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]

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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; Saraphin 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; Vijayaraghavan 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
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-splicesome formation (Behrens et al. 1993a). Therefore, the binding of the PRP8 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 TFIIB 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, spp9-1, 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 presplicesome 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 prp9ACH1 and prp9ACH2). Surprisingly, when prp9-1 cells were transformed with a high-copy-number plasmid carrying the prp9ACH2 allele, very few transformants were obtained, although transformation efficiency with the prp9ACH1 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 prp9ACH2 allele was lethal by use of a GAL-prp9ACH2 construct in which expression of the prp9ACH2 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 allele 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-prp9ACH2 plasmid or the control vector, pLG5D5, were monitored in glucose- or galactose-containing media (Fig. 3, top). In the presence of glucose or galactose, prp9-1 cells transformed with the pLG5D5 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-prp9ACH2-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-prp9ACH2 plasmid (Fig. 3, bottom). Thus, the lethality induced by the prp9ACH2 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
The prp9ΔCH2 mutant exhibits a dominant lethal phenotype in a prp9-1 strain

| Plasmid       | Efficiency |
|---------------|------------|
| pEMBLYe31 [high copy] | >1500      |
| PRP9          | 500        |
| prp9ΔCH1      | >1500      |
| prp9ΔCH2      | 3          |
| YCp50 [low copy] | 600        |
| PRP9          | >1000      |
| prp9ΔCH1      | 600        |
| prp9ΔCH2      | 7 + 40 small|
| prp9-1, ΔCH2  | >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 spp9-1 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 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 spp9-1 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 spp9-1 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, PRP9ACH2, 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 PRP9ACH2 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 aminoterminal 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 PRP9ACH2 protein has some detrimental effects on the cells, as judged by colony size and the appearance 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 PRP9ACH1 protein is unable to bind the SPP91 protein. Because the PRP9ACH1 protein is also unable to homodimerize and the prp9ACH1 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 PRP9ACH1 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 PRP9ACH2 mutant protein binds the SPP91 protein [Table 2], the dominant lethal phenotype caused by the expression of the prp9ACH2 allele in a prp9-1 or
prp9-2 background could result from titration of the SPP91 protein and the reduced homodimerization ability of the prp9ACH2 protein. prp9-1 Cells transformed with the prp9ACH2 plasmid may also contain the heterologous PRP9-1/PRP9ACH2 dimers, in addition to PRP9ACH2/PRP9ACH2 and PRP9-1/PRP9-1 homodimers. Through the use of the two-hybrid system, the PRP9ACH2 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 PRP9ACH2 protein (data not shown). These results strongly suggest that heterologous dimers can form in cells either transformed with the PRP9ACH2 or the CH2 point mutant plasmids. Thus, the observed dominant lethal phenotype of the PRP9ACH2 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 (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...
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. 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 prespliceosome 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 1993a).

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

| Combination |  β-Galactosidase activity (units) |
|-------------|----------------------------------|
| **A**       |                                  |
| (the SPP91 gene in the pGAD2F plasmid) |  |
| PRP9        | 65.9 ± 4.2                       |
| PRP9ΔCH1     | 1.3 ± 0.3                        |
| PRP9ΔCH2     | 63.9 ± 3.4                       |
| PRP9–C423    | 29.3 ± 5.6                       |
| PRP9–C426    | 26.2 ± 1.1                       |
| PRP9–H440    | 31.8 ± 2.2                       |
| PRP9–H446    | 44.6 ± 2.1                       |
| **B**       |                                  |
| (the SPP91 gene in the pMA424 plasmid) |  |
| PRP9        | 56.4 ± 3.7                       |
| PRP9ΔCH1     | <0.1                             |
| PRP9ΔCH2     | 19.4 ± 4.6                       |
| PRP9–C423    | 61.3 ± 3.9                       |
| PRP9–C426    | 31.9 ± 1.8                       |
| PRP9–H440    | 51.7 ± 4.1                       |
| PRP9–H446    | 67.4 ± 3.6                       |

Y526 cells were transformed with the indicated plasmids and assayed for β-galactosidase activity.
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**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 [Scrathin and Rosbash 1989]. (Left, lanes 1-4) Kinetic analysis of splicing complexes formation. (Middle, lanes 5-12) Inhibition of splicing reactions with 32 ~μg 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 ~μg, respectively]. (Right, lanes 13-18) Immunodepletion of a splicing extract with various amounts of preimmune [lane 13] 48 ILl or anti-PRP9 [(lanes 14-18) 48, 24, 12, 6, and 3 ILl, 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 PRP9ΔCH2 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 Choulika 1990]. The canonical TFIII A 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 homodimerization 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 PRP9ΔCH2 protein homodimerization is reduced, but heterologous dimers, e.g., prp9-1/PRP9ΔCH2, form normally. The inability of

| Proteins     | Predicted Interactions | Phenotype |
|--------------|------------------------|-----------|
| PRP9         |                        | WT        |
| PRP9ΔCH2     |                        | lethal    |
| PRP9ΔCH2     |                        | lethal    |
| PRP9-1       |                        | ts        |
| PRP9-1 and PRP9ΔCH2 |                | lethal    |

**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 prp9ΔCH2 mutant and the results of binding assays (see Discussion).
the PRP9-1/PRP9ΔCH2 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 PRP9ΔCH2 mutant does not result from a lack of homodimerization with the PRP9-1 protein but, rather, that the PRP9-1/PRP9ΔCH2 dimer is not functional. Therefore, we propose that PRP9 homodimers bind an unknown factor (Fig. 8, X factor). PRP9-1/PRP9ΔCH2 and PRP9ΔCH2/PRP9ΔCH2 dimers cannot bind the X factor and are defective. Because the PRP9ΔCH2 molecules bind the SPP91 protein with a much higher efficiency than the PRP9-1 molecules, PRP9-1/PRP9ΔCH2 and PRP9ΔCH2/PRP9ΔCH2 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.
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 pCAD2F 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 PRP3 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 Δ2 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 μl 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 Δ2 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 μl of splicing extract. After centrifugation, 4 μl of supernatant was removed and used for in vitro splicing reactions.

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