small nuclear RNA (snRNA) particles and pre-mRNA—are specifically coimmunoprecipitated under splicing conditions in the presence of 0.2 M KCl. At higher KCl concentrations, U1 snRNP dissociates from splicing complexes; nevertheless, U2 snRNA and pre-mRNA are still efficiently immunoprecipitated. Immunoprecipitation of both U1 and U2 snRNA as well as pre-mRNA is ATP-dependent and requires a pre-mRNA capable of supporting prespliceosome assembly. Analysis of the unbound complexes in native gels confirmed that prespliceosomes are specifically immunoprecipitated by anti-PRP21 antibodies. These results demonstrate that PRP21 is an integral component of the prespliceosome and establishes a stable interaction with U2 snRNP and/or pre-mRNA in that complex.**

Nuclear pre-mRNA splicing occurs in the spliceosome, a large multicomponent complex containing U1, U2, U4, U5, and U6 small nuclear ribonucleoprotein (snRNP) particles, a number of non-snRNP protein factors, and pre-mRNA. Spliceosome assembly is an ordered process that begins with binding of the U1 snRNP to the 5′ end of the intron, forming a complex known as the commitment complex. Then, U2 snRNP binds to the conserved branch site sequence in an ATP-dependent manner to form the complex known as the prespliceosome. Subsequently, U4, U6, and U5 snRNPs are added to the complex to form the spliceosome. Splicing proceeds in two cleavage/ligation steps. In the first step, the 5′ splice site is cleaved concomitant with the formation of a 2′–5′ phosphodiester bond between the 5′ end of the intron and a specific adenosine residue in the branch site sequence, producing a lariat molecule containing the intron and second exon. The second step involves the cleavage of the 3′ splice site and ligation of the two exons to produce mature mRNA (for review see refs. 1–4 and references therein).

Our current understanding of the splicing mechanism and its regulation comes largely from the identification and characterization of the factors involved. The generation of temperature-sensitive (ts) strains and other genetic approaches in *Saccharomyces cerevisiae* has greatly simplified the task of identification and cloning of a large number of genes that affect pre-mRNA processing (*PRP* genes; reviewed in refs. 1 and 4).

Yeast strains carrying the *prp21* mutation present a ts splicing defect causing accumulation of unspliced pre-mRNA at the nonpermissive temperature (5). We have cloned the *PRP21* gene and performed *in vitro* studies to investigate the role of PRP21 in the splicing process. Our results indicate that PRP21 is required for prespliceosome assembly and that it interacts with U2 snRNP and/or pre-mRNA in the prespliceosome.

MATERIALS AND METHODS

Yeast Strains and Extracts. The wild-type (wt) yeast strains used in these studies were SS330 (*MAT***a**, *ade2-101, his3Δ200, tyr1, ura3-52*) and EJ101 (*MATα, trp1, pro1-126, prb1-112, pep4-3, prc1-126*). The *prp21* strain was previously selected from a bank of ts mutants generated by *in vivo* ethyl methanesulfonate mutagenesis of SS330 (5). Yeast splicing extracts were prepared as described (6). Heat inactivation was performed by heating extract aliquots at 40°C for 6–7.5 min just before being used in splicing reactions.

Cloning and Complementation. The *PRP21* gene was isolated by transformation of the *prp21* strain with a pYCP50 yeast genomic library (7). Plasmid DNA recovered from two transformants growing at 37°C was cut with various restriction enzymes, and the fragments were subcloned into the pPHY18 yeast CEN plasmid (8), transformed into the *prp21* strain, and tested for growth at 37°C. All yeast transformations were carried out by the lithium acetate protocol (9). A 2-kilobase (kb) *Hin*dIII fragment* was sequenced by using a dideoxy sequencing kit as suggested by the manufacturer (United States Biochemical). The *PRP21* gene was contained in a smaller 1.1-kb *Hin*dIII–*Xho* I fragment that complemented the *prp21* growth defect.

Generation of Anti-PRP21 Antiserum. By using standard polymerase chain reaction (PCR) and cloning techniques, the *PRP21* open reading frame was subcloned into the *Nde* I site of pET11a (Novagen, Madison, WI) for expression under the control of T7 polymerase in *Escherichia coli* (10). Expression of *PRP21* in this system yielded a 33-kDa polypeptide in the form of insoluble inclusion bodies in quantities that amounted to about 30–50% of the total protein. PRP21 inclusion bodies were purified as described (11). PRP21 was further purified from solubilized inclusion bodies by preparative polyacrylamide/SDS gel electrophoresis. Rabbit antiserum against the electrophoretically pure PRP21 protein was prepared by Cocalico Biologicals (Reamstown, PA).

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Abbreviations: ts, temperature sensitive; PAS, protein A-Sepharose; PAS-Ab, PAS with bound antibody; snRNP, small nuclear ribonucleoprotein; wt, wild type.

*The complete sequence of the *Hin*dIII fragment determined in this work has been deposited in the GenBank data base (accession no. L07744).

Immunoaffinity purified anti-PRP21 antibodies were prepared from rabbit antiserum by acid elution of the antibodies bound to Immobilon-P membranes coated with PRP21 essentially as described (12).

Splicing Assay and Native Gel Electrophoresis. *In vitro* splicing reactions were essentially as described (13). Reactions containing 40–50% extract were incubated at 15°C and stopped by addition of a buffer containing heparin prior to native gel electrophoresis (14); however, the electrophoresis procedure was modified to increase resolution. Samples were loaded on 3% polyacrylamide gels (24 × 18 × 0.1 cm; 20:1 acrylamide/methylenebisacrylamide) in TAE buffer (40 mM Tris acetate/1 mM EDTA, pH 7.2 at 20°C) and run at 4°C for 6–8 hr at 200 V with circulating buffer.

Immunoprecipitations. Protein A-Sepharose suspension (PAS, 150 μl; Sigma) was washed twice with 1 ml of NET buffer (50 mM Tris·HCl, pH 7.5/0.1% Nonidet P-40/1 mM EDTA/0.02% sodium azide) containing 500 mM KCl (NET-500) and was resuspended in a final volume of 150 μl in NET containing 150 mM KCl (NET-150). Then, 500 μl of NET-500 and 200 μl of rabbit antiserum or purified anti-PRP21 antibodies were added and mixed gently at 4°C for 1–4 hr. Finally, the PAS beads with bound antibodies (PAS-Ab) were washed three times with 1 ml of NET-500, once with NET-150, and resuspended in 300-μl final suspension volume in NET-150.

For direct immunoprecipitations from extracts, 20-μl aliquots of PAS-Ab suspension in 300 μl of NET containing the appropriate concentration of KCl (NET-KCl) were supplemented with 1 mM dithiothreitol and 0.5 units of RNasin per μl. Then, 20 μl of yeast extract was added and mixed for 1 hr at 4°C. PAS-Ab beads were washed twice with 1 ml of the corresponding NET-KCl and once with NET for 5 min at 4°C each time and finally were resuspended in 100 μl of NET supplemented with 5 mM EDTA, 0.1% SDS, 10 μg of *E. coli* tRNA per ml, and 10 μg of proteinase-K per ml. After 10 min at 37°C, the suspension was extracted with phenol/chloroform in TE buffer (10 mM Tris·HCl/1 mM EDTA, pH 7.4) and precipitated with ethanol.

For immunoprecipitation under splicing conditions, 20-μl splicing reaction mixtures containing 100 fmol of unlabeled actin pre-mRNA were incubated 10–60 min at 15°C, and the reactions were stopped in ice by addition of 80 μl of SPL buffer (50 mM K_2HPO_4, pH 7.0/2.5 mM $MgCl_2$/1 mM dithiothreitol) containing 5 mg of heparin (Sigma H-3125) per ml and the indicated concentration of KCl. After addition of heparin, splicing complexes are stable for several hours at 4°C (J.E.A., unpublished data). Then, 20 μl of PAS-Ab suspension in the corresponding SPL-KCl buffer was added and mixed at 4°C for 30–120 min. The beads were washed twice with the corresponding SPL-KCl buffer and transferred into a new tube. Finally, the bound RNA was extracted as above. It is critical to transfer the beads into a new tube before extraction because, under these conditions, significant amounts of snRNPs, especially U1 snRNP and U5 snRNP, stick to the tube walls. However, no snRNPs bound to PAS without bound antibodies at any concentration of KCl (data not shown).

For analysis of unbound splicing complexes, a 50-μl splicing reaction with 50 fmol of actin pre-mRNA labeled with [α-^{32}P]UTP was stopped in ice with 10 μl of 60 mM K_2HPO_4 (pH 7.0) containing 3 mM $MgCl_2$, 3% PEG, 8% (wt/vol) glycerol, 25 mg of heparin per ml, and 150 mM KCl. Then 15-μl aliquots were mixed with 10 μl of PAS-Ab in SPL buffer containing 150 mM KCl. After mixing for 30 min at 4°C, the splicing complexes remaining in the supernatant were analyzed by native gel electrophoresis. The beads were washed, and the bound splicing products were extracted as above and analyzed in 8% polyacrylamide (29:1 acrylamide/methylenebisacrylamide)/7 M urea gels.

Northern Blots. Immunoprecipitated RNA from reactions containing unlabeled pre-mRNA was resolved in 8% polyacrylamide/7 M urea gels and transferred to GeneScreen membrane (NEN). The membranes were probed as described (15) with a mixture of snRNA probes generated by PCR from plasmids carrying yeast genes for snRNA or actin and were labeled with [α-^{32}P]dATP by using the random priming synthesis kit from Boehringer Mannheim.

RESULTS AND DISCUSSION

Cloning and Sequence of *PRP21*. The *PRP21* gene was isolated by complementation of the ts growth defect of the *prp21* strain with a genomic yeast library. Sequence analysis of a DNA fragment complementing the growth defect revealed an open reading frame encoding a 280-amino acid polypeptide (PRP21) with a predicted molecular mass of 33-kDa. The nucleotide sequence of *PRP21* is the same as *SPP91*, a gene identified as a second site suppressor (*spp91*) of the growth defect of a *prp9* mutant (16).

PRP21 Is Required for Prespliceosome Assembly. It was previously shown *in vivo* that the *prp21* mutation causes accumulation of unspliced pre-mRNA at a nonpermissive temperature, suggesting that PRP21 is involved in an early step of the splicing process (5). To define the role of PRP21 in splicing, we have established conditions for heat inactivation of extracts prepared from the *prp21* strain grown at the permissive temperature (*prp21* extract). We found that *prp21* extracts exhibited normal splicing activity. However, when the *prp21* extract was preheated at 40°C for as little as 6 min (Δprp21 extract), the splicing activity was abolished while the wt extract retained full activity after similar treatment (Fig. 1*A*, lanes a–f). Some residual activity was often observed in

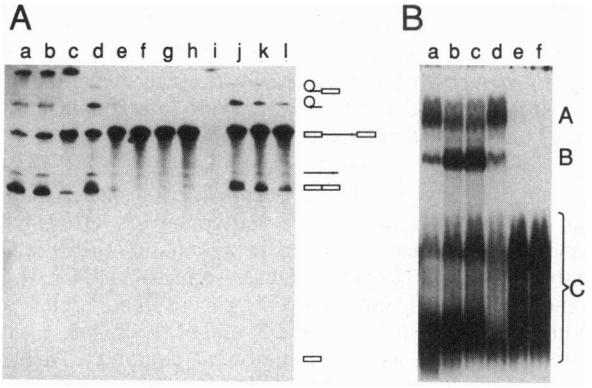

FIG. 1. Heat-inactivation *in vitro*. wt and *prp21* extracts or the 40P3 fraction were heated at 40°C and then tested for splicing activity and spliceosome formation. Splicing reactions containing labeled actin pre-mRNA were incubated at 15°C for 60 min. (*A*) Denaturing gel electrophoresis of splicing products from reactions containing wt extracts preheated for 0, 6, or 15 min (lanes a–c, respectively); *prp21* extracts preheated for 0, 6, or 15 min (lanes d–f); and 40P3 fraction preheated 0, 6, or 15 min (lanes g–i). Complementation is shown by reactions containing *prp21* extract preheated for 6 min in addition to 40P3 fraction preheated for 0, 6, or 15 min (lanes j–l). The products of the splicing reaction are represented schematically. Exons are shown as boxes, and the intron is represented by a line; circles represent RNA lariat forms. (*B*) Splicing complexes formed in reactions containing untreated or preheated extracts as in *A* were resolved in native gels. Lanes: a–c, wt extract preheated 0, 7.5, or 15 min, respectively; d–f, *prp21* extract preheated for 0, 7.5, or 15 min. Complex A contains U2, U5, U4, and U6 snRNAs in addition to pre-mRNA and splicing intermediates. Complex B contains pre-mRNA and U2 snRNA. U1 snRNA is not detected in this gel system (14); nonetheless complexes A and B are representative of spliceosomes and prespliceosomes, respectively. Complex C corresponds to heterogeneous nonspecific complexes.

Δ*prp21* extracts heated for 6–7 min and could be eliminated by extending the heat treatment by 1 or 2 additional min. However, longer treatment also resulted in some reduction of the splicing activity of wt extracts (Fig. 1*A*, lane c). Splicing is blocked at the first step of the reaction in Δ*prp21* extracts, indicating that PRP21 is a splicing factor required before 5′ splice site cleavage. Furthermore, PRP21 appears to be an exchangeable factor because the splicing activity in the Δ*prp21* extract could be restored by complementation with an inactive 40% saturation ammonium sulfate pellet fraction (40P3) prepared from wt extract (17). 40P3 exhibited complementing activity even after heat treatment for 15 min at 40°C (Fig. 1*A*, lanes j–l).

Mutations blocking the first step of splicing may either prevent spliceosome formation or allow the assembly of a nonfunctional spliceosome. To examine the role of PRP21 in spliceosome assembly, we stopped the splicing reactions with heparin and analyzed the splicing complexes formed by native gel electrophoresis as described (14). Neither prespliceosomes or spliceosomes formed in reactions with Δ*prp21* extracts (complexes B and A, respectively; Fig. 1*B*) indicating that PRP21 is required for the assembly of prespliceosomes.

PRP21 Is a Component of the Prespliceosome. Contrary to mammalian cells, the snRNPs in *Saccharomyces cerevisiae* are found in low abundance, making their biochemical characterization more difficult. Thus, little is known about their protein composition. Through the use of specific antibodies, some yeast splicing factors have been found to be tightly associated with snRNPs. By this criterion, PRP4 and PRP6 are U4/U6 snRNP proteins (18–20), while PRP8 is a U5 snRNP protein (21, 22). We have studied the interaction of PRP21 with snRNPs using rabbit antiserum raised against gel-purified PRP21 protein produced in *E. coli*. We attempted direct immunoprecipitation of snRNPs from wt extracts diluted in NET-KCl buffer and found that no significant amounts of any snRNA could be specifically immunoprecipitated under these conditions, with KCl concentrations ranging from 0.05 M to 1 M, suggesting that PRP21 is not tightly associated to snRNPs. However, we can not rule out the possibility that PRP21 might be an snRNP protein whose antigenic epitopes are not accessible to antibodies under the conditions used.

The requirement for PRP21 in early spliceosome assembly suggests that it may interact at least transiently with spliceosomal components. To address this question, we attempted immunoprecipitation of splicing complexes under splicing conditions. Splicing reactions containing unlabeled pre-mRNA were stopped with heparin, and immunoprecipitation was then carried out in splicing buffer as described in *Materials and Methods*. When anti-PRP21 antibodies were used, efficient and specific coimmunoprecipitation of the RNA components of the prespliceosome—U1 snRNA, U2 snRNA, and pre-mRNA—was observed in the presence of 200 mM KCl. In contrast, when pre-mRNA was omitted from the splicing reaction or when control antibodies from preimmune serum was used, none of these RNAs was immunoprecipitated at KCl concentrations higher than 50 mM (Fig. 2 *A* and *B*). Furthermore, a correlation between the relative amounts of U1 snRNA, U2 snRNA, and pre-mRNA immunoprecipitated was apparent, suggesting that all three RNAs are part of the same complex, most likely the prespliceosome (Figs. 2 and 3; also data not shown).

After the commitment complex is formed by U1 snRNP binding to the 5′ splice site, ATP is required for binding of U2 snRNP to the branch site to form the prespliceosome. We have tested the possibility that anti-PRP21 antisera could immunoprecipitate prespliceosomes by assaying both ATP dependence and pre-mRNA sequence requirements for immunoprecipitation of all three components. As expected, when ATP was omitted from the splicing reaction, immunoprecipitation of both U1 and U2 snRNAs as well as pre-mRNA was reduced to background levels (Fig. 2 *C* and *D*). Identical results were obtained with antibodies from crude anti-PRP21 antiserum or with immunoaffinity-purified anti-PRP21 antibodies (data not shown). Phosphor-imaging quantification of the experiments shown in Fig. 2 showed that at 200 mM KCl, the immunoprecipitation of U1 and U2 snRNAs was stimulated 50- and 70-fold, respectively, by the addition of pre-mRNA to the reactions, whereas addition of ATP stimulated immunoprecipitation of U1 snRNA, U2 snRNA, and pre-mRNA by 19-, 14-, and 37-fold, respectively. Immunoprecipitation of small amounts of prespliceosome components in the absence of ATP or pre-mRNA is likely due to the presence of endogenous ATP and pre-mRNA in the extract.

It has been shown that yeast spliceosomal complexes do not form on actin pre-mRNA mutants carrying either the 5′

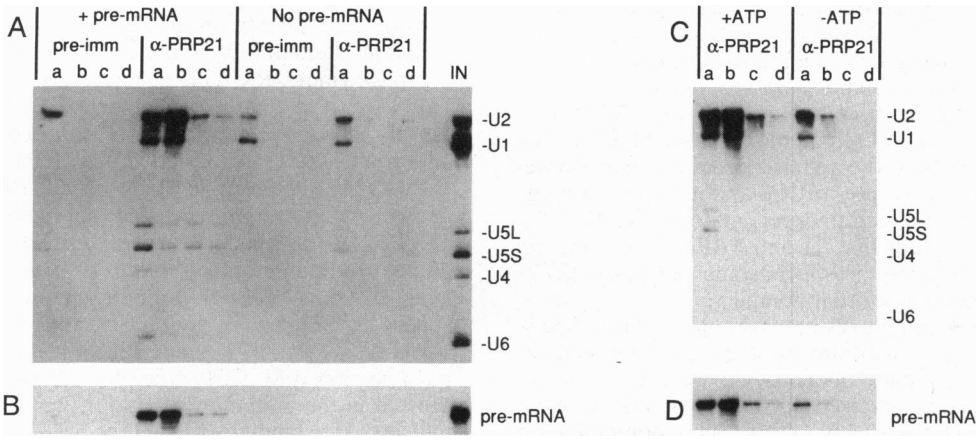

FIG. 2. Immunoprecipitation of splicing complexes. (*A* and *B*) Standard splicing reaction mixtures contained wt extract, ATP, and 100 fmol of unlabeled actin pre-mRNA (lanes + pre-mRNA) or no added pre-mRNA (lanes labeled No pre-mRNA). After incubation at 15°C, the reaction was stopped with heparin, and immunoprecipitation was carried out under splicing conditions with preimmune antibodies (lanes pre-imm) or anti-PRP21 antibodies (lanes α-PRP21) as described in the presence of KCl at 0.05 M (lanes a), 0.2 M (lanes b), 0.4 M (lanes c), and 0.6 M (lanes d). Bound RNA was extracted and analyzed by Northern blot by probing successively with a mixture of U snRNA probes (*A*) and with actin pre-mRNA probe (*B*). Pre-mRNA was the only RNA species detected with the pre-mRNA probe; thus, only that portion of the blot is shown. (C and D) Immunoprecipitations from reactions with wt extract, pre-mRNA, and ATP as above (lanes +ATP) or without ATP (lanes −ATP). Immunoprecipitations were in the presence of KCl as above with anti-PRP21 antibodies.

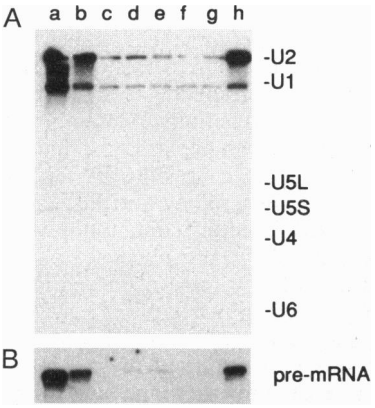

FIG. 3. Effect of pre-mRNA sequence in immunoprecipitation of prespliceosome components. Splicing reactions were as in Fig. 2 with 100 fmol of one of several actin pre-mRNA mutants. Each of the mutant pre-mRNAs also contains a 6-nucleotide deletion (Δ6) that eliminates a cryptic branch site in the intron. The Δ6 pre-mRNA behaves as its wt counterpart in splicing reactions (23). Lanes: a, wt pre-mRNA; b, Δ6 pre-mRNA; c, no pre-mRNA; d, Δ6-A1; e, Δ6-C1; f, Δ6-A257; g, Δ6-C259; h, Δ6-C303/C305. Δ6-A1 and Δ6-C1 are mutations of the guanosine at position 1 of the intron; Δ6-A257 and Δ6-C259 are mutations in the invariable TACTAAC branch point sequence; Δ6-C303/C305 is a double mutation of the 3′ splice site. All immunoprecipitations were carried out in the presence of 0.2 M KCl and the bound RNA analyzed as in Fig. 2.

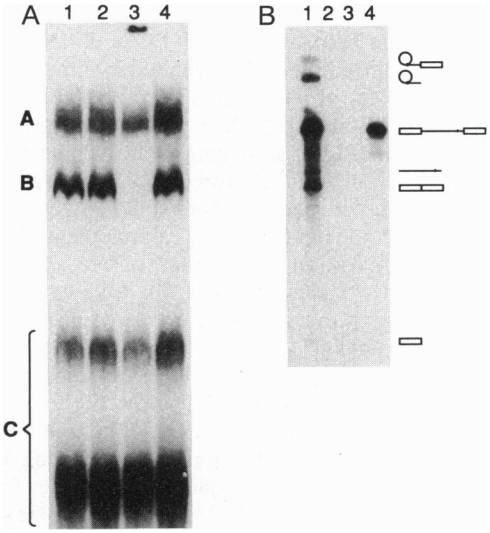

FIG. 4. Analysis of unbound splicing complexes. Standard splicing reactions containing 10 fmol of radiolabeled pre-mRNA were stopped with heparin, and splicing complexes were immunoprecipitated in the presence of 0.15 M KCl as described in text. (*A*) Autoradiography of the unbound splicing complexes separated by native gel electrophoresis of the supernatant after immunoprecipitation with PAS with no antibodies (lane 1), PAS with preimmune antibodies (lane 2), PAS with purified anti-PRP21 antibodies (lane 3), and no PAS or antibodies (lane 4). (*B*) Autoradiography of the bound splicing products from the same experiment resolved by denaturing gel electrophoresis. Lanes: 1, total RNA no PAS or antibodies; 2, PAS with no antibodies; 3, PAS with preimmune antibodies; 4, PAS with purified anti-PRP21 antibodies. Note that the lane numbers in *B* do not coincide with those in *A*.

splice site mutations A1 or C1 or the branch site mutations A257 and C259. However, spliceosomes can form on a pre-mRNA carrying a 3′ splice site double-mutation C303/C305 (14, 23, 24). We performed immunoprecipitation experiments using these mutant actin pre-mRNAs and found that mutations at either the 5′ splice site or the branch site do not support immunoprecipitation of prespliceosomal RNAs to levels higher than those found when no pre-mRNA is added. In contrast, significant amounts of U1 snRNA, U2 snRNA, and pre-mRNA are immunoprecipitated when wt or 3′ splice site mutant pre-mRNA are added (Fig. 3). These results demonstrate that coprecipitation of U1 snRNA, U2 snRNA, and pre-mRNA requires the assembly of splicing complexes and strongly suggest that immunoprecipitation of these RNAs is due to specific recognition of prespliceosomes by anti-PRP21 antibodies.

Using labeled actin pre-mRNA, we have further investigated the identity of the immunoprecipitated complexes by resolving the unbound splicing complexes in native gels (Fig. 4*A*). Prespliceosomes (complex B) are quantitatively removed from splicing mixtures treated with anti-PRP21 antibodies, while spliceosomes (complex A) remained in the supernatant. Analysis of the splicing products immunoprecipitated shows that only pre-mRNA and not splicing intermediates or mRNA are immunoprecipitated (Fig. 4*B*). Together, these results show that the anti-PRP21 antibodies can indeed immunoprecipitate prespliceosomes and that PRP21 is an integral component of that complex.

Although splicing complexes are stable for extended periods of time under the conditions used for immunoprecipitations, in none of our experiments were we able to detect splicing intermediates, excised introns, or mRNA, even when splicing proceeded for 1 hr before immunoprecipitation (Fig. 4*B* and data not shown), indicating that spliceosomes that have proceeded beyond the first step of splicing are not immunoprecipitated by anti-PRP21 antibodies. Furthermore, it is unlikely that immunoprecipitation of small amounts of pre-mRNA as well as U2, U5, and U6 snRNAs at high KCl concentrations represents mature spliceosomes, since U5 and U6 snRNA precipitation was not consistently observed (compare Fig. 2 *A* and *C*) and similar immunoprecipitation was also observed in reactions in which ATP or pre-mRNA had been omitted (data not shown). Accordingly, our native gel analysis of the unbound complexes after immunoprecipitation also indicates that spliceosomes are not recognized by anti-PRP21 antibodies. Similarly, failure to immunoprecipitate pre-mRNA and U1 snRNP when ATP was omitted from the splicing reaction indicates that anti-PRP21 antibodies do not recognize commitment complexes. However, we can not determine at this time whether PRP21 is only transiently associated with the prespliceosome or if it is also a component of other splicing complexes in which the PRP21 epitopes are not accessible to antibodies. For example, it is conceivable that after addition of the U4, U6, and U5 snRNPs to the prespliceosome, PRP21 is no longer accessible to antibodies. However, it is also possible that PRP21 leaves or becomes loosely associated with the spliceosome concomitant with the addition of the U4, U6, and U5 snRNPs.

PRP21 Is Tightly Associated with U2 snRNP or Pre-mRNA or Both. We have demonstrated that immunoprecipitation of U2 snRNA, U1 snRNA, and pre-mRNA in 200 mM KCl is due to the immunoprecipitation of prespliceosomes. However, at 400 mM KCl the coprecipitation of these three RNA species is abolished or drastically reduced. Interestingly, U2 snRNA and pre-mRNA are still efficiently immunoprecipitated at 300 mM KCl, whereas U1 snRNA is no longer present in the immunoprecipitate (Fig. 5). This observation indicates that binding of PRP21 to U1 snRNP is not required for coprecipitation of U2 snRNP and pre-mRNA and that the U1 snRNP can be selectively removed from the prespliceosome, leaving a stable U2 snRNP–pre-mRNA complex containing PRP21. This result is consistent with previous observations showing that the U1 snRNP is loosely associated while U2 snRNP maintains a stable interaction with pre-mRNA (14, 25–27). Therefore, PRP21 must interact with the U2 snRNP, the pre-mRNA, or both components of the

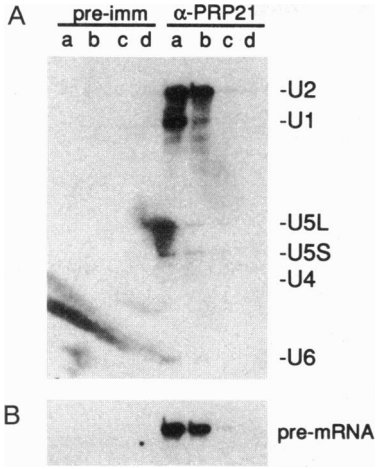

FIG. 5. Effect of KCl in immunoprecipitation of prespliceosome components. Standard splicing reactions containing 100 fmol of unlabeled pre-mRNA as in Fig. 2 were immunoprecipitated with preimmune antibodies (lanes pre-imm) or with immunopurified anti-PRP21 antibodies (lanes αPRP21) in the presence of KCl at 0.15 M (lanes a), 0.3 M (lanes b), 0.5 M (lanes c), or 1 M (lanes d). Immunoprecipitated RNA was analyzed by Northern blots as in Fig. 2.

prespliceosome. We cannot distinguish whether this is a direct interaction or whether it occurs through an interaction with other protein factors. An interaction between U2 snRNP and PRP21 is also supported by the formation of synthetic lethals (inviable double mutants) in crosses between *prp21* and strains carrying cold-sensitive mutations in the stem loop IIa of the U2 snRNA (S. Fischer-Wells and M. Ares, Jr., personal communication; S. Ruby and J.N.A., unpublished data). The stem loop IIa of U2 snRNA has been shown to be an essential structure involved in U2 snRNP binding to pre-mRNA (28, 29).

Role of PRP21 in Spliceosome Assembly. Based on our *in vitro* observations, we propose that PRP21 plays a role in U2 snRNP binding by establishing a strong interaction with the U2 snRNP or the pre-mRNA or both, and, thus, becomes an integral component of the prespliceosome. In yeast, only the products of the *PRP9* and *PRP5* genes have previously been shown to be required for prespliceosome assembly (1, 20, 30). The commitment complex containing U1 snRNP and pre-mRNA (31, 32) forms efficiently in heat-inactivated *prp9* extracts (20) and in wt extracts immunodepleted with anti-PRP9 antibodies (P. Legrain, personal communication); however, PRP9 is required for U2 snRNP binding to the pre-mRNA. Furthermore, in wt extracts, U2 snRNP is required for immunoprecipitation of pre-mRNA with an antibody against epitope-tagged PRP9 under splicing conditions (20). Those observations bear striking similarity to what we found with anti-PRP21 antibodies and, thus, suggest that PRP9 may be required at the same time as PRP21 and also may be a component of the prespliceosome. This possibility is supported by the isolation of a *PRP21* allele (*spp91*) that suppresses the *prp9* growth defect (16). A direct interaction between PRP9 and SPP91 has been shown *in vivo* (P. Legrain, personal communication). Furthermore, a physical interaction is also suggested from genetic experiments in which pairwise combinations of *prp21*, *prp9*, *prp11*, and *prp5* exhibit synergistic lethality (S. Ruby, T.-H. Chang, and J.N.A., unpublished data). Thus, the most likely explanation for the genetic and biochemical results is that PRP9 and PRP21 are required at the same time for U2 snRNP binding and that PRP21 and PRP9 interact together with the U2 snRNP or both. Further *in vitro* experiments will be necessary to unequivocally establish the functional relationship between U2 snRNP, PRP9, and PRP21.

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