Four yeast spliceosomal proteins (PRP5, PRP9, PRP11, and PRP21) interact to promote U2 snRNP binding to pre-mRNA

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We have analyzed the functions of several pre-mRNA processing (PRP) proteins in yeast spliceosome formation. Here, we show that PRP5 (a DEAD box helicase-like protein), PRP9, and PRP11 are each required for the U2 snRNP to bind to the pre-spliceosome during spliceosome assembly in vitro. Genetic analyses of their functions suggest that they and another protein, PRP21, act concertedly and/or interact physically with each other and with the stem–loop IIa of U2 snRNA to bind U2 snRNP to the pre-mRNA. Biochemical complementation experiments also indicate that the PRP9 and PRP11 proteins interact. The PRP9 and PRP11 proteins may be functioning similarly in yeast and mammalian cells. The requirement for ATP and the helicase-like PRP5 protein suggests that these factors might promote a conformational change (involving either the U1 or U2 snRNP) that is required for the association of U2 snRNP with the pre-mRNA.

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A key to understanding the mechanism and regulation of nuclear precursor messenger RNA (pre-mRNA) splicing lies in discovering the functions of numerous trans-acting factors. These factors can be grouped into two classes: the small nuclear ribonucleoprotein particles (snRNPs)—U1, U2, U4/U6, and U5—and a multitude of non-snRNP factors (for review, see Green 1991; Guthrie 1991; Ruby and Abelson 1991; Brown et al. 1992; Rymond and Rosbash 1992; Moore et al. 1993). The snRNPs and some non-snRNP factors assemble on the pre-mRNA to form the spliceosome on which splicing occurs. U1 snRNP binds first to the pre-mRNA, followed by the U2 snRNP and, finally, by the tri-snRNP U4/U5/U6 particle. Some non-snRNP factors may become integral components of the spliceosome, whereas others may only loosely or transiently associate with the snRNPs and/or the spliceosome. The functions of these non-snRNP factors are particularly intriguing as their elucidation may lead to our understanding of why the pre-mRNA splicing apparatus is so complex and requires ATP.

Pre-mRNA splicing occurs in two transesterification reactions, which are mechanistically the same as those of the group II self-splicing introns (for discussion, see Weiner 1993). As the group II selfsplicing introns require no nucleotide or protein cofactors in vitro (for review, see Jacquier 1990), it is thought that pre-mRNA splicing is likely to be catalyzed by RNA as well. The spliceosomal small nuclear RNAs (snRNAs) may have this function. Thus, it is a quandary as to the functions of the numerous snRNPs and non-snRNP proteins in pre-mRNA splicing. Some non-snRNP factors such as the mammalian ASF/SF2 (Ge et al. 1991; Krainer et al. 1991) and SC35 (Fu and Maniatis 1992), and the Drosophila transformer (Tra; Tra2 (Tian and Maniatis 1992), and sex-lethal (Sxl) (Baker 1989) proteins function in the recognition and selection of introns and splice sites. One non-snRNP protein has been proposed to regulate the fidelity of splicing (Burgess et al. 1990).

Nearly 40 proteins involved in splicing have been identified genetically in the yeast Saccharomyces cerevisiae (for review, see Guthrie 1991; Ruby and Abelson 1991; Brown et al. 1992; Rymond and Rosbash 1992). Over 20 of these proteins have been found in screens for temperature-sensitive (ts) pre-RNA processing (prp) mutants defective in pre-mRNA splicing. Among the genes sequenced so far, five—PRP2, PRP5, PRP16, PRP22, and PRP28—have deduced amino acid sequences resembling known ATP-dependent RNA helicases (for review, see Schmid and Linder 1992, Wassarman and Steitz 1992). None of these five PRP proteins has yet been shown to...
have RNA unwinding activity, but two have been shown to have RNA-dependent ATPase activity (Schwer and Guthrie 1991; Kim and Lin 1993). It is thought that these putative helicases could unwind secondary structures during splicing or that they could catalyze a more subtle ATP-dependent RNA conformational change. Several base-pairing interactions form between snRNAs (Hans et al. 1992; Sawa and Abelson 1992; Wassarman and Ruby et al. 1987; Cheng and Abelson 1987; Lamond et al. 1988). Such dynamic interactions may explain some of the functions of ATP and the putative helicases.

Many PRP proteins have been shown to be required for spliceosome assembly (Ruby and Abelson 1991; Brown et al. 1992; Rymond and Rosbash 1992; Moore et al. 1993). To understand the functional relationship of some of these proteins, we used a genetic approach to identify proteins that may interact physically or functionally with one another. By a combination of genetic and biochemical assays we have found that four proteins, PRP5, PRP9, PRP11, and PRP21, probably interact physically and/or act concertedly to promote U2 snRNP binding to the pre-mRNA during spliceosome assembly.

**Results**

**Interactions among PRP genes**

We used two in vivo genetic tests to look for potentially interacting proteins. First, we analyzed the phenotypes of haploids with various combinations of two temperature-sensitive prop mutations. Second, we tested the activities of several wild-type PRP genes in suppressing temperature-sensitive mutations in other PRP genes. In the first test, if two proteins act sequentially and/or independently in the same pathway, mutations in their genes may show an epistatic relationship; that is, in a haploid double mutant, the phenotype from only one mutation may be observed [Jarvick and Botstein 1973]. If the proteins act concertedly, are interacting physically, or are present in the same complex, mutations in their genes may act epistatically, additively, or synergistically [Huffaker et al. 1987]. When synergistic interactions occur, the haploid double mutants can be inviable at or extremely sensitive to even the permissive temperature (23°–26°C).

We analyzed the phenotypes of numerous haploid double mutants. To minimize nonspecific effects resulting from unrelated variations in strain genetic backgrounds, we created a set of nearly isogenic haploid strains by repeatedly backcrossing the strains into a single wild-type strain. These haploid temperature-sensitive mutants were then crossed with each other. The resulting heterozygous diploids were sporulated, and the meiotic progeny were germinated at 23°C. The temperature sensitivities of the progeny were determined by replica-plate testing at 26°C, 30°C, 34°C, and 37°C. Most of the crosses gave good spore viability with only a few notable exceptions: the prop3-1 mutation combined with prop4-1, prop4-1 with prop24-1, and various combinations of prop5-1, prop5-3, prop9-1, prop9-2, prop11-1, and prop21-1 mutations (Tables 1 and 2). In these crosses we observed that about one-fourth of the meiotic progeny were inviable or extremely temperature-sensitive. Most of the inviable meiotic progeny germinated and underwent one to several divisions before dying. Complementation analyses were performed on all viable meiotic products of these crosses to confirm the genotypes as well as to deduce the genotypes of the inviable progeny. Whereas most haploid single mutants grew at or below 30°C, the haploid double mutants were dead at either 23°C or 26°C (Tables 1 and 2). We were particularly interested in the combinations involving the prop5, prop9, prop11, and prop21 mutations as the pattern of synergistic interactions suggested that the respective wild-type proteins act at the same step of spliceosome assembly.

While performing these crosses we observed that the PRP9 and PRP11 genes are linked [data not shown]. To study the interactions between these two genes, we constructed a diploid strain homozygous for a prop9 mutation and heterozygous for a HIS3-disrupted prop11 null mutation. Next, we introduced either the wild-type or mutant allele of PRP11 (on a centromeric, URA3-marked plasmid) into the diploids. We then determined the genotypes and phenotypes of the meiotic progeny by replica-plate testing. With the wild-type PRP11 gene, Ura+ His+ meiotic progeny can be obtained [Table 3]. With the mutant prop11-1 allele the Ura+ His+ [prop9-1 prop11-1 and prop9-2 prop11-1 double mutant] progeny germinate and then die. Thus, either the prop9-1 or prop9-2 mutation acts synergistically with the prop11-1 mutation.

We also examined the interactions between several prop mutations and two mutations in the SNR20 gene encoding the U2 snRNA [Fig. 1]. Either the G53A or C62U mutation in stem–loop IIa of the U2 snRNA confers cold sensitivity [Ares and Igel 1990], alters the structure of the U2 snRNA in vivo, and prevents the U2 snRNP from binding to the pre-spliceosome complex [Zavanelli and Ares 1991]. The experimental design for mating the appropriate strains and analyzing their progeny is diagramed in Figure 1B. All haploid meiotic progeny with either a wild-type or mutant U2 are viable, whereas prop5-1 progeny with a mutant U2 are
Table 1. Tetrad analysis of prp crosses

| Cross (b)       | interact? (c) | 4 Viable Spores (a) | 3 Viable Spores (b) | 2 Viable Spores (c) | 1 Viable Spore (d) |
|-----------------|---------------|---------------------|---------------------|---------------------|---------------------|
|                 |               | Total Tetrads 4-0+ 3-1+ 2-2+ total | 3-0+ 2-1+ 1-2+ total | 0-2+ 1-1+ 2-0+ total | 1- 1+ total |
| predicted       |               |                     |                     |                     |                     |
| unlinked (d)    | no            | 24 4 16 4 24        | 0                   | 0                   | 0                   |
| unlinked (e)    | yes           | 24 4 16 4           | 16 16               | 4 4                 | 0                   |
| prp4-1 x prp3-1 | yes (f)       | 15 1                | 1 9 1 11            | 1 1 1 3             | 0                   |
| prp4-1 x prp24-1| yes (f)       | 10 1                | 1 2 2 2             | 1 2 3 6             | 1 1                 |
| prp5-1 x prp9-1 | yes (g)       | 14 1 4 5            | 2 2                 | 2 2 4               | 2 1 3               |
| prp5-1 x prp9-2 | yes (g)       | 12 4 4 8            | 1 1 1               | 1 1 1               | 1 1                 |
| prp5-1 x prp11-1| yes (g)       | 18 1 2 3            | 5 2 7               | 1 1 2 4             | 3 1 4               |
| prp5-1 x prp21-1| yes (g)       | 16 1 4 1 6          | 3 3 1 7             | 1 1 2 1             | 1 1                 |
| prp5-3 x prp9-1 | yes (f)       | 15 1                | 1 2 2 1             | 1 3 4               | 5 3 8               |
| prp5-3 x prp9-2 | yes (f)       | 15 1                | 1 1 8               | 9 2 9               | 2 2 1 3             |
| prp5-3 x prp11-1| yes (f)       | 17 0 2 3            | 4 2 7               | 1 1 2 1             | 6 4 10              |
| prp5-3 x prp21-1| yes (f)       | 15 1 5 1 7          | 6 2 6               | 1 1 2 1             | 0                   |
| prp9-1 x prp21-1| yes (f)       | 16 0 1 7 6          | 1 1 1 3             | 1 4 5               | 1 1                 |
| prp9-2 x prp21-1| yes (f)       | 15 0 9 9 2          | 3 1 5               | 1 1 1               | 1 1                 |
| prp11-1 x prp21-1| yes (f)     | 17 1                | 5 2 7               | 1 4 2 7             | 1 1 2               |

a Mating, sporulation, and germination were at 23°C. The temperature sensitivity (−−) or resistance (++) of the progeny at 37°C was scored.
b A diploid heterozygous for two different prp mutations was made by mating the indicated haploid mutants.
c If a synergistic phenotype is conferred by two prp mutations in the haploid, then the mutations are said to interact.
d Predicted phenotypes of progeny from a diploid heterozygous for two unlinked, noninteracting, temperature-sensitive mutations are indicated.
e Predicted phenotypes of progeny from a diploid with two mutations acting synergistically at 23°C.
f No viable double mutant obtained as detected by complementation tests.
g Double mutant is inviable at 26°C and above.
Table 2. Growth of haploid double mutants

| Gene on URA3-plasmid | Number of tetrads with temperature-sensitive, His⁺ Ura⁺ spore | Total number of tetrads |
|-----------------------|-------------------------------------------------------------|-------------------------|
| prp4-1                | + + + + nt +                                  | 15                     |
| prp5-1                | + + + +                                 | 13                     |
| prp5-3                | + + + +                                 | 14                     |
| prp6-1                | + + + +                                  | 16                     |
| prp6-3                | + + + +                                  | 16                     |
| prp8-1                | + + nt +                                 | 13                     |
| prp9-1                | + + + + +                                | 15                     |
| prp9-2                | + + + + +                                | 15                     |
| prp11-1               | + + + + +                                | 15                     |
| prp16-1               | + + + + nt +                              | 15                     |
| prp17-1               | + + + + +                                | 15                     |
| prp18-1               | + + + + +                                | 15                     |
| prp19-1               | + + + + +                                | 15                     |
| prp20-1               | + + + + +                                | 15                     |
| prp21-1               | + + + + + nt +                           | 16                     |
| prp22-1               | + + + + +                                | 14                     |
| prp24-1               | + + + + + nt +                           | 16                     |
| prp27-1               | + + + + +                               | 15                     |

[+][23] Double mutant is viable; [−26] double mutant is inviable at 23°C; [−26] double mutant is inviable at 26°C; [nt] not tested.

*prp mutations are temperature sensitive. A diploid heterozygous for two different prp mutations was made by mating the indicated haploid mutants. Mating, sporulation, and germination were at 23°C. The temperature-sensitive phenotypes of the progeny and the number of ditypes (4 - 0+ and 2 - 2+) and tetatypes (3 - 1+) from at least 10 tetrads for each cross were scored. (Data available upon request.) The viabilities of the haploid double mutants at the permissive temperature are indicated.

SNR20 encodes the U2 snRNA; G53A and C62U mutations are cold sensitive.

inviable (Experiment 2, Fig. 1C). These patterns of lethality suggest that the PRP5, PRP9, PRP11, and PRP21 proteins act concertedly or interact physically with the U2 snRNA.

We used a second type of genetic test, multicopy suppression, to examine the interaction between the PRP genes. Increasing the copy number of one gene may suppress a conditional mutation in its interacting partner gene. We introduced wild-type copies of the PRP5, PRP9, or PRP11 genes into various haploid prp mutant strains and assayed the temperature sensitivities of the transformed strains. The genes were introduced on either low-copy, centromeric, or high-copy vectors. Although we never observed complete suppression of the temperature sensitivity, we did find that a high-copy number of the PRP9 gene can partially suppress the temperature sensitivity of the prp5-1, prp11-1, or prp21-1 mutant at 30°C or 34°C, temperatures at which these mutants grow.

Table 3. Interactions between prp9 and prp11 mutations

| Genotype of diploid | Number of tetrads with temperature-sensitive, His⁺ Ura⁺ spore | Total number of tetrads |
|---------------------|---------------------------------------------------------------|-------------------------|
| prp9-1/prp9-1, PRP11/prp11::HIS3 | PRP11 10 | 15 |
| prp9-2/prp9-2, PRP11/prp11::HIS3 | PRP11 13 |
| prp9-1/prp9-1, PRP11/prp11::HIS3 | prp11-1 14 |
| prp9-2/prp9-2, PRP11/prp11::HIS3 | prp11-1 16 |
Four interacting yeast spliceosomal proteins

A

\[
\begin{array}{c}
\text{stem-loop IIa} \\
\text{GUAGUA} \\
\text{A\textsuperscript{53CG62U}} \\
\therefore \quad \text{OH} \\
\text{GUAGUA} \\
\text{3ppG-me} \\
\end{array}
\]

B

\[
\begin{array}{c}
\text{LEU2} \quad \text{SNR20} \\
\text{III} - \text{IV} \quad \text{III} - \text{IV} \\
\text{G53A} \quad \text{SNR20} \\
\text{C62U} \\
\text{Prp11-1} \\
\text{sporulate diploid} \\
\end{array}
\]

C

| genotype of heterozygous diploid | number of tetrads |
|----------------------------------|-------------------|
|                                  | with ts, Leu+ Ura+ spor | total |
| prp3-1 (wt G53A C62U) | 10 8 15 | 12 |
| prp4-1 (wt G53A C62U) | 7 14 | 4 |
| prp5-1 (Experiment 1) (wt G53A C62U) | 0 (linked) | 15 |
| prp5-1 (Experiment 2) (wt G53A C62U) | 8 16 | 11 |
| prp9-1 (wt G53A C62U) | 7 14 | 13 |
| prp9-2 (wt G53A C62U) | 7 14 | 15 |
| prp11-1 (wt G53A C62U) | 6 13 | 13 |
| prp21-1 (wt G53A C62U) | 7 16 | 12 |

Figure 1. Interactions between prp and U2 snRNA mutations. (A) The positions of the two cold-sensitive mutations G53A and C62U in the U2 snRNA are indicated. Either mutation alters the distribution of the two forms of U2 snRNA in vivo as shown for the G53A mutation, so that the form on the right is at higher concentrations in the G53A mutant (Zavanelli and Ares 1989). (B) The experimental protocol used to test interactions between a prp mutation and the G53A or C62U U2 snRNA mutation is diagramed here. The U2 snRNA is encoded by the SNR20 gene. One haploid strain has an snr20::URA3 disruption mutation and contains a LEU2-marked plasmid with the mutant (G53A or C62U), or wild-type SNR20 gene. The other haploid has a prp mutation. These two strains are mated, and the resulting meiotic progeny are analyzed by replicating tests. Here, we show the expected results if there is an interaction between the snr20-G53A and prp11-1 mutations: no temperature-sensitive, Ura3+ Leu2+ progeny will be viable. (C) The numbers of progeny with the relevant phenotypes are indicated. The results are also summarized in Table 2. The PRP5 and SNR20 genes were found to be linked in Experiment 1. Additional strains were constructed to test the interactions between these two genes [see Materials and methods] and the results of Experiment 2 are given.
poorly or not at all (Fig. 2). The suppression of the prp21-1 mutation is particularly striking; even a slight increase in copy number of PRP9 can partially suppress the prp21-1 mutation. PRP11 also can partially suppress the prp21-1 mutation. To show this we constructed a diploid heterozygous for a HIS3-disrupted prp9 null mutation and transformed the diploid with a URA3-marked plasmid with either the wild-type PRP9 or PRP11 gene. Subsequently, we obtained 13 of 16, or 0 of 16 tetrads respectively, with His \(^+\) Ura \(^+\) progeny. These results indicate that PRP11 is not functionally replacing PRP9.

In vitro heat inactivation and complementation of a prp9 mutant extract

We then analyzed the functions of the PRP5, PRP9, and PRP11 proteins by biochemical assays. [The in vitro analysis of the PRP21 protein is reported elsewhere [Arenas and Abelson 1993].] One approach to dissecting the function of a protein is to study the in vitro phenotype conferred by temperature-sensitive mutations. Splicing extracts from several temperature-sensitive prp mutants are temperature-sensitive in vitro. However, extract from the prp9-1 mutant is not temperature-sensitive in vitro under the conditions used previously [Lustig et al. 1986]. We therefore sought in vitro conditions to specifically inactivate prp9 extract.

We made active splicing extract from the prp9-1 mutant grown at the permissive temperature and then found two sets of conditions for inactivating the extract in vitro. In one set, the extract is briefly incubated in a low concentration of MgCl\(_2\) at 38°C prior to the splicing assay. The short heat treatments inactivate the prp9 extract and other prp mutant extracts but not control extracts such as that of the prp9-1 revertant, prp9R1 [Fig. 3]. The second set of inactivating conditions is to incubate the splicing reactions at 30°C instead of the normal 23°C. At this temperature, the prp9 extract does not splice pre-mRNAs, whereas the control extracts are active [data not shown]. Incubation at 30°C, however, may not be sufficient to inactivate the extract for subsequent splicing at lower temperatures. The prp9 extract with splicing buffer components but without ATP and pre-mRNA, is resistant to prolonged incubations at 30°C when subsequently assayed for splicing activity at 23°C [data not shown; see also Lustig et al. 1986]. The roles of ATP and pre-mRNA in inactivating the extract at 30°C were not investigated further.

Figure 2. Multicopy suppression of temperature-sensitive prp mutations. [A] Temperature-sensitive mutant prp strains [indicated in italics] were transformed with either a vector plasmid, or high-copy plasmids with the wild-type PRP5, PRP9, or PRP11 genes [indicated by pPRP], or a low-copy plasmid with the PRP9 gene [indicated lo-pPRP9]. [B] Equal numbers of cells of the transformants were plated in the sectors of selective medium. The transformants with a partially suppressed temperature-sensitive phenotype and the prp5-3 transformants with no such phenotype are shown here.
which the PRP5, PRP9, and PRP11 proteins act. Previously, it had been shown that the prp9 mutant or prp9R revertant were inactivated in vitro at 38°C for the times indicated. The splicing assays were then initiated by the addition of splicing buffer components, ATP, and radiolabeled pre-mRNA and incubated for 30 min at 23°C. The pre-mRNA (pre), splicing intermediates [introns–exon 2 (I-E2) and free exon 1 (E1)], and splicing products [mRNA and lariat intron (l)] were extracted, analyzed by denaturing gel electrophoresis, and visualized by autoradiography as shown here. (B) Active splicing extracts from mutants prp5-1, prp6-1, prp9-1, and prp11-1 (lanes 1–4) were heat inactivated in vitro (Δ5, Δ6, Δ9, Δ11; lanes 5–8).

The inactivated extracts were then mixed pairwise in various combinations (lanes 9–14). Splicing assays were initiated by the addition of splicing buffer components, ATP, and radiolabeled pre-mRNA and incubated for 30 min at 23°C. The RNAs were analyzed as in A.

To determine that the in vitro temperature sensitivity is attributable to the temperature-sensitive prp9 mutation and not to nonspecific inactivation of splicing components, we analyzed the effects of the mutation in two ways. First, we determined whether the heat-inactivated prp9 extract could complement inactivated extracts from other mutants. If a distinct component is inactivated in each extract, then different extracts should complement one another. If the components are not exchangeable, then the extracts will fail to complement. Extracts from prp5-1, prp6-1, prp9-1, and prp11-1 mutants were individually heat inactivated and then mixed pairwise and assayed for splicing activity at 23°C (Fig. 3B). Each inactivated extract alone has little or no splicing activity but can complement at least two other extracts. Often such complementation results in recovering more splicing activity than is present in single extracts prior to heat inactivation. One combination of extracts (prp9 and prp11) consistently complement poorly or not at all. This failure could be attributable to either nonspecifically inactivating splicing components or specifically inactivating a nondissociable complex containing the mutant protein.

To test further that the in vitro temperature-sensitive phenotype is attributable specifically to the temperature-sensitive mutation, we isolated revertants of the prp9-1 mutant and analyzed the in vitro phenotypes of each revertant for coreversion to heat resistance. Extracts made from revertants prp9R1 and prp9R2 are resistant to heat inactivation as judged by both splicing and spliceosome assembly assays (for prp9R1 see Figs. 3 and 4; for prp9R2, data not shown).

**Spliceosome assembly**

Previously, it had been shown that the prp5-1 and prp11-1 mutations inhibit spliceosome assembly, but the specific assembly steps affected were not known (Lin et al. 1987). We determined the earliest assembly steps at which the PRP5, PRP9, and PRP11 proteins act. Active or heat-inactivated mutant extracts were assayed for their abilities to form pre-spliceosome and spliceosome complexes as detected by a native gel electrophoretic assay that we developed (S.W. Ruby and J. Abelson, in prep.). The names, snRNP compositions, and order of formation of the pre-spliceosome complexes that we can detect in this assay are the following: 8 (U1 snRNP), B1 (U1/U2 snRNPs), α1 [U2/U4/U5/U6 snRNPs], α2 [U2/U5/U6 snRNPs], and β, the spliceosome [U2/U5/U6 snRNPs]. Heat inactivation of the prp5-1 or prp9-1 extract has no detectable effect on δ complex formation but inhibits U2 snRNP binding and β, formation (Fig. 4, lanes 3, 4, 9, 10, 13, 14) as does inactivation of prp11 extract (data not shown).

In an alternative approach, we analyzed the functions of the PRP5 and PRP11 proteins by an antibody inhibition assay. Pretreatment of active wild-type splicing extracts with either anti-PRP11 antibodies (Fig. 5) or anti-PRP5 antibodies (data not shown) prevents U2 snRNP binding, whereas pretreatment with preimmune sera has no effect. Pretreatment with anti-PRP4 antibody, which we used as another control because it blocks binding of the U4/U5/U6 tri-snRNP (Banroques and Abelson 1989), results in accumulation of the β, complex (Fig. 5). We conclude that the PRP5, PRP9, and PRP11 proteins are required for U2 snRNP to bind to the pre-mRNA to form the β, complex.

**Immunoprecipitation of snRNPs and pre-mRNA with anti-PRP11 antibody**

To further elucidate the role of PRP11 protein in spliceosome formation, we assayed whether PRP11 protein associates with any snRNP or only with the spliceosome. The PRP11 protein was immunoprecipitated with anti-PRP11 antibody from an active, wild-type, whole-cell splicing extract without pre-mRNA. Coprecipitating RNAs were extracted and detected in two ways: They were radiolabeled at their 3' ends (Fig. 6A), or hybridized with snRNA-specific probes (Fig. 6B).
Figure 4. Pre-spliceosome complexes formed in heat-inactivated prp mutant and control extracts. Active and heat-inactivated splicing extracts from mutants prp5-1 (lanes 1–4), and prp9-1 (lanes 9–14) and the revertant prp9R1 (lanes 5–8 and lanes 15–18) were assayed for their snRNP-binding activities. In one set of inactivation conditions [indicated by A,23], the extracts were first inactivated by incubation at 38°C for 10 min and then assayed for spliceosome formation by the addition of splicing buffer components, ATP, and radiolabeled pre-mRNA. The reactions were incubated at 23°C for 15 and 30 min: prp5 (lanes 3,4); prp9 (lanes 13,14); and prp9R1 (lanes 17,18). In the other set of inactivation conditions (assay temperature at 30°C) the splicing assays were initiated by mixing the components and radiolabeled pre-mRNA pre-equilibrated at 30°C for 2 min, and then incubated at 30°C for the times indicated. All reactions were quenched at 0°C. The snRNP/pre-mRNA complexes formed in the assays were resolved by native gel electrophoresis and visualized by autoradiography as shown here. The positions of the snRNP/pre-mRNA complexes are indicated: [U1/pre-mRNA (8), U1/U2/pre-mRNA (β1), U2/U4/U5/U6/pre-mRNA (α1), U2/U5/U6/pre-mRNA (α2)], the spliceosome (f52), and the nonspecific (ns) complexes are indicated. The order of formation of the pre-spliceosome complexes is 8, 13, eq, 3, and (S.W. Ruby and J. Abelson, in prep.). The latter four complexes are not resolved in this assay.

and U6 snRNPs specifically coprecipitate with the PRP11 protein when the immunoprecipitations are performed with low to mildly stringent conditions (50–150 mM salt washes with or without heparin). The U1 snRNP coprecipitates poorly or not at all; heparin, which reduces nonspecific associations of snRNPs in the immunoprecipitates, abolishes U1 snRNP coprecipitation [Fig. 6B]. The levels of coprecipitating U2, U4, U5, and U6 snRNPs decrease with increasing salt concentrations in the immunoprecipitation assay [Fig. 6]. These coprecipitation patterns differ markedly from those in which proteins are integral components of snRNPs. For example, anti-PRP4 antibody precipitates the U4, U5, and U6 snRNAs under the same assay conditions [Fig. 6A]. Furthermore, the PRP4 protein has been shown previously to remain snRNP-associated even in 500 mM NaCl with heparin [Banroques and Abelson 1989]. We conclude that PRP11 does associate with one or more snRNPs but that this association is salt sensitive and may be readily reversed.

Previously, we had shown that PRP11 protein associates with the spliceosome [Chang et al. 1988]. We asked here when it enters the complex as determined by immunoprecipitation assays. One consideration was that endogenous levels of ATP in the extract could be high enough to promote PRP11 binding to the pre-spliceosome if PRP11 binding was ATP dependent. We therefore used conditions to deplete the extracts of ATP (see Materials and methods). Radiolabeled premRNA was added to an active wild-type splicing extract with or without ATP. At various times thereafter, we assayed the reactions for coprecipitation of the pre-mRNA with anti-PRP11 antibody. We find that the pre-mRNA associates with the PRP11 protein in both the absence and

Figure 5. Antibody inhibition of pre-spliceosome complex formation. A wild-type splicing extract was treated with various amounts of anti-PRP11 (αPRP11) or antiPRP4 (αPRP4) antibodies, or the respective preimmune (pre) antibodies. Spliceosome formation assays were then initiated by the addition of splicing buffer components, ATP, and radiolabeled pre-mRNA. After 20 min at 23°C, the reactions were quenched at 0°C and analyzed by native gel electrophoresis. The pre-spliceosome and spliceosome complexes were visualized by autoradiography as shown here. The positions of the δ and β1 complexes are indicated.
Figure 6. Immunoprecipitation of snRNPs with anti-PRP11 antibody. [A] RNAs coprecipitating with the PRP4 or PRP11 proteins from whole-cell splicing extract treated with antibodies were radiolabeled at their 3' ends with [32P]pppCp and T4 RNA ligase and analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography as shown here. Either anti-PRP4 (lane 1), preimmune (lanes 2,4), or anti-PRP11 (lanes 3,5–8) serum was used for the immunoprecipitations. The immunoprecipitates were washed with various salt concentrations as indicated before the RNAs were extracted. In vitro-synthesized, radiolabeled U4, U5long, and U6 RNAs serve as markers. The bands corresponding to ribosomal RNAs, U2, U1, U4, U6, and the two forms of U5 [long and short] RNAs are indicated. The U6 snRNA cannot be seen in immunoprecipitates because it cannot be radiolabeled at its 3' end. [B] RNAs that coprecipitate with the PRP11 protein from whole-cell splicing extract were analyzed by denaturing gel electrophoresis, Northern blot hybridization, and autoradiography as shown here. Either anti- trimethylcap [lane 2], preimmune [lane 3] or anti-PRP11 [lanes 4–16] serum was used for the immunoprecipitations. The immunoprecipitates were washed with various concentrations of salt and heparin as indicated before the RNAs were extracted. The U1, U2, U4, U5long, U5short, and U6 snRNAs were detected by simultaneous hybridization with the snRNA-specific probes. [There was less U4 snRNA probe used relative to the other probes in this particular assay; therefore, less U4 snRNA than normal was detected. Usually we detect U4 snRNA when we detect the other snRNAs in this assay.] Total RNA extracted from whole-cell splicing extract was used for markers [lane 1].

presence of ATP [Fig. 7A]. This suggests that PRP11 protein is in the earliest detectable pre-spliceosome complex, the ~ complex, as the U1 snRNP is the only snRNP that binds to the pre-mRNA in the absence of ATP [Binderereiff and Green 1987; Ruby and Abelson 1988; Rosbash and Seraphin 1991]. Pre-mRNAs that are not active splicing substrates and bind reduced amounts of U1 snRNP [Ruby and Abelson 1988] coprecipitate poorly with the PRP11 protein [Fig. 7B]: The mean level of coprecipitating pre-mRNA with the branch point region mutation (UACaAAC) was only 25% [n = 3, S.D. = 6.3] that of wild-type pre-mRNA. The mean level of copre-

Figure 7. Immunoprecipitation of pre-mRNAs with anti-PRP11 antibody. [A] Radiolabeled wild-type pre-mRNA was added to splicing assays without [lanes 1–5,11] or with [lanes 6–10,12] ATP. At various times thereafter, the assays were immunoprecipitated with either anti-PRP11 [anti-11, lanes 1–10] or preimmune [pre, lanes 11,12] sera. Coprecipitating RNAs were extracted and analyzed by denaturing gel electrophoresis and autoradiography as shown here. Only significant levels of pre-mRNA were immunoprecipitated, splicing intermediates and products were not detectable above background [preimmune] levels. Analysis of the RNAs in parallel splicing reactions with ATP showed that the pre-mRNA was spaced [data not shown]. [B] Radiolabeled wild-type [WT or WTΔ6] or mutant [UAcA] pre-mRNA was added to splicing assays with ATP. After 5 or 10 min of incubation, the RNAs were analyzed as in A. The WTΔ6 pre-mRNA has the 6-nucleotide cryptic branchpoint sequence deleted but splices as efficiently as wild-type pre-mRNA [Vijayraghavan et al. 1986]. The mutant [UAcA] pre-mRNA is not an active splicing substrate: It binds reduced amounts of U1 snRNP [Ruby and Abelson 1988] and forms reduced amounts of the ~ complex [S.W. Ruby and J. Abelson, in prep.]. The WTΔ6 and mutant pre-mRNA have an exon 2 that is 100 nucleotides shorter than that of wild-type pre-mRNA [WT].
was also only 25% that of wild-type (data not shown). These levels are reduced but are still above the background levels (7% of the wild-type pre-mRNA; n = 5, s.d. = 5) that we obtained with a control RNA lacking an intron. We do not precipitate significant levels of splicing intermediates or products (Fig. 7A; lanes 8,9). Therefore, PRP11 may leave the spliceosome preceding the first splicing reaction, or the epitope may become inaccessible to the antibody at that time.

Discussion

Genetic evidence that PRP5, PRP9, PRP11, and PRP21 proteins interact functionally or physically

Potentially interacting proteins in several other cellular processes such as secretion (Salminen and Novick 1987; Rothblatt et al. 1989), translation [Haffter et al. 1991], cytoskeletal assembly [Novick et al. 1989; Stearns et al. 1990], transcription Arndt et al. 1989, DNA replication Hennelly et al. 1991, and morphogenesis [Bender and Pringle 1991] have been initially identified genetically. In four of these cases, it has now been shown by biochemical assays that the genetic interactions predicted actual physical or functional interactions [Adams et al. 1989, Arndt et al. 1989; Deshaies et al. 1991; Rossi et al. 1991]. For example, mutations in the SAC6 and ACT1 genes can suppress each other's defects [Adams and Botstein 1989], and the SAC6 protein binds to polymerized actin [Adams et al. 1989].

We found that the prp5, prp9, prp11, and prp21 mutations act synergistically with each other but not with other prp mutations such as prp16-2, prp17-1, and prp18-1. These latter three mutations have been shown to result in defects in the second reaction step of splicing [for review, see Guthrie 1991; Ruby and Abelson 1991; Brown et al. 1992]. Similarly, the mutations prp16-2, prp17-1, and prp18-1 have synergistic effects among each other but not with prp mutations that act at earlier or later steps [Frank et al. 1992]. This specificity argues that the synergisms we observe in the haploid double mutants are attributable to specific functional or physical interactions among the encoded gene products. The fact that the four genes, PRP5 [Dalbadie-McFarland and Abelson 1990], PRP9 [Legrain and Choulika 1990], PRP11 [Chang et al. 1988], and PRP21 [Arenas and Abelson 1993] are unique and encode structurally distinct proteins, makes it unlikely that their functions are redundant.

The specificity of the interactions among the PRP5, PRP9, PRP11, and PRP21 genes is supported further by our finding that high copies of the PRP9 or PRP11 wild-type gene can partially suppress mutations in some of the other genes of this set. The suppression of the prp5-1 and prp21-1 mutations by the wild-type PRP9 gene is particularly noteworthy. PRP9 partially suppresses the prp5-1 but not the prp5-3 mutation, an allele-specific effect, suggesting that elevated PRP9 levels neither bypass PRP5 function nor generally stabilize mutant proteins. While our work was in progress, the SPP91 gene was isolated as a second site suppressor of prp9 mutations [Chapon and Legrain 1992] and subsequently identified as PRP21 [Arenas and Abelson 1993]. SPP91 cannot functionally replace PRP9 [Chapon and Legrain 1992], therefore, the suppressor activities of SPP91 [Chapon and Legrain 1992] and PRP9 [this paper] are not attributable to functional replacement. We also find that the suppressor activity of PRP11 is weaker than that of PRP9 but, nonetheless, significant. A previous study has reported that PRP11 was unable to suppress the prp9-1 mutation [Last et al. 1987]; however, this study did not assay temperature sensitivities at the intermediate temperatures as we have done here. The suppressor activities of PRP9 and PRP11 that we see are consistent with our results that the prp5, prp9, prp11, and prp21 mutations act synergistically with each other and further support our hypothesis that the four proteins are interacting functionally or physically. Recently, possible physical interactions between the PRP9, PRP11, and PRP21 proteins have also been detected in vivo by the two-hybrid transcription activation assay [Legrain et al. 1993; Legrain and Chapon 1993].

We also genetically detected interactions between three proteins [PRP3 (Last and Woolford as cited in Ruby and Abelson 1991), PRP4 (Barroques and Abelson 1989; Bjorn et al. 1989], and PRP24 [Shannon and Guthrie 1991] known to be in the U4/U6 snRNP; either combination of mutations prp3 and prp4 or prp3 and prp24 is lethal in a haploid. We do not detect any interactions between PRP6, another U4/U6 snRNP protein [Abovich et al. 1990], and these three proteins, however, we cannot conclude that PRP6 protein does not interact with these proteins. In some instances, two proteins have been biochemically shown to interact, but these interactions cannot be detected genetically [see, e.g., Bacon et al. 1989 and Salminen and Novick 1989].

Biochemical evidence that the PRP9 and PRP11 proteins interact with each other and with the U2 snRNP

Both the PRP9 [Abovich et al. 1990] and PRP11 proteins are required for U2 snRNP to bind to the pre-spliceosome [Figs. 4 and 5]. Furthermore, the inactivated prp9-1 and prp11-1 extracts do not complement one another even though they do complement other inactivated extracts [Fig. 1B]. Our biochemical and genetic data combined suggest that these two proteins may interact physically in a complex or as parts of two complexes composed of subunits that do not exchange in the inactivated extracts. Our previous biochemical data also suggest that the PRP11 protein is in a complex. The wild-type PRP11 protein complements inactivated prp11-1 extracts only when present during heat inactivation of the mutant extract [Chang et al. 1988]. Also, the wild-type protein associates with a 30S particle in the absence of exogenous pre-mRNA. The size of this particle suggests that it could contain one or more snRNPs. Perhaps one of these is the U2 snRNP [see below].
Additional support for the idea that the PRP9 and PRP11 proteins are in a complex comes from studies of mammalian splicing factors. We were excited to hear that the mammalian homologs of PRP9 [A. Kramer, pers. comm.] and PRP11 [Bennett and Reed 1993] may have been identified. The two mammalian proteins are present in the mammalian pre-spliceosome complex A containing the U1 and U2 snRNPs and in the spliceosome [Bennett and Reed 1993]. They also comprise two of the three subunits of mammalian splicing factor SF3a (Brosi et al. 1993a). SF3a is required for the formation of pre-spliceosome complex A [Kramer and Utans 1991; Brosi et al. 1993b]. The biochemical characterization of SF3a is particularly pertinent to our suggestion that PRP9 and PRP11 are in a complex together, where they may interact physically. The fact that the three SF3a subunits copurify through many purification steps (Brosi et al. 1993a) is a good indication that these three proteins interact physically. It seems likely that PRP9 and PRP11 are part of the yeast homolog of SF3a. Either PRP5, PRP21, or some as yet unidentified protein could be the third subunit of the yeast SF3a.

We have found that the same subset of mutations (prp5, prp9, prp11, and prp21) that interact with each other also act synergistically with each other in the stem–loop IIa of the U2 snRNA. Furthermore, additional genetic studies have revealed that some, but not all, mutations in U2 also have synergistic effects with this subset of prp mutations [S. Fischer-Wells and M. Ares, pers. comm.]. These genetic data are consistent with other recent biochemical studies indicating that PRP9 and PRP11 proteins interact physically, and that they interact physically with PRP11 in the pre-spliceosome β, complex. These physical interactions, as well as the stoichiometries of the PRP9 and PRP11 proteins in the pre-spliceosome complexes, remain to be established.

We have found that PRP11 associates with pre-mRNA that are active substrates for splicing but associates poorly with mutant pre-mRNAs that do not splice and bind reduced amounts of U1 snRNP. Perhaps PRP11 binding depends on the U1 snRNP; however, we could not detect here any direct association of PRP11 with the U1 snRNP. The U1 snRNP did not precipitate with anti-PRP11 antibody in the absence of pre-mRNA [Fig. 6]. Other experiments are needed to establish whether PRP11 is binding to the pre-mRNA and/or if its binding is dependent on other factors.

Roles of PRP5 and ATP in spliceosome assembly

Here, we have shown that PRP5 protein is required for U2 snRNP to bind to the pre-mRNA to form the pre-spliceosome β, complex containing the U1 and U2 snRNPs. U2 snRNP binding is the first step in the spliceosome assembly pathway that requires ATP hydrolysis [Liao et al. 1992]. The function of the PRP5 protein may explain the roles for ATP at this step. As a member of the DEAD-box protein family [Dalbadie-McFarland and Abelson 1990], PRP5 closely resembles the translation initiation factor elf4A that has been shown to have RNA-dependent ATPase and RNA helicase activities [for review, see Wassarman and Steitz 1991; Schmid and Linder 1992]. elf4A binds to the mRNA during translation initiation, unwinds helices upstream of the AUG codon, and thereby allows the 40S ribosomal subunit to bind to the mRNA [for review, see Linder and Prat 1990]. PRP5 may have an analogous role in spliceosome assembly. PRP5 does have an RNA-dependent ATPase activity, but it is not yet known whether it has RNA-unwinding activity [C. O’Day and J. Abelson, unpubl.]. If PRP5 is an RNA helicase, it could alter either the U1 snRNP/pre-mRNA δ complex or the U2 snRNP.

Another similarity between PRP5 and elf4A suggests that factors interacting with PRP5 could modulate PRP5
activity. Our data suggest that PRP5 acts concertedly or interacts with three other proteins to promote U2 snRNP binding. eIF4A acts concertedly with eIF4B and eIF4F to bind the ribosome to mRNA (for review, see Sonenberg 1988, Linder and Prat 1990). eIF4A alone has some enzymatic activity; however, when it is in a multisubunit complex, its activity is enhanced (Ray et al. 1985, Rozen et al. 1990, Pause and Sonenberg 1992).

A second role for ATP and PRP5 could be in the recognition and selection of both the 5' and 3' pre-mRNA splice sites. The stability of the U1 snRNP bound to the pre-mRNA and the rate of ATP hydrolysis could determine which splice sites among alternative splice sites are utilized via a mechanism similar to one proposed for kinetic proofreading by the PRP16 protein in the second splicing reaction step (Burgess et al. 1990).

Previously, we (Ruby and Abelson 1988) and others (Seraphin and Rosbash 1989; Rosbash and Seraphin 1991) have suggested that some factor may mediate the interaction of the U1 snRNP with the branch point region in the δ complex and be required for forming that complex. Neither PRP5, PRP9, nor PRP11 appears to be this factor. We find that a δ complex still forms when any one of these proteins is inactivated.

Model for U2 snRNP binding to the pre-spliceosome complex

Our current working model for the functions of the PRP5, PRP9, PRP11, and PRP21 proteins is illustrated in Figure 8. We have depicted PRP5 and PRP11 as present in the δ complex because we have shown that the PRP11 (this paper) and PRP5 (C. O'Day and J. Abelson, unpubl.) proteins associate with the pre-mRNA in the absence of ATP. We do not know whether either PRP11 or PRP5 can associate with the pre-mRNA before U1 binds, but neither protein is required for U1 snRNP binding. PRP5 protein may associate only transiently with the δ complex if it functions like two other putative helicases, PRP16 (Schwer and Guthrie 1991) and PRP2 (King and Beggs 1990; Kim and Lin 1993), which interact transiently with the spliceosome at later steps. We have not included the PRP9 and PRP21 proteins in this complex on the basis of previous observations by others. PRP9 does not coprecipitate with the pre-mRNA unless the U2 snRNP is intact (Abovich et al. 1990), and an association of the U2 snRNP with the pre-mRNA in the absence of ATP in the yeast in vitro system has not been observed (Ruby and Abelson 1988; Liao et al. 1992). Anti-PRP21 antibody does not coprecipitate pre-mRNA in the absence of ATP (Arensas and Abelson 1993).

Several data suggest that PRP9, PRP11, and PRP21 proteins are present in the βi complex. We have shown here that PRP11 immunoprecipitates with the pre-mRNA in the presence of ATP. PRP21 protein is required for formation of the βi complex (Arensas and Abelson 1993). Whether PRP21 is bound to the U2 snRNP before it enters the complex is not known, but it seems unlikely as PRP21 coprecipitates with the pre-spliceosome complex but not with free U2 snRNP (Arensas and Abelson 1993).

Several aspects regarding how these four proteins act to promote U2 snRNP binding need to be elucidated. In particular, the order of events regarding the actions of these proteins and the base pairing between the U2 snRNA and the pre-mRNA branchpoint region has to be determined. This order has important implications for understanding the recognition and selection of splice sites. For example, in one possible order of events, the initial contact between the U2 snRNP and the pre-mRNA complex could occur by protein–protein and/or U1/U2 snRNP interactions. The U2 snRNA could
then base-pair with the pre-mRNA via the branchpoint region. Previous observations in yeast on the dominant in vivo lethal effects of a mutant U2 snRNA with an altered branchpoint recognition sequence could be explained by this order (Miraglia et al. 1991). An interaction between PRP9 and PRP11 could be involved in this initial recognition event.

**Materials and methods**

**Plasmids**

Five plasmids were constructed. Plasmid pCEN–prp11 was constructed by subcloning a HindIII–EcoRI fragment containing the prp11 coding region from pFL39–prp11.1 (Spachter and Friesen 1991) into a yeast shuttle vector, pRS416 (CEN–ARS–URA3) (Christianson et al. 1992). Plasmids pCEN–PRP11 and p2μ–PRP11 were generated by subcloning the 2.7-kb BamHI–HindIII fragment containing the entire coding region of PRP11 (Chang et al. 1988) into two yeast shuttle vectors, pSEY18 (2μ–URA3) and pSEY68 (CEN–URA3). Plasmid prp9::HIS3 was constructed by ligating a gel-purified 1.7-kb fragment from plasmid pJJ215 (Jones and Prakash 1990) into EcoRV-cut plasmid pPL5. Digestion of pPL5 with EcoRV removed a 700-bp fragment from the PRP9 protein-coding sequence, and the ligation was done in the presence of EcoRV enzyme to enrich for the correct construct. Plasmid pPS6 (2μ–URA3) was constructed by subcloning a 2.3-kb BamHI–HindIII containing the entire coding region of PRP9 onto the yeast shuttle vector pRS426 (2μ–URA3).

The following plasmids were obtained: pGD231 (CEN–ARS–URA3–PRP5) (Dalbadie-McFarland and Abelson 1989) and pGD532 (2μ–HIS–PRP5) from G. Dalbadie-McFarland, pPL4 (CEN–ARS–URA3–PRP9) (Leigh and Choulika 1990) and pPL5 (Leigh et al. 1991) from P. Leigh, pPL39–prp11.1 and prp9::HIS3 from K. Schappert (University of California, San Diego), pRS416 and 426 (Christianson et al. 1992) from B. Sikorski; pSEY18 and pSEY68 from S. Emr (University of California, San Diego), pJJ215 from M. Werner-Washburn (University of New Mexico, Albuquerque), YCp–LEU2–U2, YCp–LEU2–U2(G53A), YCp–LEU2–U2(C62U), and pU2::URA3 (Ares and Igel 1990) from M. Ares.

**Strains**

The relevant genotypes of strains acquired for use in this study were the following: SS328 (Mata ade2-101 his3-d200 lys2-801 ura3-52), SS330 (Mata ade2-101 his3-d200 tyr1 ural-52), SS304 (Mata prp2-1 ade2-1 his3-352 trp1-289 ura3-1-2), ts88 (Mata prp6-3), ts200 (Mata prp9-2-1-2, 20-1B (Mata prp17-1-1), 27-6C (Mata prp18-1-1), H-10a-3d (Mata prp19-1-1), 20-6a (Mata prp20-1-1), 2A (Mata prp21-1-1), 9-4a (Mata prp22-1-1), 35-3a (Mata prp24-1-1), 35-3b (Mata prp27-1-1) from U. Vijayaraghavan and M. Company (Vijayaraghavan et al. 1989), SP 4.43 (Mata prp4-1), ts257#12 (Mata prp9-1 ade2 his4 leu2 trp1 tyr1 ura1), SP16.7B (Mata prp6-6), SP11.4 (Mata prp17-1-1) from J. Beggs [see Lustig et al. 1986], IM275 (Mata prp5-3 his3-d1 leu2-3,112 lys2-801 ura3-52) from J. Beggs, ma3-1 (Mata prp3-1) from R. Wolford (see Lustig et al. 1986), IH70–wt (Mata leu2-3,112 his4-619 lys2 U2::Ura3 [YCp–LEU2–U2wt]), IH70–G53A (Mata leu2-3,112 his4-619 lys2 U2::Ura3 [YCp–LEU2–U2G53A]), and IH70–C62U (Mata leu23,112 his4-619 lys2 U2::Ura3 [YCp–LEU2–U2C62U]) from M. Ares (Ares and Igel 1990), and EJ101 (Mata pep4-3) from E. Jones [see Lin et al. 1985]. We used standard genetic techniques (Guthrie and Fink 1990) except that all matings, sporulations, and tetrads analyses were done at 23°C.

We created a set of strains with the various prp mutations in the SS330 strain background. The strains carrying the prp2-1, prp3-1, prp4-1, prp5-1, prp5-3, prp6-1, prp8-1, prp9-1, and prp11-1 mutations were crossed into SS330 or the congenic strain, SS328 and then crossed at least twice again into SS330. The original isolates with the prp6-3, prp9-2, and prp16-2 through 27-1, mutations had been generated previously in SS330 or SS328 and crossed out at least once into the parent strain (Vijayaraghavan et al. 1989). The strains carrying these mutations were crossed at least once again into SS330 or two other wild-type congenic strains, SRYWTA (Mata his3-d200 his4 leu2 trp1 tyr1 ural-3a-52) or SRYWLTb (Mata his3-d200 leu2 tyr1 ural-3a-52). These latter two wild-type strains were generated after three backcrosses of strain TS257#12 into SS330. SRYWTA and SRYWLTb were used to increase spore viability and sporulation frequency or to introduce the leu2 mutation. In the tetrad analyses where we observed reduced spore viability or sporulation frequency because of nonspecific reasons, a strain was crossed again into strain SRYWTA or SRYWLTb and then used in the analyses. A third wild-type strain, SRYWTl (Mata ade2 his3-d200 tyr1 ural-3a-52), was created by backcrossing SRYWTA again into SS330 and used in some crosses. Strains in the set used for all of the genetic analyses are designated SRY2-1a (Mata prp2-1 ade2 his3-352 trp1-289 ura1), SRY2-1b (Mata prp2-1 ade2 his3 tyr1 ura1), SRY2-1c (Mata prp2-1 ade2 his3 tyr1 ura1), SRY3-1a (Mata prp3-1 ade2 his3 tyr1 ura1), etc. A complete list of these strains and their genotypes is available on request.

Revertants prp9-1R1 and prp9-1R2 were obtained by irradiating strain TS257#12, respectively with 30 J/m² ultraviolet light and selecting for growth at 37°C. Genetic analyses showed that the prp9R1 and prp9R2 mutations are tightly linked to the PRP9 gene and, thus, probably intragenic.

We constructed several strains by transformation with the method of Ito et al. (1983). Strains SRY9-1c (Mata prp9-1 his3-d200 trp1 ural-3a-52) and SRY9-1d (Mata prp9-1 ade2 his3-d200 leu2 tyr1 ural-3a-52), or SRY9-2c (Mata prp2-2 ade2 his3-d200 tyr1 ural-3a-52) and SRY9-2d (Mata prp2-2 ade2 his3-d200 leu2 trp1 ural-3a-52) were mated to create diploid DSR1314 or DSR1373, respectively. These diploid strains were transformed with the 3.1-kb Pst1–EcoRI fragment from plasmid prp9::HIS3 to create the heterozygote TSR1127 (PRP11/prp11::HIS3, prp9-1/prp9-1) or TSR1129 (PRP11/prp11::HIS3, prp9-2/prp9-2). The prp11::HIS3 plasmid has ~700 bp of the PRP11 protein-coding sequence beginning with codon 92, substituted with the 1.8-kb BamHI–HIS3 fragment. The positions of the prp11 null mutations were confirmed by Southern blot hybridization and tetrads analyses [data not shown]. Plasmid pCEN–prp11-1 or pCEN–PRP11 was then introduced into each heterozygote by transformation to give the following strains: TSR244 (prp9-1/prp9-1), PRP11/prp11::HIS3, pCEN–PRP11), TSR243 (prp9-1/prp9-1, PRP11/prp11::HIS3, pCEN–prp11-1), TSR249 (prp9-2/prp9-2, PRP11/prp11::HIS3, pCEN–PRP11), and TSR250 (prp9-2/prp9-2, PRP11/prp11::HIS3, pCEN–prp11-1). Diploid DSR1401 was created by mating SRYWTLd with SS330. This diploid was transformed with the 3.5-kb BamHI–EcoRI fragment from plasmid prp9::HIS3 to create the heterozygote TSR1252 (PRP9/prp9::HIS3). The position of the null mutation was confirmed as described above [data not shown]. Plasmid pPL4 or pCEN–PRP11 was then introduced into this strain to create strain TSR1262 (PRP11/prp9::HIS3, pPL4) or TSR1254 (PRP9/prp9::HIS3, pCEN–PRP11).

To study the interaction between the PRP5 and SNR20 genes in vivo, we designed a diploid strain that is homozygous for Four interacting yeast spliceosomal proteins.
ppp5-1 and heterozygous for a null, URA3-disrupted chromosomal copy of the SNR20 gene. Strains SRY5-1b [Mata ppp5-1 ade - his3-d200 leu2 tyr - ura3-52] and SRY5-1c [Mata ppp5-1 ade - his3-d200 leu2 tyr - ura3-52] were mated to create diploid DSR1366. This diploid was transformed with a 4.5-kb PstI-EcoRI fragment from plasmid, psnp20::URA3 to create the SNR20/psnp20::URA3 heterozygote TSS1111. The position of the null mutation was confirmed as described above (data not shown). TSS1111 was then transformed with plasmid YCp-LEU2-U2, YCp-LEU2-U2 (G53A), or YCp-LEU2-U2 (C62U). The phenotypes of the meiotic progeny were determined by replica-plating tests. The presence of the URA3 marker in the meiotic progeny was determined by complementation analyses.

Each strain, SRY5-1b, SRY5-3a [Mata ppp5-3 ade - his3-d leu2-3,112 tyr1 - ura3-52], SRY6-1a [Mata ppp5-1 ade2-101 his3 lys2 ura3-52], SRY9-1a [Mata ppp9-1 ade2 his3-d200 tyr1 ura3-52], SRY9-2a [Mata ppp2-2 ade2-101 his3-d200 ura3-52], SRY11-1a [Mata ppp11-1 ade2-101 his3-d200 tyr1 ura3-52] or SRY21-1a [Mata ppp21-1 ade - his - tyr1 ura3-52] was transformed with plasmid pCD231, pGDS1351, plP4, psP6, pcEN-PRP11, plµPRP11, pRS423 (2µ-HIS3), or pRS426 (2µ-URA3), respectively, for the multicopy suppression assay.

In vitro splicing assays

Whole-cell splicing extracts (WCEs) were prepared from strains ts257#12, SPJ6.68, SPJ11.4, prp9-1R1, and prp9-1R2 according to Lin et al. (1985) except that we used 300 µg/ml of Zymolyase-100T [Seikagaku Kogyo]. Wild-type WCE was made from strain E101 as described previously (Lin et al. 1985). Extracts from strain RL172 were gifts from P. Fabrizio and G. McFarland (California Institute of Technology, Pasadena).

The yeast actin precursor RNA for splicing assays was synthesized in vitro by runoff transcription of a cloned template with SP6 polymerase and [32P]UTP (Amersham, Inc.) as described previously (Lin et al. 1985). The template was cut with HpaII restriction endonuclease for synthesizing the transcripts used in the native gel electrophoretic assays. The wild-type [actin Δ6], and mutant templates were cut with HpaII restriction endonuclease for synthesizing the transcripts used in the immunoprecipitation assays.

To heat-inactivate an extract by short heat treatments, MgCl2 was added to 1.5 mM, and the extract was incubated at 38°C for the times indicated. The extract was then kept on ice or frozen in liquid N2 until it was used. The splicing assays were incubated at 23°C for 30 min and processed as described (Lustig et al. 1986). For splicing assays at 30°C, the extract [40% of the reaction volume] was mixed with splicing buffer components and incubated for 2 min at 30°C, after which the reaction was initiated by the addition of 4 nmoles of [32P]labeled pre-RNA and 15 nmoles of ATP. For complementation assays, the mutant extracts were inactivated by short heat treatments at 38°C for 8 min (for prp9 and prp11 extracts) or 10 min (for prp5 and prp6 extracts). The complementation assays were as described by Lustig et al. [1986].

Immunoprecipitations

Immunoprecipitations of the snRNPs from WCEs with anti-PRP11 or preimmune sera were done essentially as described (Banroques and Abelson 1987), except that 8 µl of serum was used in the experiments. Five microliters of wild-type WCE was incubated in splicing buffer without ATP or pre-mRNA at 23°C for 25 min. Sodium heparin (Sigma) was then added as indicated, and the mixture was next added to the antibody immobilized on protein A-Sepharose for immunoprecipitation on a rocking platform at 4°C for 1 hr. The Sepharose was then washed with 50 mM Tris-HCl (pH 7.4), 0.05% NP-40, and the indicated concentration of NaCl (NET buffer). The coprecipitated RNAs were extracted and analyzed as described (Banroques and Abelson 1987).

For pre-mRNA coprecipitation studies, anti-PRP11 and preimmune antibodies were immobilized on protein A-Sepharose as described above except that the Sepharose was washed with either splicing buffer [60 mM KPO4 at pH 7, 3 mM MgCl2, 3% PEG-8000, 8% glycerol, 1.5 mM dithiothreitol] with 1 mg/ml of ammonium heparin (Sigma), or NET with the indicated salt concentration. Twenty microliters of 40% (vol/vol) WCE in splicing buffer was first incubated for 10 min at 23°C to deplete the WCE of endogenous ATP. In some experiments, 2 mM glucose was added to the mixture to completely deplete the endogenous ATP (Horowitz and Abelson 1993). The splicing assay was initiated by adding 16 fmole of pre-mRNA and 30 nmoles of ATP as indicated. The splicing reactions were quenched at 0°C with the addition of 1 µg/µl of ammonium heparin and then added to the immobilized antibody. After 1 hr of incubation at 4°C on a rocking platform, the immunoprecipitates were washed with either splicing buffer or the appropriate NET. The coprecipitated RNAs were extracted and analyzed as described above and measured with a Molecular Dynamics PhosphorImager.

Native gel electrophoresis

A 10-µl splicing reaction with radiolabeled pre-mRNA was quenched by adding it to 10 µl of ice-cold 50 mM HEPES-K+ (pH 7.4), 2 mM (CH3COO)2Mg [R buffer, Seraphin and Rosbash 1988] with 2 µg/µl of carrier RNA. Carrier RNA was prepared from mouse intestine as described (Auffray and Rougeon 1980). The sample was incubated on ice for 10 min, and 5 µl of loading buffer [200 mM Tris-phosphate (pH 8.0 at 0°C), 50% (vol/vol) glycerol] was added. The samples were fractionated on a 3.2% polyacrylamide gel (50:1 acrylamide/bisacrylamide) in 48 mM Tris-phosphate (pH 8.0 at 0°C), 1.5 mM (CH3COO)2Mg [TPM8 buffer] at 4°C for 16-20 hr at 5.5-6.7 V/cm. The gel was transferred to 3MM Whatman paper, and the complexes were visualized by autoradiography.

For assaying antibody inhibition of pre-spliceosome complex formation, antibody from anti-PRP11 or preimmune sera was prepared by affinity chromatography with protein A-Sepharose as described previously (Banroques and Abelson 1989). From 4 to 100 µg of antibody was added to WCE and incubated on ice for 30 min. Splicing buffer components, ATP and radiolabeled pre-mRNA were then added and the reactions were incubated for 20 min at 23°C. The reactions were quenched at 0°C and analyzed by native gel electrophoresis.

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