Interactions between PRP9 and SPP91 splicing factors identify a protein complex required in prespliceosome assembly

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The PRP9 protein is a yeast splicing factor implicated in the early steps of spliceosome assembly whose sequence contains an amino-terminal putative leucine zipper structure and two carboxy-terminal motifs reminiscent of zinc fingers. Here, we show that the deletion of the second carboxy-terminal motif results in a dominant lethal phenotype. This observation, combined with an in vivo-binding assay for protein-protein interactions, reveals the presence of two distinct binding sites on the PRP9 protein. The carboxy-terminal region contributes to the PRP9 homodimerization, whereas the amino-terminal region binds the SPP91 splicing factor. Further experiments suggest that other factors bind to PRP9 and SPP91 proteins. Finally, we demonstrate that the PRP9 protein acts after the formation of the U1 snRNP-pre-mRNA complex. The existence of a protein complex including the PRP9 factor is discussed.

[Key Words: PRP; yeast; GAL4 fusion protein; pre-mRNA splicing]

Received March 15, 1993; revised version accepted April 28, 1993.

Nuclear introns are excised from pre-mRNA precursors via two *trans*-esterification reactions taking place in a multimolecular complex called the spliceosome (for review, see Green 1991; Guthrie 1991; Rymond and Rosbash 1992; Moore et al. 1993). This mechanism has been analyzed primarily in vitro and is conserved between the yeast *Saccharomyces cerevisiae* and higher eukaryotes. The spliceosome forms on the newly transcribed pre-mRNA by the ordered addition of soluble proteins and the four ribonucleoprotein particles, containing, respectively the U1, U2, U4 and U6 (U4/U6), and U5 snRNAs (Frendewey and Keller 1985; Konarska and Sharp 1986; Pikielny et al. 1986; Cheng and Abelson 1987; Legrain et al. 1988; Seraphin and Rosbash 1989; Barabino et al. 1990; Kramer and Utans 1991; Michaud and Reed 1991). The initial U1 small nuclear ribonucleoprotein (snRNP) interaction with the pre-mRNA results in the formation of a commitment complex, the U2 snRNP then binds to this complex and, ultimately, the spliceosome assembles by the addition of the U4/U6 and U5 snRNPs as a pre-formed tri-snRNP.

Although multiple splicing factors have been identified, very little is known about the protein-protein interactions that occur during spliceosome assembly. Most of the identified interactions involve RNA-RNA base-

pairing between snRNAs, between pre-mRNA sequences, or between snRNA and the pre-mRNA (for review, see Moore et al. 1993), or interactions between proteins and RNAs (Bordonne et al. 1990; Scherly et al. 1990; Whittaker and Beggs 1991; Frank et al. 1992; Liao et al. 1993). In higher eukaryotes, several factors, such as U2AF, ASF/SF2, SF1, SF3, and SC35 proteins, have been shown to be required during the early steps of spliceosome assembly in association with U1 and U2 snRNPs (Ge et al. 1991; Krainer et al. 1991; Kramer and Utans 1991; Fu and Maniatis 1992b; Zamore et al. 1992), but relationships between these various factors remain unknown.

In *S. cerevisiae*, genetic studies have identified at least 30 PRP genes, including the PRP9 gene, involved at various steps during spliceosome assembly or splicing reactions (Hartwell et al. 1970; Vijayraghavan et al. 1989). The functions of the PRP proteins have been identified primarily by the use of heat-sensitive *prp* alleles, for which in vitro and in vivo splicing defects have been characterized. In addition to a splicing defect, we observed that the *prp9-1* mutant exhibits an increased pre-mRNA nuclear export into the cytoplasm (Legrain and Rosbash 1989), suggesting that the PRP9 protein may act during the early steps of the spliceosome assembly pathway. In addition, the PRP9 protein is required in vitro for the formation of the pre-spliceosome, which contains the U1 and the U2 snRNPs, but is not tightly associated

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with the U2 snRNP (Abovich et al. 1990). A mammalian PRP9 homolog has been identified that is associated with the 17S U2 snRNP (Behrens et al. 1993b) and is also required for pre-spliceosome formation (Behrens et al. 1993a). Therefore, the binding of the PRP9 protein to the early splicing complexes could be an essential step for the in vivo commitment in the splicing pathway.

The PRP9 gene product contains two cysteine/histidine motifs (CH motifs) loosely related to zinc finger motifs of the TFIIIA type and a putative leucine zipper domain (Legrain and Choulika 1990). Such motifs can be involved in protein–protein interactions. Recently, we isolated an extragenic suppressor, *spp91-1*, of the heat-sensitive *prp9-1* mutation. The combination of the *prp9-1* mutation and a decreased expression of the *SPP91* gene results in a synthetic lethal phenotype. These observations suggested possible interactions between PRP9 and SPP91 proteins (Chapon and Legrain 1992).

In this paper we show that the *prp9* mutant deleted for the second CH motif exhibits a dominant lethal phenotype that leads us to postulate several binding properties for the PRP9 protein. By using an in vivo binding assay (Fields and Song 1989; Chien et al. 1991), we have identified and characterized a direct interaction between the PRP9 and SPP91 proteins and, in addition, have demonstrated that the PRP9 protein homodimerizes. These studies strongly suggest that the PRP9 and SPP91 proteins form a multimolecular complex, probably in association with additional factors. Finally, we show that the U1 snRNP–pre-mRNA complex forms in the absence of the PRP9 protein. Taken together, the properties of the PRP9 and SPP91 proteins suggest that the PRP9/SPP91 complex acts in the formation of the U1 snRNP/U2 snRNP/pre-mRNA prespliceosome complex.

Results

Deletion of the CH2 region of the PRP9 protein confers a dominant lethal phenotype in a *prp9-1* genetic background

The PRP9 protein contains two motifs, referred to as CH1 and CH2 (Fig. 1), reminiscent of Cys₂His₂ zinc fingers. The function of these motifs was addressed previously by a complementation assay of the *prp9-1* heat-sensitive mutation with genes mutated in the cysteine and histidine codons (Legrain and Choulika 1990). The PRP9 proteins mutated in either the cysteine or histidine residues of the CH2 motif do not complement the *prp9-1* mutation. By use of a plasmid-shuffling strategy, these

CH2 mutants were shown to be null alleles of the *PRP9* gene (data not shown). To investigate further the function of the CH domains, deletions covering each of these motifs were made (referred to as *prp9ΔCH1* and *prp9ΔCH2*). Surprisingly, when *prp9-1* cells were transformed with a high-copy-number plasmid carrying the *prp9ΔCH2* allele, very few transformants were obtained, although transformation efficiency with the *prp9ΔCH1* plasmid or the vector alone was normal (Table 1). This low efficiency of transformation was dose dependent, because the number of transformants increased slightly when a low-copy-number plasmid was used. We verified that expression of the *prp9ΔCH2* allele was lethal by use of a *GAL-prp9ΔCH2* construct in which expression of the *prp9ΔCH2* allele is directed from a galactose-inducible promoter. With this construct, *prp9-1* cells are viable when plated on glucose-containing medium, and lethality is observed after transfer of the cells to galactose-containing medium (Fig. 2). This dominant lethal phenotype was observed only in *prp9-1* or *prp9-2* cells (Vijayraghavan et al. 1989) and not in wild-type cells or in *prp9-1* cells cotransformed with a wild-type *PRP9* allele (data not shown). In addition, the double mutant carrying the *ts1* mutation and the CH2 deletion did not affect the transformation efficiency of *prp9-1* cells (Table 1).

The growth rates of *prp9-1* cells transformed with the *GAL-prp9ΔCH2* plasmid or the control vector, pLGSD5, were monitored in glucose- or galactose-containing media (Fig. 3, top). In the presence of glucose or galactose, *prp9-1* cells transformed with the pLGSD5 vector have very similar growth rates (left panel). In contrast, 12 hr after transfer to galactose-containing medium (i.e., two doubling times), the growth rate of *GAL-prp9ΔCH2*-transformed cells decreases (right panel). The splicing efficiencies in these transformed *prp9-1* cells were measured at various times after galactose induction. Northern blot analysis of transcripts of an intron-containing gene, the *RP51A* gene, showed that as early as 1 hr after the transfer to galactose-containing medium, the splicing of this RNA was severely impaired in cells transformed with the *GAL-prp9ΔCH2* plasmid (Fig. 3, bottom). Thus, the lethality induced by the *prp9ΔCH2* allele is a consequence of a splicing defect.

The dominant lethal phenotype observed with the CH2 deletion mutant suggests that the PRP9 protein has at least two functional domains, because a dominant lethal phenotype may be viewed as the result of a competition between an inactive protein that retains one function and the active protein that contains at least two

Figure 1. The PRP9 protein. Positions and changes corresponding to the *prp9-1* (*ts1*) and *prp9-2* (*ts2*) mutations are indicated, as well as the location of the deletion mutants (Δ CH1 and Δ CH2, hatched boxes) and the conserved residues in the zinc fingers. A putative leucine zipper motif (Zip) is indicated.

Table 1. The *prp9ΔCH2* mutant exhibits a dominant lethal phenotype in a *prp9-1* strain

Plasmid	Efficiency
pEMBLye31 (high copy)	>1500
<i>PRP9</i>	500
<i>prp9ΔCH1</i>	>1500
<i>prp9ΔCH2</i>	3
YCp50 (low copy)	600
<i>PRP9</i>	>1000
<i>prp9ΔCH1</i>	600
<i>prp9ΔCH2</i>	7 + 40 small
<i>prp9-1, ΔCH2</i>	>1000

Cells were transformed and grown at 25°C. Transformation efficiency is expressed as the number of colonies obtained with 1 μg of plasmid DNA.

functional sites (Herskowitz 1987). One of the interactions of the PRP9ΔCH2 protein that remains unaltered involves the region containing the mutated temperature-sensitive residues, because the dominant lethal phenotype is observed in *prp9-1* and *prp9-2* cells but not in isogenic wild-type cells. The SPP91 protein is a likely candidate as a PRP9 protein partner, because the *spp91-1* allele was isolated as a suppressor of the *prp9-1* mutation.

PRP9 and SPP91 proteins interact through the amino-terminal domain of the PRP9 protein

To analyze the possible interactions of the PRP9 protein, we used the two-hybrid system (Fields and Song 1989; Chien et al. 1991), which scores for in vivo interactions

Figure 2. Growth phenotype of the *prp9ΔCH2* mutant. Cells were streaked on glucose- or galactose-containing medium and grown at 25°C. The wild-type (WT) and *prp9-1* (*prp9ts*) strains are isogenic (Chapon and Legrain 1992) and were transformed with the *prp9ΔCH2* (ΔCH2) plasmid or the pLGSD5 (pSD5) control vector. **Figure 3.** Splicing defect associated with the dominant lethal phenotype of the *prp9ΔCH2* mutant. *prp9-1* cells were transformed with plasmids pLGSD5 (left) or *prp9ΔCH2* (right). (Top) Growth curves in glucose (+) or galactose (−) containing media. (Bottom) Northern analysis was carried out with a *RP51A* gene probe on RNAs prepared from cells after transfer (0, 1, 5, and 9 hr) to glucose- or galactose-containing media. The positions of the *RP51A* pre-mRNA (pre) and mRNA are indicated.

between two proteins overproduced in *S. cerevisiae*. In this assay, two proteins are fused to the DNA-binding domain and to the activation domain of the yeast transcriptional factor GAL4, respectively. An interaction between these proteins brings together the two GAL4 domains, allowing the formation of a functional GAL4 complex, which activates the expression of a *lacZ* reporter gene. The interaction of the PRP9 and SPP91 proteins was assayed, in the two possible combinations, pGAD2F:PRP9/pMA424:SPP91 and conversely (Fig. 4A). As indicated by the blue color of the colonies, the SPP91 protein interacts strongly either with the PRP9 protein or the PRP9ΔCH2 protein. In contrast, the *prp9-1* mutation severely reduces the interaction between the PRP9 and SPP91 proteins, which becomes barely detectable.

This observation led us to test whether the *spp91-1* mutation suppresses the *prp9-1* phenotype by restoring the interaction between the PRP9-1 and SPP91-1 mutant proteins (Fig. 5). At 30°C, β-galactosidase activities in cells transformed with the PRP9 and SPP91 hybrid constructs varied between 50 and 100 units. These activities were reduced to nearly background levels by the *prp9-1* mutation. β-Galactosidase activity was increased by the *spp91-1* mutation in combination A (~5 units compared with <1 unit for the PRP9-1 and SPP91 interaction), although no effect of this mutation was detectable in the other combination. Because the *prp9-1* mutant exhibits a temperature-sensitive phenotype, these experiments

Figure 4. In vivo dimerization of PRP9 and SPP91 proteins and homodimerization of the PRP9 protein. GGY1::171 cells were transformed with a pGAD2F derivative plasmid [GAL4-activating domain (*bottom* line)] and a pMA424 derivative plasmid [GAL4 DNA-binding domain; (*top* line)] harboring PRP9, PRP9-1, PRP9ΔCH2, or SPP91 hybrid proteins. (A) Interaction between the PRP9 and SPP91 proteins (PRP9–SPP91 dimer). The heterodimerization assay is presented for the two combinations, with PRP9, PRP9-1, and PRP9ΔCH2 proteins produced from the pGAD2F plasmid and SPP91 protein from the pMA424 plasmid and conversely. (B) Homodimerization of the PRP9 protein. Two negative controls with pGAD2F or pMA424 vectors are included.

were also performed with cells grown at 22°C (Fig. 5, right panel). At this temperature, PRP9 and SPP91 interaction still results in high levels of β -galactosidase activity, which is reduced in the presence of the *prp9-1* mutation. The levels of β -galactosidase activity are consistently lower at 22°C than at 30°C, which is also seen for the wild-type GAL4 protein (data not shown). The restoration of high levels of β -galactosidase activity by the *spp91-1* mutation, however, is more pronounced than at 30°C (Fig. 5, cf. PRP9-1/SPP91 and PRP9-1/SPP91-1 interactions). Similar data were also obtained with the *prp9-2* mutation (data not shown), consistent with the observation that the *spp91-1* mutation also suppresses this temperature-sensitive mutation (Chapon and Legrain 1992), which maps to residue 177 (see Materials and methods; Fig. 1). These results clearly demonstrate that the positions corresponding to the *prp9-1* and *prp9-2* mutations, which are located on the amino-terminal domain of the PRP9 protein, contribute to the binding between the PRP9 and SPP91 proteins.

The PRP9 protein homodimerizes through a domain containing the CH2 motif independent of the SPP91-binding domain

Experiments utilizing the two-hybrid system also established that the PRP9 protein has the capacity to form homodimers (Figs. 4B and 6A). The *prp9-1* and *prp9-2* mutations did not affect homodimerization, whereas deletion of the CH1 domain completely abolished dimer formation. Deletion of the CH2 domain reduced the homodimerization efficiency by a factor of 4. Overproduction of the PRP9ΔCH2 protein has some detrimental effects on the cells, as judged by colony size and the ap-

pearance of white revertant colonies (see also Fig. 4A). In addition, fusion proteins were also produced from the point mutants in the CH2 motif at the cysteine- and histidine-conserved residues (Legrain and Choulika 1990). The β -galactosidase activities measured with these hybrid proteins were reduced by at least one order of magnitude, this effect being more pronounced with the cysteine mutations, demonstrating that the CH2 point mutations severely affect PRP9 protein homodimerization.

The effect of the mutations in the CH1 or CH2 regions of the PRP9 protein on the interaction with the SPP91 protein was also tested (Table 2). Neither the CH2 deletion nor the CH2 point mutations significantly affect binding to the SPP91 protein (Table 2). The β -galactosidase activities differed at the most by a factor of two from the values measured for the wild-type PRP9 hybrid proteins. In contrast, the PRP9ΔCH1 protein is unable to bind the SPP91 protein. Because the PRP9ΔCH1 protein is also unable to homodimerize and the *prp9ΔCH1* mutant does not exhibit a dominant lethal phenotype, the inability to bind the SPP91 protein probably results from a large alteration of the protein structure, which inactivates the PRP9ΔCH1 protein. These experiments establish that the CH2 motif is an essential element for the PRP9 protein dimerization but not for the binding to the SPP91 protein.

The CH2 motif of the PRP9 protein is required for interaction with an additional factor

Because the PRP9ΔCH2 mutant protein binds the SPP91 protein (Table 2), the dominant lethal phenotype caused by the expression of the *prp9ΔCH2* allele in a *prp9-1* or

Figure 5. Analysis of interactions between PRP9 and SPP91 proteins. Y526 cells were transformed with two plasmids derived from pGAD2F (top line) or pMA424 (bottom line), respectively. Cells were grown at 30°C or 22°C. The two combinations (A and B) are shown for each interaction. Error bars are indicated (see Materials and methods).

prp9-2 background could result from titration of the SPP91 protein and the reduced homodimerization ability of the *prp9ΔCH2* protein. *prp9-1* Cells transformed with the *prp9ΔCH2* plasmid may also contain the heterologous PRP9-1/PRP9ΔCH2 dimers, in addition to PRP9-ΔCH2/PRP9ΔCH2 and PRP9-1/PRP9-1 homodimers. Through the use of the two-hybrid system, the PRP9-ΔCH2 protein dimerized with PRP9 and PRP9-1 proteins with wild-type efficiencies (Fig. 6B). Similarly, the CH2 point mutants form heterologous dimers with the PRP9 or the PRP9-1 protein as efficiently as the PRP9ΔCH2 protein (data not shown). These results strongly suggest that heterologous dimers can form in cells either transformed with the *PRP9ΔCH2* or the CH2 point mutant plasmids. Thus, the observed dominant lethal phenotype of the *PRP9ΔCH2* mutant probably does not result from the reduced ability of the corresponding protein to homodimerize, but from the loss of an additional PRP9 function that is associated with the CH2 domain.

The PRP9 protein acts in the spliceosome assembly after the formation of the U1 snRNP-pre-mRNA commitment complex

The specific step at which the PRP9 protein is required before the binding of the U2 snRNP is not established. We investigated the function of the PRP9 protein in the

spliceosome assembly in further detail by in vitro assays. Anti-PRP9 antibodies were raised and used either to inhibit splicing reactions or to immunodeplete splicing extracts. These reactions were analyzed by electrophoresis in non-denaturing conditions, which resolves the commitment complexes (containing the U1 snRNP) from the later complexes (Fig. 7, lanes 1–4; Seraphin and Rosbash 1989). Preincubation of a splicing extract with anti-PRP9 antibodies prior to the addition of the pre-mRNA specifically inhibits the spliceosome formation (lanes 6–12, cf. with lane 5). Concomitantly with the decrease of spliceosome formation, accumulation of commitment complexes was observed. To determine whether the PRP9 protein is required for the formation of the commitment complexes, immunodepletion experiments were performed with anti-PRP9 antibodies. Immunodepletion of the PRP9 protein from splicing extracts prevented the formation of spliceosomes, whereas the commitment complexes accumulated (lanes 13–18). These results establish that the PRP9 protein acts after the binding of the U1 snRNP to the pre-mRNA and is required for the binding of the U2 snRNP to this commitment complex.

Discussion

In *S. cerevisiae*, the PRP9 protein is required for U2

Figure 6. Homodimer formation between PRP9 proteins. (A) PRP9 protein homodimers. Wild-type (solid bar), heat sensitive (dark shaded bar), CH2 point mutants (light shaded bar), and deletion mutants (hatched bars) homodimers are presented. (B) Heterologous PRP9 dimers. The dimers between the PRP9 Δ CH2 protein (produced from a pGAD2F plasmid) and the wild-type PRP9 or the PRP9-1 proteins are presented. Error bars are indicated (see Materials and methods).

snRNP binding to the spliceosome but is not tightly associated with this snRNP. The interaction of this factor with the pre-mRNA, however, depends on the integrity of the U2 snRNA (Abovich et al. 1990). In HeLa cell extracts, anti-PRP9 antibodies cross-react with a protein that is a component of the U2 17S snRNP (Behrens et al.

Table 2. Mutations in the PRP9 CH2 motif do not interfere with the binding to the SPP91 protein

	β -Galactosidase activity (units)
Combination A (the <i>SPP91</i> gene in the pGAD2F plasmid)	
PRP9	65.9 $\pm$ 4.2
PRP9 Δ CH1	1.3 $\pm$ 0.3
PRP9 Δ CH2	63.9 $\pm$ 3.4
PRP9-C423	29.3 $\pm$ 5.6
PRP9-C426	26.2 $\pm$ 1.1
PRP9-H440	31.8 $\pm$ 2.2
PRP9-H446	44.6 $\pm$ 2.1
Combination B (the <i>SPP91</i> gene in the pMA424 plasmid)	
PRP9	56.4 $\pm$ 3.7
PRP9 Δ CH1	<0.1
PRP9 Δ CH2	19.4 $\pm$ 4.6
PRP9-C423	61.3 $\pm$ 3.9
PRP9-C426	31.9 $\pm$ 1.8
PRP9-H440	51.7 $\pm$ 4.1
PRP9-H446	67.4 $\pm$ 3.6

Y526 cells were transformed with the indicated plasmids and assayed for β -galactosidase activity.

1993a). Here, we establish that the PRP9 protein acts after the formation of the commitment complexes. Thus, it is very likely that the PRP9 protein contributes to the binding of the U2 snRNP to the pre-mRNA/U1 snRNP complex, probably in conjunction with several additional proteins. In higher eukaryotes, the U2AF, SC35, ASF/SF2, SF1, and SF3 proteins (Ge et al. 1991; Krainer et al. 1991; Kramer and Utans 1991; Fu and Maniatis 1992a; Zamore et al. 1992) are also implicated in this step, but little is known concerning their sequential requirement (Bennett et al. 1992a). In addition, some heterogeneous nuclear RNPs (hnRNPs) could also be involved in this process (Bennett et al. 1992b). Recently, further purification of the SF3 activity has led to the characterization of a factor, SF3a, required for the pre-spliceosome assembly, which contains three protein components, including the PRP9 homolog (R. Brosi and A. Krämer, pers. comm.).

Several proteins, such as those mentioned above, are required for the binding of the U2 snRNP to the commitment complexes; these proteins may interact together and play a role in prespliceosome formation as a multimolecular complex. Here, we show evidence for such protein-protein interactions between the PRP9 and SPP91 splicing factors. Deletion of the CH2 region of the PRP9 protein leads to a dominant lethal phenotype in *prp9-1* and *prp9-2* strains. The restriction of the expression of the dominant lethal phenotype to *prp9-1* or *prp9-2* mutants and the isolation of *spp91-1* as a suppressor of these mutations strongly suggested that the SPP91 and PRP9 proteins interact. We confirmed this interaction by use of the two-hybrid system (Fields and Song

Figure 7. Inhibition of spliceosome formation and immunodepletion of splicing extracts with anti-PRP9 antibodies. In vitro splicing reactions performed with labeled pre-mRNA were analyzed by native gel electrophoresis (Seraphin and Rosbash 1989). (Left; lanes 1–4) Kinetic analysis of splicing complexes formation. (Middle; lanes 5–12) Inhibition of splicing reactions with 32 μ g of preimmune serum immunoglobulins (lane 5) or various amounts of anti-PRP9 immunoglobulins ([lanes 6–12] 32, 16, 8, 4, 2, 1, and 0.5 μ g, respectively). (Right; lanes 13–18) Immunodepletion of a splicing extract with various amounts of preimmune ([lane 13] 48 μ l) or anti-PRP9 ([lanes 14–18] 48, 24, 12, 6, and 3 μ l, respectively) sera. In lanes 5–18, splicing reactions were carried out for 20 min. Commitment complexes (CC) and later splicing complexes (SP) are indicated at left.

1989; Chien et al. 1991). To our knowledge, this is the first demonstration of a protein–protein interaction between two splicing factors. The binding of U2AF to its 35-kD-associated protein has been reported, however the 35-kD protein is not required for in vitro splicing reactions (Zhang et al. 1992). The *prp9-1* and *prp9-2* mutations identify two residues important for the binding of the SPP91 protein. They are located on flanking sides of a putative leucine zipper structure, a classical dimerization motif (Landschulz et al. 1988). No such motif, however, is present in the SPP91 sequence. Recently, the SPP91 protein was shown to be identical to the PRP21 protein, a splicing factor not tightly associated with any snRNP in the absence of spliceosomes but required for the formation of U2 snRNP–pre-mRNA complexes (J. Arenas and J. Abelson, pers. comm.). Interactions between PRP proteins have been suggested, on the basis of genetic evidence such as synthetic lethality, suppression of heat-sensitive mutations by overexpression of a wild-type gene, or selection of second-site suppressors (Last et al. 1987; Jamieson et al. 1991; Strauss and Guthrie 1991), but in none of these cases have direct interactions been demonstrated. Dominant lethal mutants have also been reported for a U2 snRNA mutant in the branchpoint binding sequence (Miraglia et al. 1991) and also for a *prp16* mutant (Schwer and Guthrie 1992).

The homodimerization of the PRP9 protein requires intact CH1 and CH2 regions. The PRP9 Δ CH2 protein retains a reduced ability to homodimerize, which may be attributed to the presence of the CH1 domain. The CH1 and CH2 regions of the PRP9 protein, as well as those found in three other splicing factors, the yeast PRP6, PRP11 proteins and the human U1C protein, are loosely related to those found in zinc finger proteins (Legrain and Chouluka 1990). The canonical TFIIIA zinc fingers are involved in DNA and RNA binding (Darby and Joho 1992; Theunissen et al. 1992), but more generally, zinc fingers are considered as basic structural protein elements (Kaptein 1991). Our results strongly suggest that the CH2 motif may be directly involved in the ho-

modimerization process but not necessarily as sites of direct contact.

The PRP9 protein behaves as a modular protein with two independent domains. The interactions between the various PRP9 and SPP91 proteins are summarized in Figure 8. The heat-sensitive phenotype of the *prp9-1* mutant is attributable to a weak interaction of the PRP9 protein with the SPP91 protein. The PRP9 Δ CH2 protein homodimerization is reduced, but heterologous dimers, e.g., *prp9-1*/PRP9 Δ CH2, form normally. The inability of

Figure 8. Model for the identified and predicted interactions of the PRP9 protein.

Figure 8. Model for the identified and predicted interactions of the PRP9 protein. The existence of an unidentified X factor is predicted by the observation of the dominant lethal phenotype of the *prp9 Δ CH2* mutant and the results of binding assays (see Discussion).

the PRP9-1/PRP9 Δ CH2 dimer to bind the SPP91 protein through the PRP9-1 monomer cannot account for the dominant lethal phenotype because the *prp9-1* mutant only displays a temperature-sensitive phenotype. These results indicate that the dominant lethal phenotype of the PRP9 Δ CH2 mutant does not result from a lack of homodimerization with the PRP9-1 protein but, rather, that the PRP9-1/PRP9 Δ CH2 dimer is not functional. Therefore, we propose that PRP9 homodimers bind an unknown factor (Fig. 8, X factor). PRP9-1/PRP9 Δ CH2 and PRP9 Δ CH2/PRP9 Δ CH2 dimers cannot bind the X factor and are defective. Because the PRP9 Δ CH2 molecules bind the SPP91 protein with a much higher efficiency than the PRP9-1 molecules, PRP9-1/PRP9 Δ CH2 and PRP9 Δ CH2/PRP9 Δ CH2 dimers will titrate out the SPP91 protein from the PRP9-1/PRP9-1 dimers, leading to the lethal phenotype. In contrast, the CH2 point mutants, which do not exhibit a dominant lethal phenotype, may form functional heterologous dimers with the PRP9-1 protein.

Several suggestions as to the identity of the X factor can be proposed. The U2 snRNP itself might bind to the PRP9 protein by either protein–protein and/or protein–RNA interactions. The presence of zinc finger-related structures in the homodimerization domain of the PRP9 protein is suggestive of a binding to nucleic acids, for example, to the U2 snRNA. The U1C protein, however, contains a similar domain that is involved in the binding to the U1 snRNP, but this binding is probably mediated through protein–protein interactions between U1–70K and U1C proteins (Nelissen et al. 1991). Similarly, the X factor could be a protein associated with the U2 snRNP. No such protein has yet been identified in yeast.

The PRP9/SPP91 complex may allow the bridging between U1 and U2 snRNPs in the splicing complexes. It is probable that the SPP91 protein also binds another splicing factor. In addition to PRP9 and SPP91 proteins, other splicing factors, such as PRP5 and PRP11 proteins (Chang et al. 1988; Dalbadie-McFarland and Abelson 1990), are required early in the spliceosome assembly pathway. Recently, we have shown that the PRP11 protein is associated with the PRP9/SPP91 complex (P. Legrain and C. Chapon, unpubl.). Thus, a multimolecular complex of splicing factors may be implicated in the formation of the U1 and U2 snRNP-containing splicing complex.

Materials and methods

Yeast strains and manipulation

Yeast media were prepared and genetic manipulations were performed as described (Rose et al. 1990). The wild-type strains, MGD353-13D [*MATa ura3-52 trp1-289 leu2-3, -112 ade2 arg4*] and MGD353-46D [*MATa ura3-52 trp1-289 leu2-3, -112 his3 Δ 1 cyhR*] and their *prp9-1* counterparts, Cy105 and Cy103, respectively, have been described previously (Chapon and Legrain 1992). A temperature-sensitive mutant, ts200, was isolated during a search for conditional splicing mutants and identified as a *prp9* allele (Vijayraghavan et al. 1989). We renamed this mutant allele, *prp9-2* because another temperature-sensitive allele,

prp9-1, had been characterized previously (Legrain et al. 1991). Strains GGY1::171 [*Δ gal4 Δ gal80 ura3::GAL1-lacZ his3 leu2*] (Gill and Ptashne 1987) and Y526 [*Δ gal4 Δ gal80 ura3::GAL1-lacZ his3 leu2 trp1*] were provided by M. Werner (CEA, France).

Characterization of the *prp9-2* mutation

The *prp9-2* mutation was mapped by plasmid repair, and the mutated DNA was cloned by PCR and sequenced. A single mutation which converts the glycine residue at position 177 into an aspartate residue, was found. The temperature-sensitive phenotype caused by this change was confirmed by in vitro mutagenesis of the wild-type PRP9 gene.

Plasmid construction

Molecular biology manipulations were performed as described (Sambrook et al. 1989). The plasmid pPL5 (Legrain et al. 1991), which contains the PRP9 gene, was used to derive the Δ CH1 and Δ CH2 deletions, by digestion and religation of two *SalI* sites introduced by in vitro mutagenesis at positions indicated by the corresponding amino acid residues in Figure 1. The GAL-*prp9 Δ CH2* plasmid contains a PCR-derived *prp9 Δ CH2* DNA fragment cloned at the *Bam*HI site of the plasmid pLGSD5, downstream of the GAL10 promoter (Guarente et al. 1982). PCR-derived DNA fragments encoding the appropriate PRP9 or SPP91 open reading frames were inserted at the *Bam*HI site of the pGAD2F and pMA424 plasmids (Ma and Ptashne 1987; Chien et al. 1991) and partially sequenced. Six mutations, resulting from PCR mutagenic activity, were found in >18,000 nucleotides sequenced. Three additional mutations were found in the nucleotides containing the oligonucleotide primer sequences, giving a rate of error of ~0.5%. These mutations are unlikely to affect the results observed, because both combinations give essentially similar results. The PRP9 Δ CH1 constructs, which did not display any binding activity, were sequenced in toto.

In vivo splicing defect analysis

Transformed cells were grown at 25°C in selective medium lacking uracil and containing 2% lactate, 2% glycerol, and 0.05% glucose. Exponentially growing cells were transferred to glucose (2%)- or galactose (2%)-containing media. Regular dilutions were made to keep the culture exponential (1–3 OD₆₀₀ units). Cells were harvested at various times, and RNA was prepared (Pikielny and Rosbash 1985). Northern blot analysis using a RP51A DNA probe detecting both mRNA and pre-mRNA was performed as described previously (Chapon and Legrain 1992).

β -Galactosidase assays

Protein–protein interaction in the two-hybrid system can be monitored on plates in the presence of X-gal or in liquid culture by assaying the β -galactosidase activity present in the cells. Plate assays were performed at 37°C with a 3- to 5-ml overlay containing 0.5% agar, 0.5 M phosphate buffer (pH 7.0) 1% SDS, 6% dimethylformamide, and 0.02% X-gal. Quantitative assays were performed on liquid cultures. Exponentially growing cells were harvested (~1 OD₆₀₀/ml) and β -galactosidase assays were performed as described previously except that the incubation temperature was 30°C (Legrain and Rosbash 1989). A β -galactosidase unit corresponds to 10³ $\times$ OD₄₂₀ units per minute of reaction for 1 ml of culture at 1 OD₆₀₀ unit. Under these con-

ditions, cells transformed with a plasmid harboring the GAL4 protein provide ~1000 (Y526 strain) or 2000 (GGY1::171 strain) units, and the sensitivity of the assay is ~0.1 unit. Cells transformed with only one of the PRP9- or SPP91-derived plasmids and either the pGAD2F or pMA424 vector contained <0.5 unit (e.g., see Fig. 4B). Assays were done in duplicate for three independent transformants. Means of values and standard deviations are indicated.

Preparation of anti-PRP9 antibodies

A recombinant PRP9 protein deleted for the first 25 amino acid residues was produced in *Escherichia coli* using the pET3c expression vector (Studier and Moffatt 1986; Rosenberg et al. 1987), gel-purified, and injected into rabbits. These antibodies react, by western blot, with a 65-kD product, identified as the PRP9 protein by use of cells overexpressing the PRP9 gene (Behrens et al. 1993a).

In vitro splicing reaction

Splicing extract preparation, transcription of the $\Delta 2$ splicing substrate derived from the *RP51A* gene, and native gel electrophoresis were performed as described (Seraphin and Rosbash 1989). The immunoglobulin fraction from sera was purified by affinity chromatography on protein A-Sepharose. Inhibition of the splicing reaction was performed as follows: 4 μ l of splicing extract was mixed with various amounts of immunoglobulins for 30 min at 4°C. The splicing reactions were initiated by the addition of the $\Delta 2$ substrate in the appropriate buffer and incubations performed under the standard conditions. The immunodepletion experiment was performed as follows: Protein A-Sepharose beads were incubated in NET150 buffer (150 mM NaCl, 50 mM Tris-HCl at pH 7.4, 0.05% NP-40) with various amounts of serum at 4°C for 2 hr. After two washes in NET150 buffer, two washes were performed in the splicing extract buffer (Lin et al. 1985). The beads were then incubated for 1 hr at 4°C with 8 μ l of splicing extract. After centrifugation, 4 μ l of supernatant was removed and used for in vitro splicing reactions.

Acknowledgments

We are greatly indebted to G. Bordenave for his expertise and help in immunization procedures, to S. Fields and M. Werner for plasmids and strains used for the two-hybrid system and to U. Vijayraghavan for the gift of the *prp9-2* strain. We thank J. Arenas, J. Abelson, R. Brosi, and A. Krämer for communicating unpublished results. We also thank G. Chanfreau, A. Jacquier, A. Korman, A. Plessis, and C. Transy for numerous stimulating discussions and for critical reading of the manuscript. Finally, we particularly acknowledge the continuous support and interest of B. Dujon in this work. This work was partly supported by the Université Pierre et Marie Curie and the Centre National de la Recherche Scientifique (URA 1149). F.G. is ATER at the Université Pierre et Marie Curie.

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Genes Dev. 1993, **7**:

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