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The Yeast *PRP6* Gene Encodes a U4/U6 Small Nuclear Ribonucleoprotein Particle (snRNP) Protein, and the *PRP9* Gene Encodes a Protein Required for U2 snRNP Binding

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***PRP6* and *PRP9* are two yeast genes involved in pre-mRNA splicing. Incubation at 37°C of strains that carry temperature-sensitive mutations at these loci inhibits splicing, and in vivo experiments suggested that they might be involved in commitment complex formation (P. Legrain and M. Rosbash, Cell 57:573–583, 1989). To examine the specific role that the *PRP6* and *PRP9* products may play in splicing or pre-mRNA transport to the cytoplasm, we have characterized in vitro splicing and spliceosome assembly in extracts derived from *prp6* and *prp9* mutant strains. We have also characterized RNAs that are specifically immunoprecipitated with the *PRP6* and *PRP9* proteins. Both approaches indicate that *PRP6* encodes a U4/U6 small nuclear ribonucleoprotein particle (snRNP) protein and that the *PRP9* protein is required for a stable U2 snRNP-substrate interaction. The results are discussed with reference to the previously observed in vivo phenotypes of these mutants.**

The pre-mRNA splicing pathways are identical in yeast (*Saccharomyces cerevisiae*) and mammalian systems (for a review, see reference 9). In both systems, the first step of splicing consists of cleavage at the 5′ splice junction and lariat formation, whereas the second step consists of cleavage at the 3′ splice junction and exon ligation. The cleavage and ligation steps are preceded by a stepwise assembly process during which the pre-mRNA associates with a large number of *trans*-acting factors, including four small nuclear ribonucleoprotein particles (snRNPs) (5, 8, 13, 22).

In the yeast system, many of these *trans*-acting factors are identified as *PRP* gene products (32). The *PRP* genes (formerly the *RNA* genes) (11) were originally connected to pre-mRNA splicing because it was found that incubation of several of these temperature-sensitive mutant strains at nonpermissive temperatures led to an increase in pre-mRNA levels and a decrease in mRNA levels from intron-containing genes (23). This connection has been strengthened by the observation that heating extracts derived from some of these strains has mutant-specific effects on splicing and spliceosome assembly (4, 20). The general strategy of comparing extracts derived from mutant strains, complementing mutant extracts, and examining mutant gene products and mutant snRNPs provides a valuable tool for studying pre-mRNA splicing in vitro in the yeast system.

In contrast to the in vitro systems, the study of splicing in vivo is difficult, as comparable assays do not yet exist. Yet it is likely that the assembly process in the nucleus closely resembles what takes place during splicing in vitro, namely, the stepwise association of multiple splicing factors with the pre-mRNA to generate a mature complex (the spliceosome) within which the cleavage and ligation steps take place. For many pre-mRNA substrates, it is also generally considered that this association is quite efficient such that most pre-mRNA molecules are properly spliced. An efficient assembly process presumably avoids pre-mRNA degradation within the nucleus as well as transport of unspliced transcripts to the cytoplasm.

With these considerations in mind, a previous study examined the fate of inefficiently spliced pre-mRNAs (17). The results indicated that in some cases a reduction in splicing efficiency leads to a dramatic increase in pre-mRNA translation. The splicing efficiency reduction was achieved in two different ways, by introducing *cis*-acting mutations into the substrate and by examining a few strains that carried mutations in splicing factors. The three mutant strains in which pre-mRNA translation was the most convincing carried a mutation in U1 snRNA or temperature-sensitive mutations in the genes *PRP6* and *PRP9*. Our preferred hypothesis was that these three gene products, U1 snRNA and the PRP6 and PRP9 proteins, participate in the early events of spliceosome assembly and thereby contribute to stable pre-mRNA commitment to the splicing pathway; RNA transport to the cytoplasm, resulting in pre-mRNA translation, constitutes a default process that is normally inhibited in the case of efficiently assembled pre-mRNAs.

Support for this hypothesis must come from in vitro studies in which individual steps of spliceosome assembly can be distinguished. Indeed, a good deal is known about the role of U1 snRNP in spliceosome assembly, and that evidence supports the notion that U1 snRNP associates with the pre-mRNA in an ATP-independent manner at an early stage of spliceosome assembly (25, 29). Subsequently U2 snRNP joins the complex, followed by U4/U6 and U5 snRNPs (2, 5, 13, 16, 22). The role of the *PRP6* and *PRP9* gene products, however, is completely unknown. The experiments described in this report define the role of these two gene products during in vitro spliceosome assembly. They indicate that the *PRP9* product is essential for U2 snRNP binding and that the *PRP6* protein is a U4/U6 snRNP protein. The results suggest that pre-mRNA transport into the cytoplasm can be enhanced by mutants at different stages in the assembly process.

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MATERIALS AND METHODS

Yeast strains and plasmids. Two *prp9* mutant strains, JM664 (17) and ts100 (30); the four *prp6* mutant strains SpJ6.66 (17), ts39 (30), ts88 (30), and ts284 (30); SpJ 4.41 (17), a *prp4* mutant; and SpJ8.31 (17), a *prp8* mutant, have been previously described.

Plasmids PL4 and PL1 (16a) carry genomic inserts in YCP50 (24) containing the wild-type genes *PRP9* and *PRP6*, respectively. Strain JM664 was transformed with PL4, and with the vector alone to generate strains 9^+ and 9YCP, respectively.

Strain SpJ6.66 was transformed with PL1 and with the vector alone to generate strains 6^+ and 6YCP, respectively.

BS-Y46 (wild type), BS-Y82 (*Gal*-U1) and BS-Y88 (*Gal*-U2) have been previously described and characterized (29).

Construction of epitope-tagged *PRP9* and *PRP6* genes. The nine-amino-acid epitope derived from the influenza virus hemagglutinin protein (HA) recognized by the monoclonal antibody 12CA5 (31) was inserted into both PRP9 and PRP6, either at the N terminus after the second amino acid or at the C terminus before the last amino acid.

For *PRP9*, a *Hin*dIII-*Sna*BI fragment containing the complete gene (16a) was subcloned from PL4 into pTZ19U. For *PRP6*, two subclones were generated from PL1. One was a *Cla*I-*Xba*I fragment containing the 5′ end of the gene cloned into pTZ19U, and the other was an *Acc*I-*Eco*RI fragment containing the 3′ end cloned into pTZ18U. The sequence for the HA epitope was inserted by in vitro mutagenesis by the method of Kunkel (15). After mutagenesis, the *PRP9* (wild type), *PRP9*-5′HA, and *PRP9*-3′HA genes were cloned back into YCP50. For *PRP6*, the 5′ and 3′ insertions were cloned back into YCP50 to create *PRP6* (wild type), *PRP6*-5′HA, *PRP6*-3′HA, and *PRP6*-5′-3′HA; the latter contained two epitopes, one at each end. All five tagged genes were reintroduced into their respective *prp* mutant strains by transformation (12), and the temperature-sensitive phenotype was rescued in all cases. The expression of each tagged gene was tested by Western immunoblotting of total protein (data not shown). The only plasmid that appeared not to express the tagged protein was *PRP9*-5′HA; since this plasmid rescued the temperature-sensitive phenotype of *prp9*, the protein was probably clipped near the N terminus, eliminating the HA epitope. For the preparation of tagged extracts and immunoprecipitations, the strains *PRP9*-3′HA and *PRP6*-5′-3′HA were used.

Extract preparation. A glass bead miniextract procedure, as described by Seraphin and Rosbash (29), was used throughout these experiments, with the following modifications: 100 ml of cells were grown to an optical density at 600 nm of 2 to 3, and 230 optical density units of cells was routinely processed for extract. Cells were grown at 25°C and subjected to a final 1 h at 37°C to manifest the mutant *prp* phenotype. *PRP9*- and *PRP6*-rescued strains, harboring either the original genomic clones or the epitope-tagged genes, were grown continuously at 37°C. For the *prp4* (SpJ4.41) and *prp8* (SpJ8.31) strains, the control extracts were prepared from aliquots of the cultures kept at 25°C (permissive temperature), while the other aliquots were incubated at 37°C for 1 h before extract preparation. The U1- and U2-depleted extracts were prepared from strains BS-Y82 and BS-Y88, respectively, after 16 h of growth at 30°C in medium containing 4% glucose, as described previously (29).

Splicing reactions. Unless specifically noted, standard (10-μl) reactions containing 4 μl of extract were carried out as previously described (21).

The substrate Δ2 and runoff transcription to the *Dde*I site were as described previously (22) except that the transcript was prepared at a specific activity of 3×10^5 cpm/ng.

The two-step reactions for chasing commitment complexes into spliceosomes were performed as described (29). Briefly, the radioactive substrate prepared as described above was incubated with ATP, splicing salts, and 2 μl of the first extract for 10 min at 25°C. A 50-fold molar excess of the same substrate at a specific activity of 120 cpm/ng was then added in a volume of 1 μl, and the incubation continued for 1 min; then 2 μl of the second extract was added, followed by incubation for 10 min at 25°C. Native gel electrophoresis of splicing complexes was performed on vertical gels in 0.5× TBE as described previously (29).

Streptavidin-agarose selection. Splicing complexes formed on a biotinylated Δ2 substrate were bound to streptavidin-agarose as described previously (27). After proteinase K treatment, the RNA was analyzed for the presence of U2, U4, and U6 snRNAs by primer extension as described previously (28) except that three labeled oligonucleotide primers complementary to nucleotides 100 to 121 of U2, 72 to 92 of U4, and 28 to 54 of U6 were added simultaneously to the reaction mixtures. The cDNA products were separated on 5% acrylamide–7 M urea gels.

Extract fractionation. A splicing extract prepared from the wild-type strain (BS-Y46) was adjusted to 300 mM KCl and centrifuged at 356,000 × *g* for 2 h at 4°C in a Beckman TL-100 centrifuge, using a TLA-100.1 or TLA-100.2 rotor as described previously (26). The pellets were resuspended in dialysis buffer (21) containing 0.1% Nonidet P-40. The supernatants were dialyzed for 1 h against the same buffer and used to complement the splicing of mutant extracts.

Immunoprecipitation. The monoclonal antibody 12CA5 (31) was purified from ascites fluid (a generous gift of Ian Wilson) on GammaBind G (Genex) according to the manufacturer's recommendations.

(i) snRNPs associated with *PRP9* and *PRP6*. HA-tagged or control extracts (50 μl) were preincubated without substrate in a final volume of 125 μl containing splicing salts and ATP for 10 min at 25°C. Aliquots of 25 μl were immunoprecipitated for 2 h at 4°C in 0.5 ml of NET (0.05 M Tris [pH 7.4], 0.05% Nonidet P-40, NaCl concentrations as indicated) containing 20 μl of GammaBind Plus beads (Genex) that had 4 μg of the purified 12CA5 antibody prebound as described previously (1). After four washes with 1 ml of the same buffer, the beads were proteinase K treated and phenol extracted. After ethanol precipitation, the RNA was electrophoresed on 4% acrylamide–7 M urea gels. Electroblotting, UV cross-linking, and hybridization were carried out as described previously (18) except that blotting was done for 1 h at 420 mA. Radioactive probes for U1, U2, U4, U5, and U6 snRNAs were generated by random-primer labeling of plasmids with a kit (Boehringer Mannheim) as recommended by the manufacturer. Hybridization was carried out in 20 ml with 10^6 cpm of each of the five snRNA probes per ml.

(ii) Radioactive substrate. Samples (40 μl) of splicing reaction mixtures, containing 16 μl of either a tagged extract or a control extract (without the epitope) plus 3 ng of radioactive Δ2 substrate, were incubated for 30 min at 25°C. Parallel reactions were carried out in which the same extracts had been depleted in vitro of U2 snRNP by preincubation with an oligonucleotide complementary to U2 snRNA, as previously described (14). After incubation, immunoprecipitation was carried out as described above for snRNA

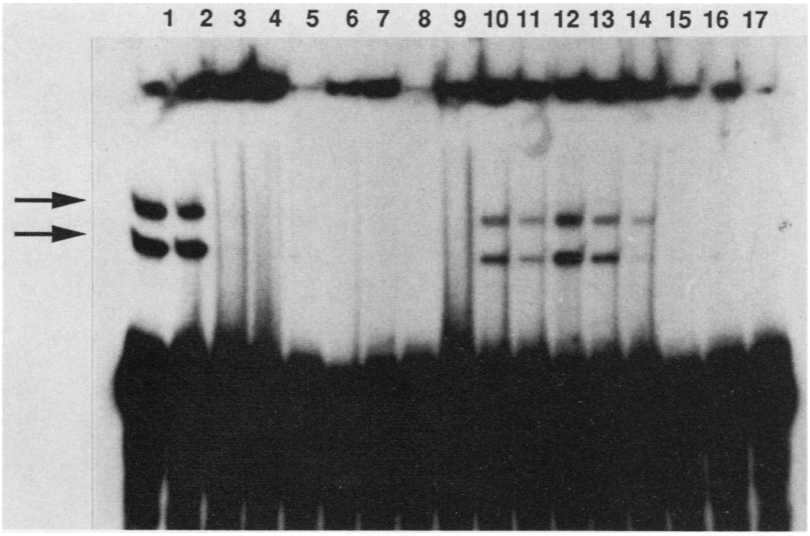

FIG. 1. Splicing assay and mutant extract complementation. Splicing reactions (10 μl) were carried out with either 4 μl of a single extract (lanes 1 to 8) or 2 μl each of the indicated extracts tested for complementation (lanes 9 to 17). The splicing products were analyzed on a 15% acrylamide–7 M urea gel. Lanes: 1 and 2, extracts derived from strains 9^+ and 6^+; 3 and 5, strains 9YCP and 6YCP; 4, ts100; 6 to 8, ts39, ts88, and ts284; 9, the two *prp9* mutant extracts 9YCP and ts100; 10 to 13, 9YCP complemented with the *prp6* mutant extracts 6YCP, ts39, ts88, and ts284; 14, 6YCP complemented with ts100; 15 to 17, 6YCP with the *prp6* mutant extracts ts39, ts88, and ts284. It should be noted that in our hands ts100 was not rescued by PL4 (N. Abovich, unpublished observations), so there is some doubt about whether ts100 carries a temperature-sensitive *prp9* allele. The arrows point to two splicing products, the lariat intermediate (upper band) and the lariat intron (lower band); the mRNA is invisible under the degradation products of the pre-mRNA.

analysis except that binding and washing were carried out in 0.5 ml of NET (containing 100 mM NaCl). Bovine serum albumin was included during binding at 0.6 mg/ml.

After proteinase K treatment and phenol extraction, the radioactive RNA was analyzed on a 15% acrylamide–7 M urea gel.

RESULTS

To define the roles of the *PRP9* and *PRP6* gene products, we analyzed splicing and spliceosome assembly in extracts made from mutant strains that carry the *prp6* or *prp9* temperature-sensitive mutation. With two exceptions, the strategy was similar to that previously used to analyze *prp* mutant strains in vitro. First, cells were heated at the nonpermissive temperature prior to extract preparation, rather than heated in vitro, to effect a mutant phenotype (4, 20). Second, splicing extracts were prepared by a glass bead miniextract procedure, previously used to characterize splicing extracts depleted in vivo of U1 and U2 snRNPs (29).

Heating *prp6* and *prp9* mutant strains for 1 h at the nonpermissive temperature prior to preparing extracts (subsequently referred to as heated extracts) dramatically inhibited in vitro splicing (Fig. 1). Two independent *prp9* mutants (lanes 3 and 4; but see legend to Fig. 1) and four independent *prp6* mutants (lanes 5 to 8) were examined; all had similar phenotypes. Isogenic strains were created by transforming the original *prp9* and *prp6* mutant strains with the *PRP9* and *PRP6* genes, respectively (16a). Heated extracts from these two strains spliced well (lanes 1 and 2), indistinguishably from standard wild-type strain-derived extracts that are insensitive to heating for 1 h at 37°C (data not shown). Heated extracts from strains transformed only with the YCP vector (lanes 3 and 5) were, as expected, indistinguishable from the mutant extracts.

The effects of the mutants on splicing were specific, since inactive extracts from the two mutants complemented well in vitro. All extracts of *prp6* strains complemented all extracts from *prp9* strains (Fig. 1, lanes 10 to 14). In contrast, inactive extracts from independent mutations of a single gene failed to complement (lanes 9 and 15 to 17). The data indicate that heating of the cells prior to extract preparation inactivates splicing in a mutant-specific fashion.

To look for possible mutant effects on spliceosome assembly, complexes formed in the different extracts were compared by nondenaturing electrophoresis. As previously shown (29), U1 snRNP is required for the subsequent addition of U2 snRNP. In the absence of U1 snRNP, no snRNP-containing splicing complexes were detectable; in the absence of U2 snRNP, the U1 snRNP-containing commitment complexes predominated (Fig. 2A, lanes 2 and 3). The *prp9* heated extract was unable to form significant quantities of U2 snRNP-containing spliceosomes (lane 5). As a consequence, the phenotype was identical to that observed in a U2 snRNP-depleted extract (compare lanes 3 and 5). The effect was specific because the isogenic control wild-type strain showed no phenotype (lane 4), other mutant genes showed different effects (e.g., Fig. 3), and heating was required to manifest a robust phenotype.

These observations suggest that the *PRP9* product might add to, or interact with, the commitment complex subsequent to its formation. Alternatively, the *PRP9* gene product might be a component of the commitment complex; i.e., a *prp9* mutant complex would be unable to interact productively with a factor or factors required for U2 snRNP addition (perhaps even U2 snRNP itself). In this case, commitment complexes formed in a *prp9* extract should be unable to progress to spliceosomes upon the subsequent addition of a *PRP9*-containing extract.

To distinguish between these two possibilities, a chase experiment was performed (Fig. 2B). Commitment complexes formed in a *prp9* heated extract were assessed for their ability to progress along the spliceosome pathway after

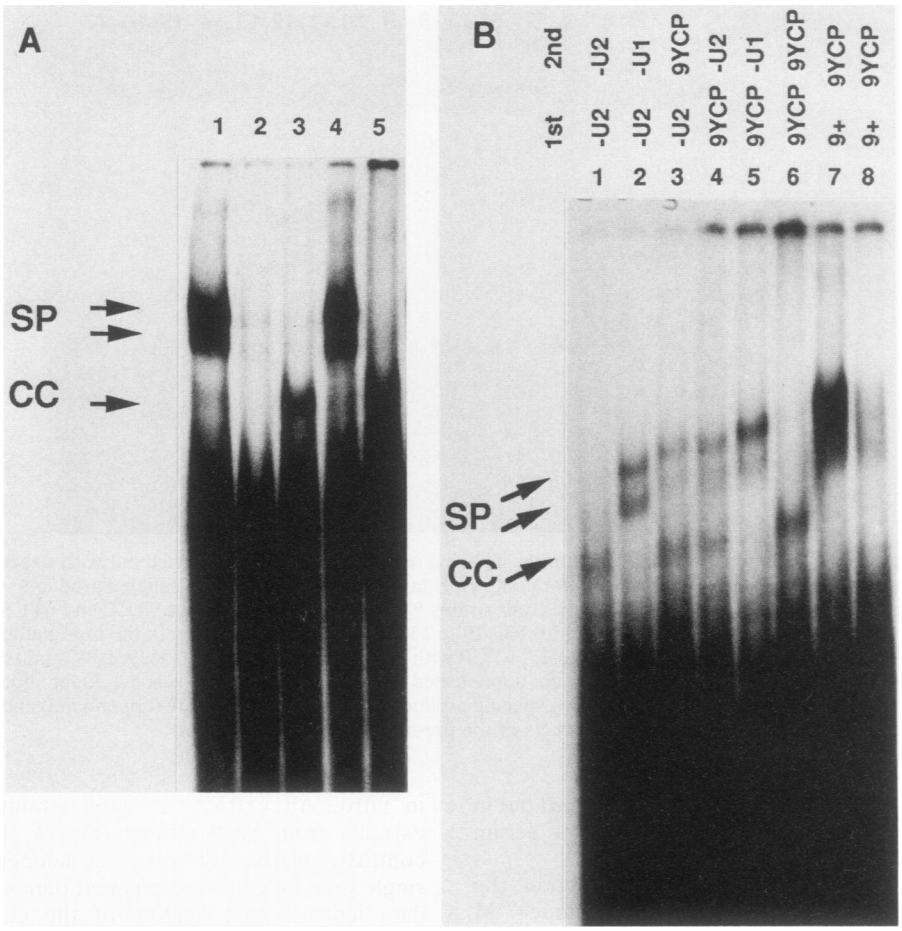

FIG. 2. Native gel electrophoretic analysis of splicing complexes. (A) *prp9* mutant extracts accumulate commitment complex. Standard splicing reactions were carried out as described in the legend to Fig. 1 and analyzed by electrophoresis on native gels as described in Materials and Methods. Lanes: 1, wild-type extract; 2, U1-depleted extract; 3, U2-depleted extract; 4, 9^+; 5, 9YCP. The arrows show the positions of commitment complex (CC) and spliceosomes (SP). (B) The commitment complexes formed in a *prp9* extract are chased into spliceosomes. Two-step reactions were performed as described in Materials and Methods. The extracts used for the first and second steps are indicated above the lanes. In lane 8, a 50-fold molar excess of cool substrate was added together with the hot substrate as a control.

the subsequent addition of different extracts. As previously described (18, 29), an excess of low-specific-activity radioactive substrate was added before the second extract. By diluting the specific activity of the uncommitted pre-mRNA substrate, this protocol restricts the analysis to substrate that is committed to the splicing pathway during the first incubation, i.e., to the commitment complexes formed with the high-specific-activity substrate. The data indicate that commitment complexes formed in a *prp9* heated extract are indistinguishable from those formed in a U2 snRNP-depleted extract, as both commitment complex populations were well chased by the addition of a U1 snRNP-depleted extract (Fig. 2B, lanes 2 and 5). The result suggests that PRP9, like U2 snRNP, acts after formation of the U1 snRNP-containing commitment complex.

The *prp9* heated extract was somewhat effective in chasing the commitment complexes formed in a U2 snRNP-depleted extract (Fig. 2B, lane 3). Similarly, a U2 snRNP-depleted extract was somewhat effective in chasing the commitment complexes formed in a *prp9* heated extract (lane 4). These results can be contrasted with the completely ineffective readdition of the same extract for the second incubation (lanes 1 and 6). The data suggest that the *PRP9* product and U2 snRNP complement poorly, perhaps reflecting an interaction among these two components.

In contrast to the dramatic assembly phenotype of the heated *prp9* extract, the heated *prp6* extract showed no qualitative assembly phenotype (Fig. 3, lane 3). Although less spliceosome is apparent (lane 3), this result is frequently obtained with a variety of mutant extracts with no readily interpretable pattern (data not shown). The absence of an assembly phenotype suggests that the *prp6* mutants do not affect spliceosome assembly or that there are aspects of the assembly pathway not readily detected by these procedures. An indication that the latter consideration may be relevant is the finding that heated extract from other well-characterized mutant snRNP extracts also had no qualitative phenotype (lanes 4 to 7). These two mutants, *prp8* and *prp4*, have been shown to affect U5 snRNP and U4/U6 snRNP, respectively (1, 3, 19). Since these mutant extracts failed to splice and complement each other well (they also complemented other mutant extracts; data not shown), it may be that these procedures do not distinguish between U2 snRNP-containing complexes and those that contain U2 snRNP plus additional snRNPs, e.g., U5 snRNP and U4/U6 snRNP or U5 snRNP and U6 snRNP.

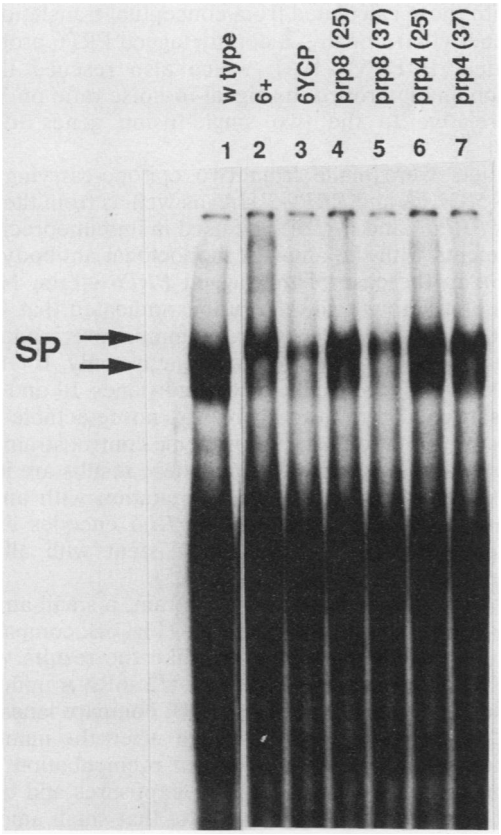

FIG. 3. Splicing complexes formed in *prp6*, *prp4*, and *prp8* mutant extracts. Standard splicing reactions were carried out and analyzed as for Fig. 2A. Lanes: 1, wild-type extract; 2, 6^+; 3, 6YCP; 4, SpJ8.31 extract prepared from cells grown at 25°C (permissive temperature); 5, same as lane 4 except that cells were grown for 1 h at 37°C before extract preparation; 6, SpJ4.41 extract (25°C); 7, SpJ4.41 extract (37°C). SP, Spliceosomes.

Since all of the described spliceosome complexes that follow U2 snRNP addition contain U5 and U4/U6 snRNPs or U5 and U6 snRNPs (2, 13, 22), we decided to test the effect of the *prp6* and *prp9* mutations on U4/U6 snRNP addition, as well as on U2 snRNP addition, by an independent method. A biotinylated substrate was added to different extracts, and the snRNAs associated with the substrate were assayed by selection on streptavidin-agarose and primer extension (Fig. 4). Although primers complementary to three regions of U5 were tested in this assay, none of them yielded a detectable signal at the concentration of RNA selected in this assay. In fact, weak signals could be detected only if 10 μg of total yeast RNA was used (data not shown), a result consistent with the extensive secondary structure of U5 snRNA (10).

U2 snRNP could not bind to the substrate in a heated *prp9* extract (Fig. 4; compare lanes 1 to 3), consistent with the results shown above. As expected from the assembly gels (Fig. 3), neither of the other two heated mutant extracts (*prp6* and *prp4*; lanes 4 and 5) showed an effect on U2 snRNP binding. In contrast, all three heated mutant extracts (*prp9*, *prp6*, and *prp4*; lanes 3 to 5, respectively) showed an effect on U6 snRNP addition compared with the control extract (lane 2); indeed, U6 snRNA levels were indistinguishable from what was recovered from binding to an inactive splicing substrate (compare lanes 3 to 5 with lane 1). In the case of *prp4*, the result serves as a positive control, given that the *PRP4* gene encodes a U4/U6 snRNP protein (1, 3).

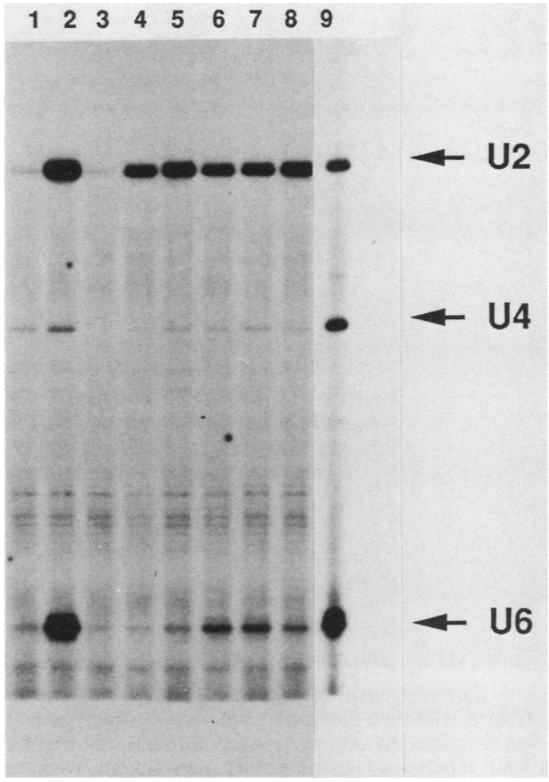

FIG. 4. Analysis of snRNAs in splicing complexes formed with various *prp* mutant extracts. Splicing reactions (50 μl) were carried out with 18 ng of cold biotinylated substrate and 20 μl of extract (lanes 1 to 5) or 10 μl each of two complementing extracts (lanes 6 to 8). The reactions were stopped and incubated with streptavidin-agarose. After proteinase K treatment of the bound complexes, RNA was phenol extracted and used for primer extensions. Primer extensions were performed for U2, U4, and U6 simultaneously, and the products were analyzed on 5% acrylamide–7 M urea gels as described in Materials and Methods. All reactions contained 18 ng of biotinylated Δ2 substrate except that shown in lane 1, which contained 18 ng of a biotinylated substrate of a similar size, derived from the first exon of the *Drosophila per* gene. Lanes: 1 and 2, wild-type extract; 3, 9YCP; 4, 6YCP; 5, *prp4*; 6, 9YCP plus 6YCP; 7, 9YCP plus *prp4*; 8, 6YCP plus *prp4*; 9, 0.5 μg of yeast total RNA.

In vitro complementation of the three mutant extracts increased the amount of recovered U6 snRNA (lanes 6 to 8), similar to what was observed for in vitro complementation of splicing (Fig. 1). Although U4 snRNA could not be successfully assayed because of the relatively high signal recovered from the inactive splicing substrate (lane 1), the results indicate that both the *prp9* and *prp6* mutations prevent the stable addition of U6 snRNP during spliceosome assembly. In the former case, this is presumably an indirect consequence of the absence of U2 snRNP binding, required for the subsequent binding of U4/U6 and U5 snRNPs.

The experiments shown in Fig. 2 to 4 indicate that the *prp9* mutation prevents stable U2 snRNP binding and that the *prp6* mutation prevents stable U6 snRNP (presumably stable U4/U6-U5 snRNP) binding. However, they do not indicate whether the wild-type gene products are snRNP proteins (for example, a U2 snRNP protein in the case of the *PRP9* product and a U4/U6 snRNP protein in the case of the *PRP6* product) or whether they are non-snRNP factors required at these two different stages of spliceosome assembly. To examine this issue, a centrifugation step was used to sepa-

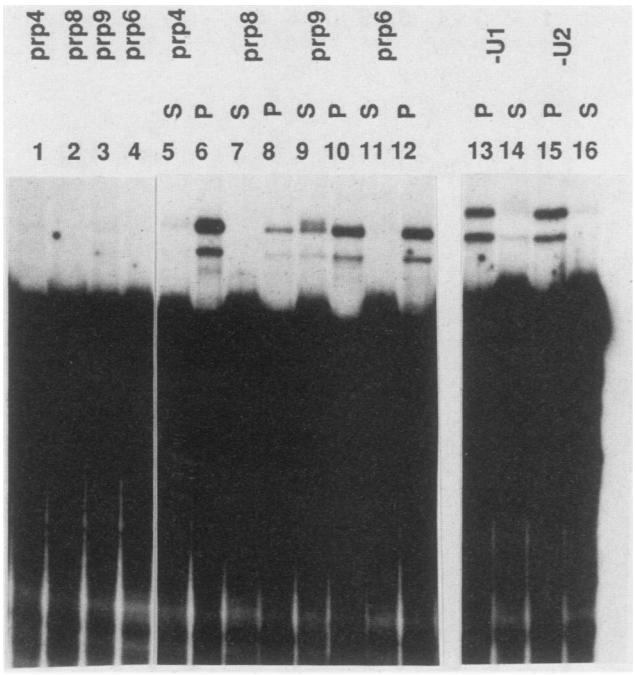

FIG. 5. Complementation of mutant extracts with fractions derived from a wild-type extract. A wild-type extract from BS-Y46 cells was fractionated as described in Materials and Methods into pellet and supernatant fractions. These fractions were used to complement standard 10-μl splicing reactions containing 2 μl of the indicated extracts, and the products were analyzed on 15% acrylamide–7 M urea gels. Lanes: 1 to 4, 4 μl of the indicated extracts; 5 to 16, 2 μl of the respective extract and equivalent amounts of pellet (P) or supernatant (S) fractions as indicated. -U1 and -U2 refer to extracts prepared from BS-Y82 and BS-Y88 grown for 16 h in glucose, thereby depleted of U1 and U2 snRNAs.

rate a wild-type extract into two fractions, an snRNP pellet fraction and a non-snRNP supernatant fraction (Fig. 5).

The fractionation was successful in the sense that only the pellet fraction complemented snRNP-defective extracts, i.e., a U1 snRNP-depleted extract, a U2 snRNP-depleted extract, a heated *prp4* (U4/U6 snRNP mutant) extract, and a heated *prp8* (U5 snRNP mutant) extract (Fig. 5, lanes 13, 15, 6, and 8, respectively). Similarly only the pellet fraction complemented the heated *prp6* mutant extract (lane 12), consistent with the idea that *PRP6* might encode an snRNP protein. The results were less straightforward for *prp9* (lanes 9 and 10), since both the pellet and supernatant fractions complemented this extract.

To examine snRNP association of the PRP6 and PRP9 proteins in a more direct way, the PRP6 and PRP9 proteins were epitope tagged to make them accessible to existing antibody reagents (6, 7; P. Kolodziej and R. A. Young, Methods Enzymol., in press). A nine-amino-acid epitope from the HA influenza virus (31) was added to the amino-terminal and carboxy-terminal regions of both the *PRP9* and *PRP6* coding regions. Both *PRP9* fusion genes (*PRP9*-5′HA and *PRP9*-3′HA) rescued the *prp9* mutation, and both *PRP6* fusion genes (*PRP6*-5′HA and *PRP6*-3′HA) rescued the *prp6* mutation. Western blotting experiments with yeast strains carrying the tagged proteins revealed a PRP6-5′HA and a PRP6-3′HA band of approximately 98 kDa and a PRP9-3′HA band of approximately 55 kDa (and no band in the case of PRP9-5′HA, presumably because of proteolytic digestion of the epitope). These apparent molecular masses are consistent with those calculated from conceptual translation of the two genes (16a). Finally, a doubly tagged PRP6 protein was generated (PRP6-5′-3′HA), which also rescued the *prp6* mutation and improved the signal-to-noise ratio on Western blots relative to the two single-fusion genes (data not shown).

Extracts were made from two epitope-carrying strains (*PRP6*-5′-3′HA and *PRP9*-3′HA) as well as from the control strains *PRP6*$^+$ and *PRP9*$^+$ and used in immunoprecipitation experiments with the anti-HA monoclonal antibody 12CA5 (Fig. 6). In the case of the tagged *PRP6* strain, Northern (RNA) blotting with snRNA probes indicated that U4, U5, and U6 snRNAs were specifically immunoprecipitated (Fig. 6A, lane 9). At higher ionic strength, only U4 and U6 snRNAs were specifically recovered (lanes 10 and 11). At these salt concentrations, there was no detectable snRNA immunoprecipitation from the isogenic control strain lacking the tagged epitope (lanes 2 to 6). These results are identical to those reported for immunoprecipitation with anti-PRP4 reagents (1, 3) and suggest that *PRP6* encodes a U4/U6 (perhaps U4) snRNP protein, consistent with all of the previous characterization (Fig. 3 to 5).

In the case of the tagged *PRP9* strain, a small amount of U2 snRNA was immunoprecipitated (Fig. 6B; compare lanes 9 and 10 with lanes 4 and 5). Unlike the results with the tagged *PRP6* protein (Fig. 6A), this U2 snRNA immunoprecipitation was salt sensitive (Fig. 6B; compare lanes 11 and 10). Identical results were obtained when the immunoprecipitations were carried out without preincubation or ATP addition (data not shown). Longer exposures and quantitation of these and other data indicate that small amounts of the other snRNAs were also immunoprecipitated specifically from the tagged *PRP9* strain.

If PRP9 is associated with U2 snRNP during spliceosome formation, the anti-HA monoclonal antibody might immunoprecipitate the pre-mRNA substrate from a tagged *PRP9* extract. To test this possibility, radioactive pre-mRNA was incubated in a *PRP9*-tagged extract with or without prior U2 snRNA inactivation by oligonucleotide-directed RNase H digestion (Fig. 7, lanes 8 and 11). The immunoprecipitation results show that PRP9 was associated with pre-mRNA in a U2 snRNA-dependent manner. Similar results were obtained with a *PRP6*-tagged extract (lanes 10 and 12). Combined with the assembly phenotype of the heated *prp9* extract, these data indicate that the PRP9 protein associates with U2 snRNP and the pre-mRNA substrate at an early stage of spliceosome assembly.

DISCUSSION

The data presented in this report lead to the conclusion that the PRP6 protein is a U4/U6 snRNP protein and that the PRP9 protein is required for U2 snRNP addition during spliceosome assembly. The *PRP9* gene product is the first identified yeast protein that interacts, directly or indirectly, with U2 snRNP.

Our approach was similar to that previously used to analyze extracts derived from temperature-sensitive splicing mutants, namely, to compare active and inactivated extracts (4, 20) and to use antibody reagents directed against the splicing factors to identify associated snRNAs (1, 3, 19). However, a number of modifications were introduced to facilitate and improve existing procedures. Most notably, we heated the cells prior to extract preparation rather than heating the extracts made at permissive temperatures. This strategy was dictated by two considerations. First, we could

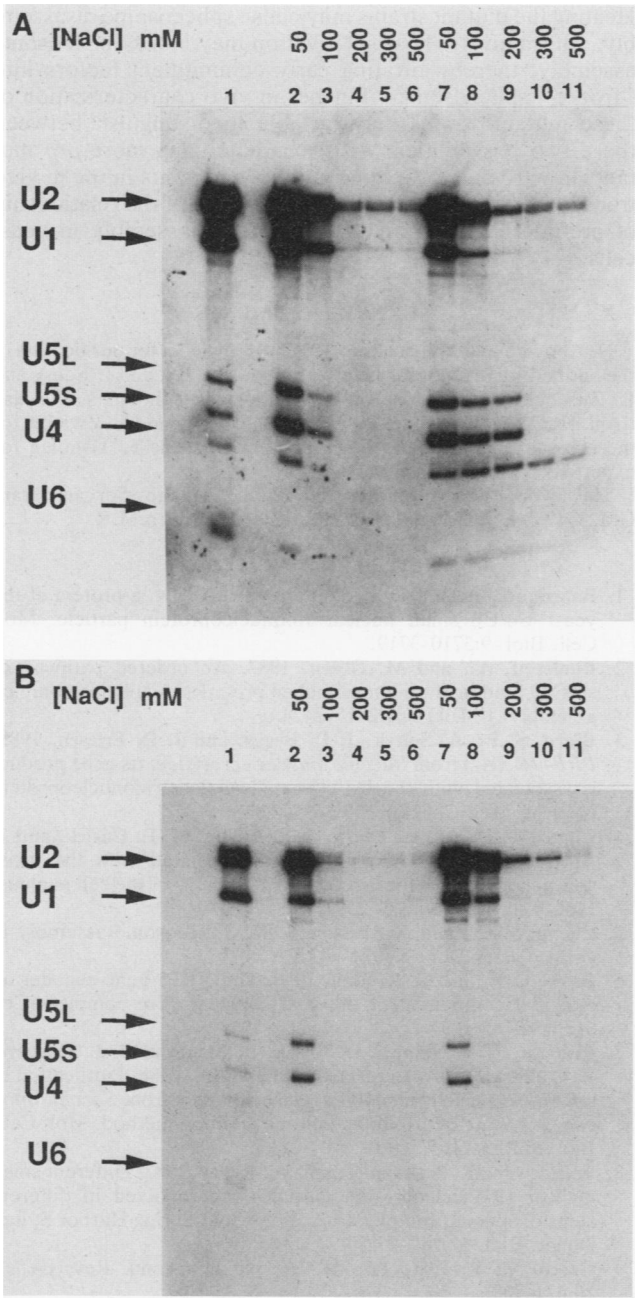

FIG. 6. Immunoprecipitation of snRNPs associated with PRP6 and PRP9. Preincubation of the extract and immunoprecipitations are described in Materials and Methods. The equivalents of 10 μl of extract were immunoprecipitated at the salt concentrations indicated above the lanes. The immunoprecipitated complexes were treated with proteinase K and phenol extracted. The RNAs were separated on 4% acrylamide–7 M urea gels, which were electroblotted and probed with labeled snRNA probes as described in Materials and Methods. (A) U4/U6 snRNPs coprecipitated with PRP6-HA. Lanes: 1, 0.5 μg of yeast total RNA; 2 to 6, extract derived from the *PRP6*$^+$ strain; 7 to 11, extract derived from the *PRP6*-5′-3′HA extract. (B) snRNP coprecipitated with PRP9-3′HA. Lanes: 1, 0.5 μg of yeast total RNA; 2 to 6, extract derived from the *PRP9*$^+$ extract; 7 to 11, extract derived from the *PRP9*-3′HA-tagged extract.

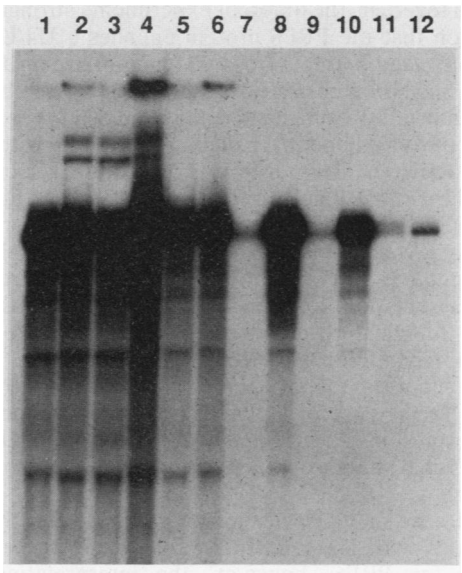

FIG. 7. Immunoprecipitation of pre-mRNA associated with PRP9 and PRP6. Splicing reactions and immunoprecipitations were carried out as described in Materials and Methods, and the RNA was analyzed on a 15% acrylamide–7 M urea gel. Lanes: 1 to 6, aliquots of the reaction mixtures before immunoprecipitation; 7 to 12, the immunoprecipitated samples; 1 to 4, standard splicing reactions with *PRP9*$^+$, *PRP9*-3′HA, *PRP6*$^+$, and *PRP6*-5′-3′HA, respectively; 5 and 6, the same extracts as in lanes 2 and 4 except that U2 snRNP had been predigested with a complementary oligonucleotide.

use the same simple protocol for many different temperature-sensitive mutants rather than develop a mutant-specific protocol (with variations of time, temperature, salt concentration, etc.) for each strain. Second, inactivation should be more complete and, importantly, might more accurately reflect the mechanism by which the mutation inactivates splicing in vivo upon a shift to 37°C. Indeed, these protocols permitted us to study two mutants, *prp6* and *prp9*, that had previously been refractory to specific heat inactivation in vitro (20).

Additionally, a glass bead miniextract procedure was used with which large numbers of extracts from multiple strains or from cells manipulated physiologically prior to extract preparation could be more easily processed (29). It is very likely that the later events of spliceosome assembly are less easily visualized by this approach, as we have not been able to distinguish by gel electrophoresis splicing complexes that contain U2 snRNP from those that contain U2 snRNP plus the snRNPs that are added subsequently, U4/U6 and U5 snRNPs. The inability to visualize the addition of the late snRNPs is probably related to the extract preparation protocol rather than the electrophoresis assay, because splicing is also significantly reduced in these miniextracts relative to what is commonly observed in large extracts (data not shown). We suspect that these miniextracts contain a relatively low activity of a late factor, necessary for the stable addition of U4/U6 and U5 snRNPs to the spliceosome (perhaps a low activity of one of these two snRNPs). This hypothesis explains all of the relevant observations: the low splicing activity in these extracts, the inability to visualize the addition of these late snRNPs by electrophoresis, and the inability to detect an assembly pattern in heated extracts different from that in mutants deficient in one of these late snRNP activities.

Taken together, the different experiments strongly support the notion that the PRP6 protein encodes a U4/U6 snRNP protein. Heated extracts from this mutant are unable to add the late snRNPs yet carry out the earlier assembly events indistinguishably from heated isogenic wild-type extracts. PRP6 activity is found in a pellet fraction along with all of the snRNP activities. The tagged PRP6 protein associates with pre-mRNA in a U2 snRNP-dependent fashion. Finally, the tagged PRP6 protein immunoprecipitates U4/U6 snRNA. At relatively low salt concentrations, U5 snRNA is also immunoprecipitated, an observation that presumably reflects the known association between U4/U6 snRNP and U5 snRNP (5, 13). Identical results have been reported for snRNA immunoprecipitation with the PRP4 protein (1, 3), which further supports the assignment of PRP6 to the U4/U6 snRNP. In the case of PRP4, the characterization suggests that the protein is principally associated with U4 snRNP (33). Detailed studies of this kind remain to be carried out for PRP6.

In the case of PRP9, the results suggest that the protein cooperates with U2 snRNP. In contrast to the conclusion reached for PRP6, however, the interpretation is less straightforward. The heated PRP9 extracts are unable to add U2 snRNP to the U1 snRNP-containing commitment complexes, suggesting that either U2 snRNP or an activity required for stable U2 snRNP addition is inactivated. Such an activity might normally interact with the substrate prior to U2 snRNP addition (i.e., the preferred mechanism for a U2AF-like activity [26, 34]). Alternatively, it might be associated with U2 snRNP prior to the U2 snRNP-substrate interaction (e.g., as an integral U2 snRNP protein), or it might interact with and be required to stabilize the U2 snRNP-substrate complex. As there is a substantial amount of PRP9 activity that does not pellet with snRNPs at 0.3 M KCl, it is unlikely, in our view, that PRP9 is a tightly associated snRNP protein like PRP6, operationally defined by its salt-insensitive association with snRNA. This interpretation is substantiated by the partial in vitro complementation of a heated *prp9* extract with a U2-depleted extract; were PRP9 a permanent, tightly associated U2 snRNP protein, there should be essentially no activity in the U2-depleted extract. Consistent with this view is the relatively salt sensitive nature of the U2 snRNA immunoprecipitation with the tagged PRP9 protein (Fig. 6B).

The U2 snRNA dependence of the substrate immunoprecipitation (Fig. 7) argues against a PRP9 activity that interacts with the substrate prior to U2 snRNP. A similar situation might obtain in mammalian cells for U2AF-substrate-U2 snRNP interactions, despite in vitro experiments demonstrating that U2AF can interact with the substrate prior to U2 snRNP addition (34). We cannot exclude the possibility that U2 snRNP binding stabilizes, or exposes the epitope on, an already formed PRP9–pre-mRNA complex. In any case, the dramatic assembly phenotype of the heated extract suggests that a PRP9-U2 snRNP interaction is required to potentiate U2 snRNP activity.

Given this characterization, how do we interpret the strong in vivo phenotype that caused us to focus on these two gene products? Consistent with another study (16a), these two mutants probably cause pre-mRNA transport because they interfere with spliceosome assembly. However, the data show that the *PRP9* and *PRP6* gene products both act in vitro after formation of the U1 snRNP-containing commitment complex, indicating that a consistent relationship between commitment in vitro and early commitment in vivo is unlikely. If these two gene products are also relatively late factors in vivo, there are two simple possibilities to account for their strong pre-mRNA transport phenotype. Heating the mutant strains may cause spliceosome disassembly. Alternatively, heat inactivation may block spliceosome assembly, thereby titrating early commitment factors into "frozen" spliceosomes. Further in vivo characterization of these mutant strains may be able to distinguish between these two possibilities. Also, characterizing more *prp* mutants in vitro and examining more *prp* mutants in the in vivo transport assay may give a clearer picture of the relationship of pre-mRNA transport to spliceosome assembly in yeast cells.

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