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Functional association of essential splicing factor(s) with PRP19 in a protein complex

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We have previously shown that the yeast PRP19 protein is a spliceosomal component, but is not tightly associated with small nuclear RNAs. It appears to associate with the spliceosome concomitant with or just after dissociation of the U4 small nuclear RNA during spliceosome assembly. We have found that PRP19 is associated with a protein complex in the splicing extract and that at least one of the associated components is essential for splicing. Taking advantage of the epitope tagging technique, we have isolated the PRP19-associated complex by affinity chromatography. The isolated complex is functional for complementation for the heat-inactivated *prp19* mutant extract, and consists of at least seven polypeptides in addition to PRP19. At least three of these can interact directly with the PRP19 protein. We also show that the PRP19 protein itself is in an oligomeric form, which might be a prerequisite for its interaction with these proteins.

Key words: protein complex/PRP19/splicing factors

Introduction

Splicing of pre-mRNA involves orderly interactions of small nuclear ribonucleoprotein particles (snRNPs) and other protein factors to assemble the spliceosome (Pikielny *et al.*, 1986; Bindereif and Green, 1987; Cheng and Abelson, 1987; Konarska and Sharp, 1987; Lamond *et al.*, 1988). A key to understanding the mechanism of the splicing reaction lies in understanding the functions of these *trans*-acting factors. Five small nuclear RNAs (snRNAs) are known to be involved in the splicing reaction (for reviews, see Maniatis and Reed, 1987; Steitz *et al.*, 1988; Guthrie, 1991; Moore *et al.*, 1993). Their roles in spliceosome assembly have been extensively studied in both yeast and mammals. Base-pairing mediated interactions between snRNPs, and between snRNPs and the pre-mRNA, appear to play important roles in assembly of the spliceosome (Zhuang and Weiner, 1986, 1989; Parker *et al.*, 1987; Wu and Manley, 1989, 1991; Hausner *et al.*, 1990; Datta and Weiner, 1991; Newman and Norman, 1991, 1992; Madhani and Guthrie, 1992; Reich *et al.*, 1992; Sawa and Abelson, 1992; Sawa and Shimura, 1992; Wassarman and Steitz, 1992).

Assembly of the spliceosome is a multi-step process and involves sequential binding of the snRNAs to the pre-mRNA in the order U1, U2 then U4/U6 plus U5 as a preformed tri-snRNP. After all five snRNAs have become associated with the pre-mRNA, U4 becomes only loosely bound (Pikielny *et al.*, 1986; Bindereif and Green, 1987; Cheng and Abelson, 1987; Konarska and Sharp, 1987), and does not participate in the subsequent splicing reactions (Yean and Lin, 1991), suggesting unwinding of the U4/U6 duplex. A large conformational rearrangement of the spliceosome occurs concomitant with U4 dissociation as the modes of interaction between the pre-mRNA and snRNAs change. New base-pairings between U5 and the pre-mRNA, and between U6 and the 5′-splice site region of the pre-mRNA are detected (Wassarman and Steitz, 1992).

Increasing amounts of information on the functions of protein factors have also accumulated recently. The SR proteins are a family of evolutionarily conserved pre-mRNA splicing factors, which share an RNA recognition motif and a serine/arginine-rich domain, identified in higher eukaryotic cells (Mayeda *et al.*, 1992; Zahler *et al.*, 1992). These proteins, including SC35, SF2/ASF, and U2AF and *Drosophila* splicing regulators, have been demonstrated to play a role in regulating differential splicing of different introns (Ge and Manley, 1990; Krainer *et al.*, 1990; Valcárcel *et al.*, 1993; Zahler *et al.*, 1993), possibly by forming specific committed complexes with pre-mRNAs in a concentration-dependent manner (Fu, 1993). Several other protein factors, including intron-binding protein (Gerke and Steitz, 1986; Tazi *et al.*, 1986), polypyrimidine tract-binding protein (PTB) (Garcia-Blanco *et al.*, 1989), heterogeneous nuclear RNP C protein (Choi *et al.*, 1986), and PTB-associated splicing factor (PSF) (Patton *et al.*, 1993), are also involved in pre-mRNA splicing. More than 20 proteins are found to be associated with mammalian spliceosome (Bennett *et al.*, 1992). However, the function of these proteins is not known.

Identification of proteins essential for pre-mRNA splicing in yeast has been greatly facilitated by the power of yeast genetics. Nearly 40 genes encoding splicing factors, named *PRP* for precursor RNA processing, have been identified in screens for temperature-sensitive mutants defective in pre-mRNA splicing, or by isolation of suppressors of mutations in *PRP* or snRNA genes or in an intron (for reviews, see Ruby and Abelson, 1991; Rymond and Rosbash, 1992).

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Most of the *PRP* genes have been cloned and several of their gene products characterized biochemically. PRP4 and PRP6 are part of the U4/U6 snRNP (Banroques and Abelson, 1989; Bjørn *et al.*, 1989; Abovich *et al.*, 1990), and PRP8 and PRP18 are part of the U5 snRNP (Lossky *et al.*, 1987; Horowitz and Abelson, 1993a). PRP8 and PRP11 are integral components of the spliceosome (Chang *et al.*, 1988; Whittaker *et al.*, 1990). PRP2 is required for the first cleavage-ligation reaction, but is dispensable for assembly of the spliceosome (Lin *et al.*, 1987). PRP16, PRP17/SLU4 and PRP18 are required only for the second step of the splicing reaction (Schwer and Guthrie, 1991; Frank *et al.*, 1992; Horowitz and Abelson, 1993b). SLU7, identified by screening for synthetic lethals, is also involved in the second cleavage-ligation reaction, and can mediate 3′ splice site choice (Frank and Guthrie, 1992). PRP22 is required for the release of the mature message from the spliceosome (Company *et al.*, 1991). Both PRP2 and PRP16 have been demonstrated RNA-dependent NTPase activity (Schwer and Guthrie, 1991, 1992; Kim *et al.*, 1992). PRP16 is further proposed to play a role in promoting the fidelity of mRNA splicing in an ATP hydrolysis-dependent manner (Burgess and Guthrie, 1993), reminiscent of NTPases believed to enhance accuracy in other macromolecular biological processes (Thompson, 1988).

Interactions between splicing components have been studied genetically. It has been suggested that PRP16, PRP17/SLU4, PRP18, SLU7 and the U5 snRNA interact functionally. The U5 snRNP may play a role in coordinating a set of factors required for the second catalytic step of the splicing reaction (Frank *et al.*, 1992). Interactions between PRP9, PRP11 and PRP21/SPP91 have also been observed (Legrain and Chapon, 1993). It was further proposed that in an early step of the spliceosome assembly process, PRP5, PRP9, PRP11 and PRP21/SPP91 interact to promote U2 snRNP binding to the pre-mRNA (Ruby *et al.*, 1993). A PRP9-related splicing factor, SF3a, has consistently been identified in the mammalian system, and has been demonstrated to interact with U2 snRNP in the presence of another splicing factor, SF3b (Brosi *et al.*, 1993). Thus, in addition to the base-pairing mediated RNA−RNA interaction, interactions between proteins that occur during spliceosome assembly also play important roles in splicing.

We have previously shown that the yeast PRP19 protein is essential for the pre-mRNA splicing reaction *in vitro*. It is not tightly associated with snRNAs, but is associated with the spliceosome during the splicing reaction (Cheng *et al.*, 1993; Tarn *et al.*, 1993a). We have also shown that PRP19 becomes associated with the spliceosome concomitant with or just after dissociation of U4 from the spliceosome (Tarn *et al.*, 1993b), suggesting a possible role for mediating conformational rearrangement at this step of the spliceosome assembly process. Unlike most of the other PRP proteins, PRP19 contains no discernible motif, and has no homology to sequences in the GenBank/EMBL database (Cheng *et al.*, 1993).

In an attempt to purify the PRP19 protein for functional studies, we found that in the splicing extract, the PRP19 protein is associated in a protein complex with other components. At least one of these associated components is also essential for splicing. We isolated the PRP19-associated complex by affinity chromatography, and identified at least seven polypeptides, in addition to PRP19, in this complex. Three of these proteins appear to interact with PRP19 directly. We also show here that the PRP19 protein is in oligomeric form, which might be a prerequisite for its interaction with other components.

Results

Overproduction of the PRP19 protein does not overproduce the activity that complements the PRP19-immunodepleted extract or the heat-inactivated prp19 mutant extract

In order to purify the PRP19 protein, PRP19 was first expressed in *Escherichia coli* under the control of T7 RNA polymerase (Studier *et al.*, 1990). However, the *E.coli*-produced PRP19 protein failed to complement the PRP19-immunodepleted extract or the heat-inactivated *prp19* mutant extract (data not shown). It is possible that PRP19 expressed in *E.coli* was not properly folded or modified to assume its active structure. Alternatively, if PRP19 is physically associated with other components in the extract, immunodepletion of PRP19 or heat-inactivation of the *prp19* mutant extract may result in depletion or inactivation of another essential splicing factor(s) acting in concert.

To distinguish between these alternatives, a yeast strain which carries a multi-copy plasmid, YEP.PRP19, and overproduces the PRP19 protein (Cheng *et al.*, 1993) was used to assay for overproduction of the complementation activity. The amount of the PRP19 protein produced from the overproducer and non-overproducer strains was first assessed by immunoblot analysis with anti-PRP19 antibody. As shown in Figure 1A, PRP19 was not detected with up to 10 μl of the extract from a regular strain, whereas PRP19 was detected with 1 μl of the extract from the same strain harboring YEP.PRP19. This shows that the PRP19 protein was overproduced at least 10-fold in this overproducer strain.

Extracts prepared from these two strains were then serially diluted and used for complementation of the PRP19-immunodepleted extract or the heat-inactivated *prp19* mutant extract. We previously constructed a yeast strain in which the PRP19 protein is tagged with the HA epitope recognized by the monoclonal antibody 12CA5 (Tarn *et al.*, 1993a). We demonstrated that the splicing activity of extracts prepared from such a strain can be depleted by preincubation of the extract with 12CA5 antibody-coupled protein A−Sepharose (Tarn *et al.*, 1993a). The epitope-tagged extract, after preincubation with 12CA5 antibody-coupled protein A−Sepharose, was used as the PRP19-depleted extract in these experiments.

As shown in Figure 1B, while the complementing extracts by themselves at the highest amount used in the complementation assays (1 μl) gave no splicing activity (last two lanes), the complementation activity for the PRP19-immunodepleted extract decreased with decreasing amounts of extracts from both the regular and the overproducer strains. Nevertheless, the amounts of the complementation activity were similar in these two extracts. When the extracts were assayed for complementation for the heat-inactivated *prp19* mutant extract, the same results were obtained: both the regular and the overproducer strains showed similar amounts of complementation activity as shown in Figure 1C. These results indicate that although it overproduced the PRP19 protein >10-fold, the strain harboring plasmid YEP.PRP19 did not overproduce the

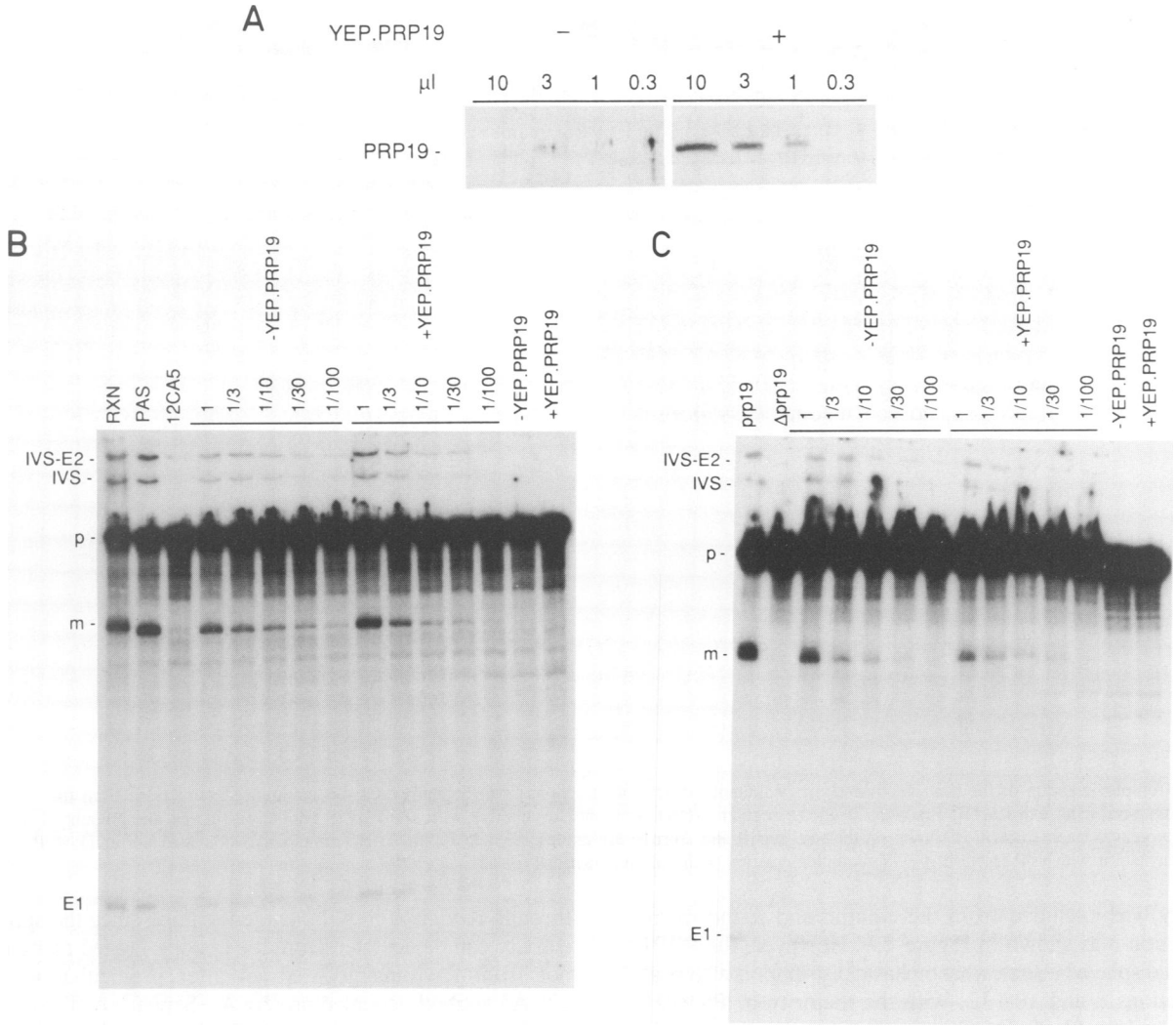

Fig. 1. Complementation of the PRP19-immunodepleted extract or the heat-inactivated *prp19* mutant extract with extracts overproducing or not overproducing the PRP19 protein. (**A**) Immunoblot analysis of extracts prepared from yeast strains harboring (+) or not harboring (−) plasmid YEP.PRP19 with anti-PRP19 antibody. (**B**) Extracts prepared from yeast strains harboring (+YEP.PRP19) or not harboring (−YEP.PRP19) plasmid YEP.PRP19 were serially diluted in buffer D and used for complementation of the PRP19-immunodepleted extract. The amounts of the complementing extracts used after dilution were equivalent to 1, 1/3, 1/10, 1/30 and 1/100 μl. An epitope-tagged strain was used to prepare the extract for depletion of PRP19 with monoclonal antibody 12CA5 (see text). Lane 1, the splicing reaction of the extract without treatment (RXN); lane 2, the extract was preincubated with protein A−Sepharose (PAS); lane 3, the extract was preincubated with the 12CA5 antibody to deplete PRP19; lanes 4−8, complementation of the depleted extract with various amounts of extracts prepared from a regular strain; lanes 9−13, complementation of the depleted extract with various amounts of extracts prepared from the PRP19-overproducing strain; lane 14, the splicing assay of 1 μl of the regular extract; lane 15, the splicing assay of 1 μl of the overproducer extract. (**C**) The same as (B) except the heat-inactivated *prp19* mutant extract was used for the complementation assay. Lane 1, the splicing assay of the *prp19* mutant extract (prp19); lane 2, the splicing assay of the heat-inactivated *prp19* mutant extract (Δprp19).

activity that complemented the PRP19-immunodepleted extract or the heat-inactivated *prp19* mutant extract.

Separation of the PRP19-complementing activity from the majority of the PRP19 protein as a complex of higher molecular weight in the PRP19-overproducing extract

A possible explanation for the above observation is that PRP19 is associated with some other essential splicing factor(s), which was depleted from the extract by the antibody in concert with PRP19 in the PRP19-immunodepleted extract. As a consequence, addition of the PRP19 protein alone could not restore the splicing activity of the depleted extract. In the *prp19* mutant extract, this PRP19-associated complex may dissociate after heat treatment, and cannot be reconstituted to form the functional complex even upon addition of the wild-type PRP19 protein. In this case, fractionation of the splicing extract from the overproducer strain may separate the complementation activity as a complex form from the uncomplexed PRP19 protein.

Extracts prepared from both the PRP19-overproducing strain and the regular strain were precipitated with 40% ammonium sulfate, and the pellet fraction (40P), enriched for the PRP19 complementation activity, was chromatographed on a Sepharose CL-6B column. The resulting fractions were assayed for complementation for the heat-inactivated *prp19* mutant extract, and for the PRP19 protein by Western blot analysis. When the extract prepared from the overproducer strain was used, the complementation

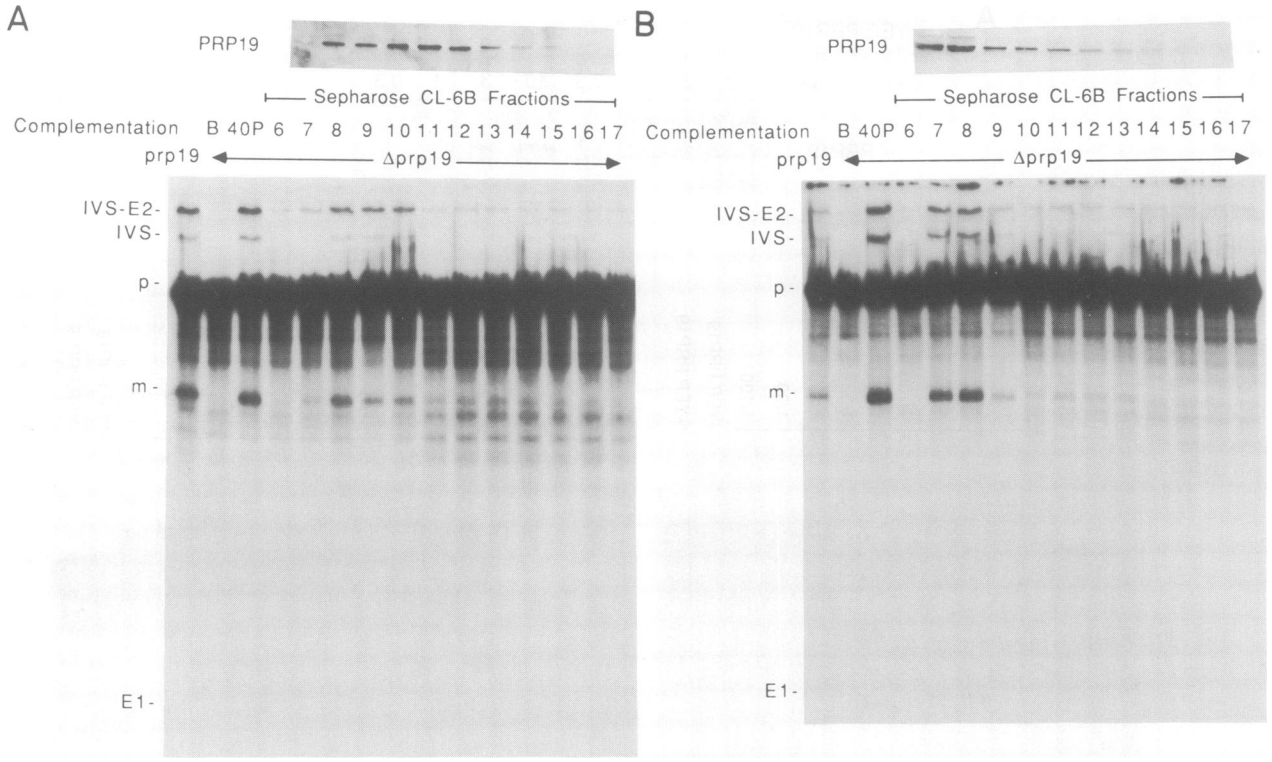

Fig. 2. Complementation of the heat-inactivated *prp19* mutant extract with fractions from Sepharose CL-6B column. (**A**) Fractions from the PRP19-overproducing extract. (**B**) Fractions from the regular extract. Immunoblot analysis with anti-PRP19 antibody to reveal the amount of the PRP19 protein in each fraction is shown on the top. prp19 and Δprp19 are as described in Figure 1; B, complementation with buffer D; 40P, complementation with 40P fraction; 6–17, complementation with column fractions 6–17.

activity was excluded from the column and found mainly in fraction 8 as shown in Figure 2A. However, the PRP19 protein displayed a somewhat biphasic distribution with peaks in fractions 8 and 10–12, with the majority of PRP19 in fractions 10–12. This indicates that the complementation activity was separated from the majority of the PRP19 protein in the PRP19-overproducing extract, and was fractionated as a higher molecular weight protein. In contrast, when a regular extract was used, the complementation activity and the PRP19 protein cofractionated, both of which were excluded from the Sepharose CL-6B column as shown in Figure 2B such that both the complementation activity and the PRP19 protein were fractionated in fractions 7 and 8. These results indicate that the complementation activity resides in a larger complex than the free PRP19 protein. This complex may consist of other essential splicing factor(s) in association with PRP19.

Isolation of the PRP19-associated complex by affinity chromatography

To study the function of PRP19, it is important to isolate the PRP19-associated complex. The obvious difficulty with purification of yeast splicing factors without overproduction is the low abundance of these factors due to limited numbers of intron-containing genes in the yeast *Saccharomyces cerevisiae*. A yeast strain in which the PRP19 protein is tagged with a hemagglutinin epitope recognized by the monoclonal antibody 12CA5 (Tarn *et al.*, 1993a) was used to facilitate isolation of the PRP19-associated complex. We have previously shown that the 12CA5 antibody specifically recognizes the tagged PRP19 protein in the splicing extract through recognition of the tagged epitope since the splicing activity can be depleted from the epitope-tagged extract but not from the untagged extract after preincubation with 12CA5 antibody-coupled protein A–Sepharose (Tarn *et al.*, 1993a). We anticipated that the PRP19-associated complex could be isolated from this epitope-tagged extract as a functional form by binding to the 12CA5 antibody-conjugated Sepharose column followed by elution with the peptide of the 12CA5 epitope.

We began by testing for isolation of the spliceosome using this technique. The splicing reaction was carried out using the epitope-tagged extract and the reaction mixture was precipitated with 12CA5 antibody-coupled protein A–Sepharose. After extensive washing to remove unbound material, the precipitate was incubated with the 12CA5 peptide for elution of the bound materials. As shown in Figure 3, the splicing intermediates and the pre-mRNA were precipitated by the 12CA5 antibody as demonstrated previously (Tarn *et al.*, 1993a). After incubation with the 12CA5 peptide, ~80% of the precipitated RNAs became soluble in the supernatant fraction, and 20% of the RNAs retained in the precipitate. In the control experiment, when the peptide was not included during incubation, all the precipitated RNAs remained insoluble. This indicates that the epitope-tagged PRP19 protein in association with the spliceosome can bind to 12CA5 antibody-coupled protein A–Sepharose, and is eluted by incubation with the 12CA5 peptide.

To isolate the PRP19-associated complex from the epitope-tagged strain, the IgG fraction of the 12CA5 ascites fluid was first isolated by chromatography on the protein

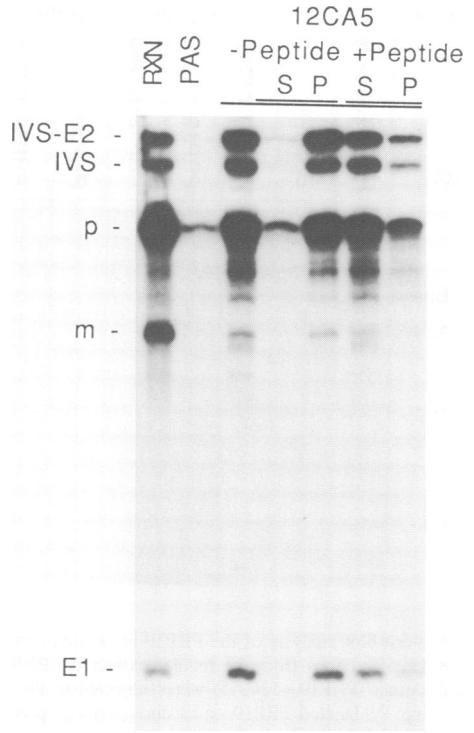

Fig. 3. Affinity isolation of the spliceosome. The spliceosome was formed in the epitope-tagged extract, and precipitated with 12CA5 antibody-coupled protein A−Sepharose. The precipitate was incubated in the absence (−Peptide) or presence (+Peptide) of the 12CA5 epitope. RNA from both pellet (P) and supernatant (S) fractions was analyzed. RXN, the splicing reaction; PAS, precipitation with protein A−Sepharose.

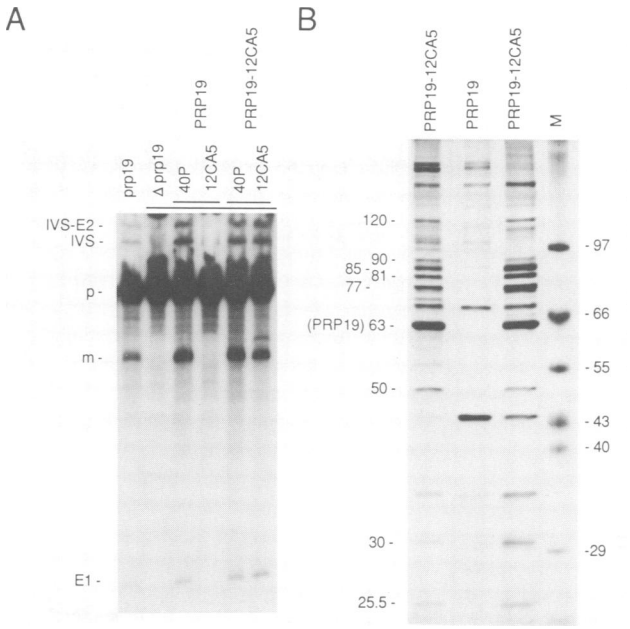

Fig. 4. Affinity purification of the PRP19-associated complex. The 40P fraction prepared from the regular strain (PRP19) or the epitope-tagged strain (PRP19-12CA5) was applied to the 12CA5 antibody column, and the bound complex was eluted with the 12CA5 peptide. (**A**) Complementation assays of the isolated fractions using the heat-inactivated *prp19* mutant extract. *prp19* and *Δprp19* are as described in Figure 1. PRP19 and PRP19-12CA5 represent the untagged and tagged extracts, respectively. 40P and 12CA5 represent complementation of heat-inactivated prp19 extracts with 40P and affinity isolated fractions, respectively. (**B**) Analysis of protein compositions of isolated fractions by SDS−PAGE. M, molecular weight marker. The approximate molecular weights of the polypeptides specifically present in the tagged extract are indicated.

A−Sepharose column, and then conjugated to CNBr-activated Sepharose. Splicing extracts prepared from the epitope-tagged strain were precipitated with 40% saturated ammonium sulfate and the pellet fraction was applied to the 12CA5 antibody column. After recycling four times and extensive washing of the column, the bound materials were eluted with 0.6 mM of the 12CA5 peptide. If the eluted fraction contained the intact PRP19-associated complex, then it should be able to complement the PRP19-immunodepleted extract or the heat-inactivated *prp19* mutant extract. As a control, a parallel experiment was performed using the untagged extract. One expects complementation with the 40P fraction from both the tagged and untagged extracts, but with the affinity isolated fraction only from the tagged extract. The result is shown in Figure 4A. Indeed, the affinity isolated fraction was functional for complementation of the heat-inactivated *prp19* mutant extract only from the epitope-tagged extract and not from the untagged extract, while the 40P fractions from both the tagged and untagged extracts were functional. This demonstrates that the affinity isolated fraction contained all the components, very likely as a stable complex associated with PRP19, required for the complementation activity.

For identification of components associated with the PRP19 protein, the protein compositions of the affinity isolated fractions from both the tagged and untagged extracts were examined by SDS−PAGE. As shown in Figure 4B, several polypeptides were present in the affinity column fractions from both tagged and untagged extracts. PRP19 and several other polypeptides were present only in fractions from the tagged extract. For comparison, two preparations of the affinity isolated fraction from the tagged extract are shown here. Polypeptides of estimated sizes 120, 90, 85, 81, 77, 50, 30 and 25.5 kDa were identified that are probably associated with PRP19. None of these proteins reacted with anti-PRP19 antibody (data not shown), suggesting that they are not proteolytic fragments of PRP19. Northern blot analysis revealed no detectable amount of snRNA associated with the affinity isolated fraction (data not shown), consistent with our previous results that PRP19 is not a component of snRNPs (Tarn *et al.*, 1993a).

To confirm further that the proteins isolated together with PRP19 from the antibody column are associated with PRP19 as a complex, the fraction isolated from the antibody column was subjected to sedimentation analysis on 10−30% glycerol gradients. Fractions collected from the gradient were analyzed for their complementation activity and for their protein composition by SDS−PAGE. As shown in Figure 5A, the complementation activity for the heat-inactivated *prp19* mutant extract sedimented as a broad peak mainly between fractions 4 and 14. When analyzed for the protein composition, as shown in Figure 5B, most of the PRP19 protein sedimented near the top of the gradient as a nonfunctional form. Only a fraction of PRP19 sedimented as larger complexes. Proteins of 120, 90, 85, 81 and 77 kDa apparently cosedimented with the complementation activity, and were also enriched in fractions 4−14. The 50 kDa

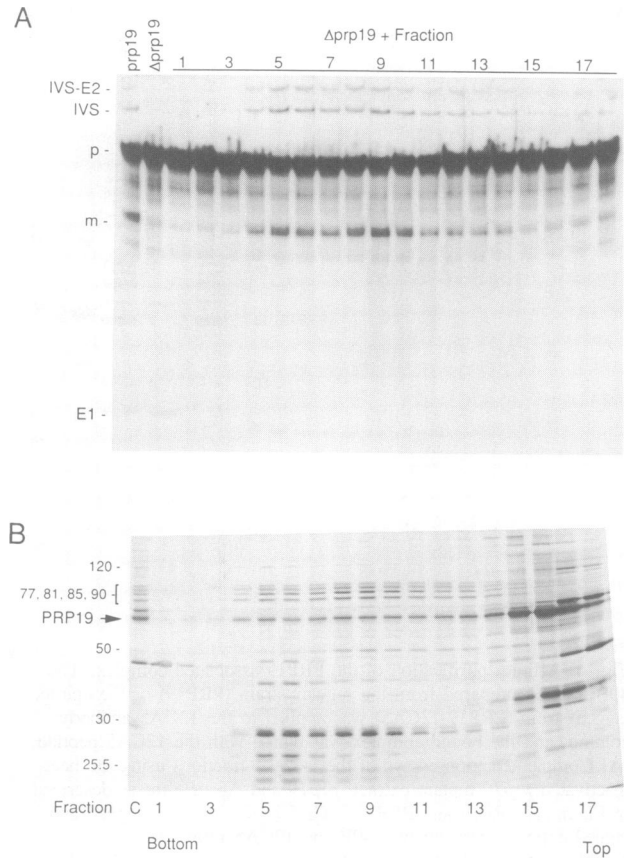

Fig. 5. Sedimentation analysis of the isolated PRP19-associated complex on a 10–30% glycerol gradient. (**A**) Complementation assays of the gradient fractions using the heat-inactivated *prp19* mutant extract. *prp19* and *Δprp19* are as described in Figure 1. (**B**) Analysis of protein compositions of gradient fractions by SDS–PAGE. The approximate molecular weights of the polypeptides cosedimenting with the complementation activity are indicated.

protein, however, did not cosediment with the complementation activity, suggesting that it was not associated with PRP19. On the gradient, several low molecular weight proteins, while not clearly detected when isolated from the affinity column, were enriched in fractions 5–10, particularly in fractions 5 and 6. Comparison with proteins from the purified yeast ribosome and Northern blot analysis of the gradient fractions using rDNA as probes suggest that these proteins are ribosomal proteins (data not shown). Thus, fractions 5 and 6 may represent association of the PRP19-associated complex with the ribosome, and the PRP19-associated complex itself was mainly in fractions 8–10. In these conditions, the spliceosome also sediments in fractions 8–10 (data not shown). Therefore, the PRP19-associated complex has a size comparable to that of the spliceosome. The 30 and 25.5 kDa proteins, although migrating closely to two of the ribosomal proteins, also appeared to cosediment with the complementation activity. Therefore, we tentatively conclude that the 120, 90, 85, 81 and 77 kDa proteins, and maybe also the 30 and 25.5 kDa proteins, are probably associated with PRP19 in a protein complex, although we cannot exclude the possibility that some of the small proteins are proteolytic fragments of the larger ones. At least one of these proteins is also essential for splicing.

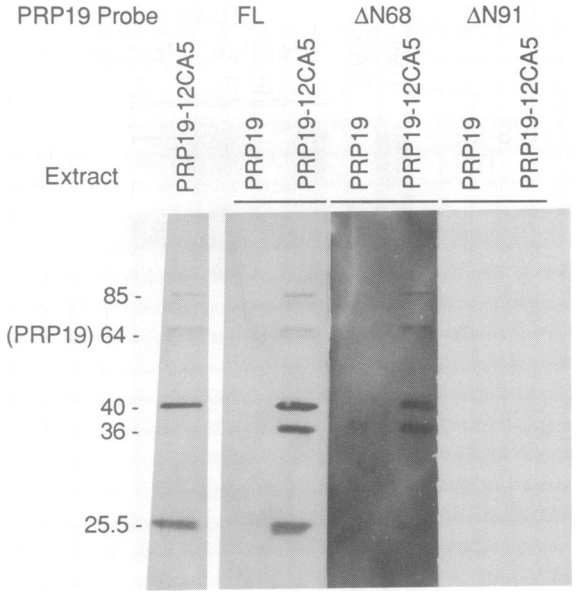

Fig. 6. Far Western blot analysis of the PRP19-associated complex. The affinity isolated fractions from the regular extract (PRP19) or the epitope-tagged extract (PRP19–12CA5) were subjected to far Western blot analysis using ^{35}S-labeled PRP19 or its derivatives as probes. The approximate molecular weights of proteins interacting with the probe are indicated. FL, full-length PRP19; ΔN68, deletion of 68 amino acid residues from the N-terminus of PRP19; ΔN91, deletion of 91 amino acid residues from the N-terminus of PRP19.

Identification of proteins directly interacting with PRP19 by far Western blot analysis

The proteins co-isolated with PRP19 from the affinity column may interact with PRP19 directly or via other protein(s) in the complex. To identify proteins directly interacting with PRP19, proteins isolated from the antibody column were subjected to far Western blot analysis (Ferrell and Martin, 1991), using *in vitro* translated, ^{35}S-labeled PRP19 as a probe. Proteins were fractionated by SDS–PAGE and then transferred to PVDF membranes. After denaturation and renaturation, the membrane was incubated with the ^{35}S-labeled PRP19 protein, which will bind to those proteins that could directly interact with PRP19.

As shown in Figure 6, while none of the proteins isolated from the untagged extract interacted detectably with PRP19, five of those isolated from the epitope-tagged extract (estimated sizes 25.5, 36, 40, 64 and 85 kDa) interacted with the labeled PRP19 protein. The 64 kDa protein is PRP19, which is in an oligomeric state when not associated with other proteins (see below). The 36 kDa protein did not appear in all preparations (see the first lane for a different preparation) and, therefore, may represent a proteolytic fragment of some other protein. The 25.5 and 85 kDa proteins were also detected by silver staining as shown in Figure 4B. Nevertheless, the 40 kDa protein seen on the far Western blot was not detected by silver staining. This is not surprising since it is known that not all proteins are stained by silver. Therefore, the 25.5, 40 and 85 kDa proteins are among those in the PRP19-associated complex interacting with PRP19 directly.

Two N-terminally truncated forms of PRP19 were constructed to examine the importance of the N-terminal region of the PRP19 protein in its interaction with other

proteins. When the proteins in the PRP19-associated complex were probed on a far Western blot with PRP19 whose N-terminal 68 amino acids are deleted (ΔN68), all five proteins that interacted with the full-length PRP19 were also detected (Figure 6). This indicates that the first 68 amino acid residues of the PRP19 protein are not essential for the interaction. However, when the first 91 amino acid residues of PRP19 were deleted (ΔN91), almost no interaction was detectable. Furthermore, when excess amounts of the unlabeled full-length, ΔN68 or ΔN91 PRP19 proteins purified from *E.coli* were used for competition with the labeled full-length probe, only the full-length and ΔN68, but not the ΔN91 proteins competed away the interactions (data not shown). This indicates that some sequences between amino acid residues 68 and 91 are important for the interaction of PRP19 with other proteins.

The PRP19 protein is in an oligomeric state

Initial characterization of the PRP19 protein overproduced in yeast showed that this protein, when not associated with the functional complex, behaves as if its molecular weight was much higher than that calculated (data not shown). It is possible that the overproduced protein, although not in a functional complex, may be associated with a subset of the proteins in the functional complex, or with other proteins in the yeast extract. We then partially purified the PRP19 protein from *E.coli*, and determined its molecular weight by gel filtration chromatography on the Sepharose CL-6B column. Fractions collected from the column were subjected to Western blot analysis for detection of PRP19. Protein molecular weight markers for gel filtration, thyroglobulin (669 kDa), apoferritin (443 kDa) and β-amylase (200 kDa), were used for the calibration. As shown in Figure 7A, the PRP19 protein has a mol. wt of ~230 kDa. The partially purified PRP19 protein was also examined by electrophoresis on a size exclusion nondenaturing polyacrylamide gel (Andersson *et al.*, 1972) and estimated to have a mol. wt of ~220 kDa (data not shown). The *in vitro* translated PRP19 protein or the protein purified from the yeast overproducer strain showed the same molecular weight on gel filtration (data not shown). These results suggest that the PRP19 protein may be in an oligomeric state, since monomeric PRP19 is 56.5 kDa, when not associated with the functional complex. Alternatively, the protein may be in an extensively elongated structure that gives a much higher apparent molecular weight on gel filtration or size exclusion gel electrophoresis.

To assess the sequence dependence of the protein structure, several truncated forms of PRP19 were examined for their chromatographic behavior on gel filtration. These proteins were expressed in *E.coli* and partially purified by chromatography on a phosphocellulose column. As shown in Figure 7B, full-length PRP19 was excluded nearly completely from a Bio-gel A 0.5m column, as expected. Proteins with up to 68 amino acid residues of the N-terminal region deleted (ΔN22, ΔN46 and ΔN68) retained similar chromatographic behavior, suggesting no drastic change in protein structure. However, when 91 N-terminal amino acid residues were deleted, the protein behaved as if it were much smaller, i.e with its calculated molecular weight. This suggests that some sequences between amino acid residues 68 and 91 are responsible for such a structural change. It

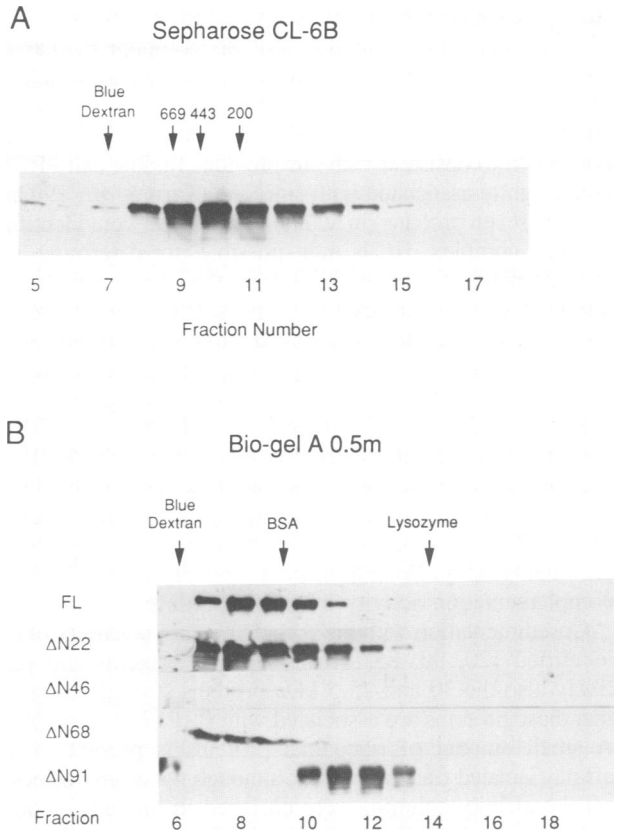

Fig. 7. Gel filtration chromatography of PRP19 and its derivatives expressed in *E.coli*. (**A**) Chromatography of PRP19 on a Sepharose CL-6B column. Eluted positions of molecular weight markers for gel filtration are indicated. (**B**) Chromatography on a Bio-gel A 0.5m column of PRP19 and PRP19 with different lengths of the N-terminal region deleted.

is less likely that increasing the size of the deletion from 68 to 91 amino acid residues resulted in a large conformational change of the monomeric PRP19 protein rather than disruption of intermolecular interaction between PRP19 proteins. Therefore, we conclude that the anomalous chromatographic behavior of the PRP19 protein was due to oligomerization, and that the N-terminal sequence between amino acid residues 68 and 91 is essential for oligomerization. Since we have previously shown that this part of the sequence was also essential for interaction of PRP19 with other proteins in the PRP19-associated complex, it is possible that oligomerization is a prerequisite for interaction of PRP19 with other proteins.

Discussion

The PRP19 protein is essential for splicing of pre-mRNAs both *in vivo* and *in vitro*. Biochemical characterizations reveal that PRP19 is not a component of snRNPs, but is associated with the spliceosome during the splicing reaction. It appears to associate with the spliceosome concurrently with or just after dissociation of U4 snRNA. A major conformational rearrangement of the spliceosome occurs during this transition. New base-pairings between U5-pre-mRNA and U6-pre-mRNA are detected (Wassarman and Steitz, 1992). PRP19 may play a role in mediating such structural change.

In an attempt to purify the PRP19 protein for functional

study, we found that PRP19 is associated as a complex with at least one other essential splicing component. Taking advantage of the epitope-tagging technique, we have isolated this complex by affinity chromatography. The isolated complex contained no detectable amount of snRNAs (data not shown), consistent with our previous finding that PRP19 is not tightly associated with snRNAs (Tarn *et al.*, 1993a). At least seven proteins, in addition to PRP19, were identified in this complex. It is not possible to determine the stoichiometry of these proteins from the silver stained gels, although PRP19 appeared to be present in a much greater amount than the other associated proteins. Sedimentation analysis of the complex on glycerol gradients revealed that most of the PRP19 protein sedimented near the top of the gradient as free protein. However, a fraction of PRP19 cosedimented with those proteins associated with PRP19 in the complex and the complementation activity for the heat-inactivated *prp19* mutant extract. Apparently, a large fraction of the PRP19-associated complex dissociated during affinity chromatography. We estimated a recovery of $<5\%$ of the complementation activity by this procedure.

Cosedimentation with the complementation activity of the identified 120, 90, 85, 81 and 77 kDa proteins and very likely also the 30 and 25.5 kDa proteins strongly suggests that these proteins are associated with PRP19 as a complex. A small amount of ribosomal proteins is present in the affinity isolated fraction, which, although not clearly detected off the affinity column, were enriched in limited fractions in the glycerol gradient. This made identification of the 30 and 25.5 kDa proteins in the gradient fractions more difficult. Detection of the 25.5 kDa protein on the far Western blot further supports specific association of this protein with the PRP19-associated complex.

Sedimentation analysis revealed that the PRP19-associated complex has a size comparable to that of the spliceosome. Since PRP19 is a spliceosomal component, this raises the question whether the isolated PRP19-associated complex is the endogenous spliceosome. Northern blot analysis revealed that the isolated PRP19-associated complex does not contain detectable amounts of the spliceosomal snRNAs (data not shown). Such analysis was performed with ~ 0.5 pmol of the complex, at least 50-fold over the detectable level of snRNAs in the spliceosome. Therefore, we conclude that the PRP19-associated complex is not the endogenous spliceosome.

Why the PRP19-associated complex sedimented as a broad peak on glycerol gradients is not clear. Since there was a trace amount of ribosome in the PRP19-associated complex isolated from the affinity column, it is possible that those fractions of higher density on the glycerol gradient (fractions 5 and 6) were ribosome bound. In this case, this nonspecific association of ribosome apparently did not interfere with the complementation activity of the PRP19-associated complex.

Far Western blot analysis identified the 25.5 kDa and 85 kDa proteins and a previously unidentified 40 kDa protein directly interacting with PRP19. The 40 kDa protein, although not detected by silver staining, appeared to interact with PRP19 with high affinity and gave a very strong signal comparable to that of the 25.5 kDa protein on far Western blot. The 85 kDa protein interacted with PRP19 with somewhat lower affinity and gave a weaker signal. The PRP19 protein was also detected on far Western blot to interact with itself although with a weak signal. This is not surprising in view of the fact that PRP19 is in an oligomeric form. During incubation with the membrane, subunit exchange may have occurred, promoting formation of ^{35}S-labeled PRP19 containing oligomers on the membrane. Nevertheless, compared with the 40 and 25.5 kDa proteins, the signal of PRP19 was fairly weak on the far Western blot even though the PRP19 protein was most abundant in the affinity isolated fraction. This may be due to a limited amount of subunit exchange. We cannot exclude the possibility that the small proteins are fragments of the larger ones.

Interactions of PRP19 with the 25.5, 40 and 85 kDa proteins appeared to be direct and independent of other proteins. It is possible that some other proteins also interact with PRP19 directly, but were not detectable by far Western blot analysis. Interaction of these proteins with PRP19 may be stabilized only through interaction with other proteins, or may be too weak to be detected using this method. Therefore, the three proteins identified on the far Western blot may represent only a subgroup of proteins directly interacting with PRP19.

Initial complementation assays using the PRP19-immunodepleted extract or the heat-inactivated *prp19* mutant extract showed that overproduction of the PRP19 protein did not overproduce the complementation activity. We have purified the PRP19 protein to homogeneity from a PRP19-overproducing strain by a chromatographic procedure similar to that used for the PRP19-associated complex (Sheu and Cheng, unpublished results). The purified PRP19 protein also gave no complementation activity for either the depleted extract or the heat-inactivated *prp19* mutant extract (data not shown). It is conceivable that the splicing activity of the immunodepleted extract cannot be restored simply by addition of an excess amount of PRP19 as depletion of PRP19 with antibodies resulted in depletion of the entire PRP19-associated complex. In the case of the heat-inactivated *prp19* mutant extract, it is possible that the PRP19-associated complex was inactivated after heat treatment and was not reconstituted even upon addition of the wild-type PRP19 protein. Similar observation has been reported for PRP11 where the splicing activity of the heat-inactivated *prp11* mutant extract cannot be restored upon addition of the wild-type PRP11 protein. However, the *prp11* extract becomes heat-resistant when the wild-type PRP11 protein was added prior to heat treatment (Chang *et al.*, 1988). A PRP11-associated 30S complex was also detected by sedimentation analysis (Chang *et al.*, 1988). It was suggested that in the *prp11* mutant extract, the PRP11-associated complex dissociates upon heat treatment, and cannot be reconstituted upon addition of the wild-type PRP11 protein. However, in the presence of the PRP11 protein, subunit exchange occurs during incubation and the PRP11-associated complex becomes insensitive to heat treatment (Chang *et al.*, 1988). In the case of PRP19, reconstitution failed to occur even when the wild-type PRP19 protein was added to the *prp19* mutant extract prior to heat treatment (data not shown). Furthermore, the isolated PRP19 associated complex did not complement the heat-inactivated *prp11* mutant extract (data not shown). These results suggest that the PRP19-associated complex is distinct from the PRP11-associated 30S complex and that subunit exchange did not occur during incubation.

Protein–protein interaction has recently been demonstrated to be important for splicing of pre-mRNAs

by genetic studies. PRP16, PRP17/SLU4, PRP18 and SLU7, all of which are involved in the second cleavage-ligation reaction, are shown to interact with each other and with U5 snRNA functionally (Frank *et al.*, 1992). Interactions between PRP5, PRP9, PRP11 and PRP21/SPP91 to promote U2 snRNP binding to the pre-mRNA at an early step of the spliceosome assembly process were also observed (Legrain and Chapon, 1993; Ruby *et al.*, 1993). PRP18 is further shown biochemically to associate with U5 snRNA (Horowitz and Abelson, 1993a). In the mammalian system, a PRP9-related splicing factor SF3a is shown to interact with U2 snRNP in the presence of another splicing factor, SF3b, to generate a structure similar to 17S U2 snRNP (Brosi *et al.*, 1993). Here we demonstrate physical association of PRP19 with at least seven other proteins in a protein complex. It is not known whether all of these proteins are essential splicing factors. However, at least one of them is also essential for splicing. Biochemically, this is the first demonstration of a non-snRNP protein complex involved in pre-mRNA splicing. The fact that the wild-type PRP19 protein does not complement the heat-inactivated *prp19* mutant extract strongly suggests that interactions between some, if not all, of these proteins are important for the splicing reaction.

The PRP19 protein is in an oligomeric form when overproduced in yeast, expressed in *E.coli*, or synthesized *in vitro* in the reticulocyte lysates. This was based on characterization of the proteins by gel filtration and by electrophoresis on size exclusion nondenaturing polyacrylamide gel. The protein becomes monomeric upon deletion of 91 amino acid residues from the N-terminus, while deletion of 68 amino acid residues does not affect oligomerization. Apparently, the region between amino acid residues 68 and 91 is essential, but may not be sufficient, for oligomerization. The oligomeric state of PRP19 in the identified protein complex is not clear. Consistent with the results that deletion of 91, but not 68, amino acid residues from the N-terminus of PRP19 abolished its interaction with other proteins by far Western blot analysis, it is possible that oligomerization is a prerequisite for interaction of PRP19 with other proteins. Therefore, PRP19 may also exist as an oligomeric form in the PRP19-associated complex.

During spliceosome assembly, PRP19 becomes associated with the spliceosome concurrent with or just after dissociation of U4 snRNA. Since the size of the PRP19-associated complex is comparable to that of the spliceosome, it is unlikely that the intact PRP19-associated complex binds to the spliceosome. Dissociation of the complex may be necessary for releasing PRP19 to bind to the spliceosome. It is not clear whether some other protein(s) in this complex is also associated with the spliceosome in concert with PRP19. The oligomeric state of the PRP19 after binding to the spliceosome also awaits further investigation.

The functional role of either the PRP19 protein or the PRP19-associated complex is not known. We have previously demonstrated that PRP19 is associated with the spliceosome just after or simultaneously with dissociation of U4. In other words, U4 and PRP19 are mutually exclusive for their presence in the spliceosome (Tarn *et al.*, 1993a,b). Dissociation of U4 is accompanied by a large conformational rearrangement including formation of new base-pairing between snRNAs and the pre-mRNA (Wassarman and Steitz, 1992). PRP19 may play an important role during this transition. It is not clear whether other components associated with PRP19 are also involved in this same step or other steps of the spliceosome assembly process. One preliminary experiment showed that splicing with extracts depleted of PRP19 by antibodies failed to accumulate large amounts of the U4-containing splicing complex (data not shown). This suggests that some component in the PRP19-associated complex might be required for binding of the U4/U6−U5 tri-snRNP to the spliceosome. Currently, we are trying to isolate the PRP19-associated complex in large amounts for identification of these components. Isolation of genes encoding proteins directly interacting with PRP19 is also under way.

Materials and methods

Antibodies

The 12CA5 ascites fluid was purchased from Berkeley Antibody Company. Anti-PRP19 antibody was raised against the PRP19 protein expressed in *E.coli* as described (Cheng *et al.*, 1993).

Construction of N-terminal deletions of PRP19

Site-directed mutagenesis were performed to introduce an *Nco*I site at various positions near the N-terminus of the PRP19 protein. Oligonucleotides GCGACTTTTCCATGGTAGTCCTGGAT, CTATTTCTTCCATGGTTAAGGGCTC, TTAACGTAGCCATGGTTGTAGACTC and AAGTTTTCGACCATGGTAGCATCCCA were used for constructing ΔN22, ΔN46, ΔN68 and ΔN91, respectively. The *Nco*I fragments of the *PRP19* gene (Cheng *et al.*, 1993) were then inserted into the *Nco*I site of plasmid PAR3039-1 (Banroques and Abelson, 1989) for expression of various N-terminally deleted PRP19 in *E.coli*.

For *in vitro* translation of the PRP19 protein and its derivatives, oligonucleotide-directed mutagenesis was performed to introduce a *Pst*I site 62 nucleotides upstream of the initiation codon (for full-length) and an *Eco*RI site 9 or 81 nucleotides upstream of the second ATG (for ΔN68 and ΔN91, respectively) in the coding sequence, using oligonucleotides CTTGGAAAGTCTGCAGCTTTTAAAA, GCCATGGTTGAATTCTCTGTTAGT and CGAGCATTATGAATTCCCATTCAT, respectively. The *Pst*I−*Bam*HI fragment of the *PRP19* gene was then inserted into the *Pst*I and *Bam*HI sites of plasmid pGEM-1 (Promega), and the *Eco*RI−*Bam*HI fragment into the *Eco*RI and *Bam*HI sites of plasmid pGEM-2 (Promega) for *in vitro* transcription with SP6 RNA polymerase (Promega).

Preparation of splicing extracts and substrates

Yeast whole cell extracts and the 40P fraction were prepared according to Cheng *et al.* (1990). Actin precursors were synthesized *in vitro* using SP6 RNA polymerase according to Cheng and Abelson (1987).

Splicing assays

Splicing assays were carried out according to Lin *et al.* (1985), using uncapped actin pre-mRNA as the substrate. For complementation, each 10 μl of the reaction mixture contained 4 μl of the heat-inactivated or depleted extract and 3 μl of the complementing extracts or fractions. The *prp19* mutant extract was heat-inactivated by preincubation of the extract at 37°C for 20 min. The PRP19-depleted extract was prepared from the epitope-tagged strain as described (Tarn *et al.*, 1993a). Complementation with the PRP19-depleted extract was carried out at 23°C for 30 min, and with the heat-inactivated *prp19* extract at 15°C for 2 h.

Gel filtration chromatography

For the PRP19 protein, the partially purified PRP19 protein isolated from *E.coli* (Cheng *et al.*, 1993) was chromatographed on a Sepharose CL-6B or Bio-gel A 0.5m column pre-equilibrated with 20 mM HEPES, pH 7.9, 50 mM NaCl, 0.2 mM EDTA and 20% glycerol (buffer D). Protein molecular weight markers (from Sigma) were dissolved or diluted in buffer D at final concentrations of 15 or 30 mg/ml, and chromatographed in the same way.

For the PRP19-associated complex, to the 40P fraction prepared from the overproducer or regular extract was added KPO_4, pH 7.0, to a final concentration of 60 mM. Two hundred microliters of 40P were applied to a 5 ml column of Sepharose CL-6B pre-equilibrated with Buffer D containing 60 mM KPO_4, pH 7.0 (Buffer DK).

Affinity chromatography

The purified 12CA5 antibody was coupled to CNBr-activated Sepharose 4B at final protein concentrations of 3–5 mg/ml according to the instruction by Pharmacia. To ~150–200 mg of 40P was added KPO_4, pH 7.0, to a final concentration of 60 mM, and applied to 0.1 ml of the 12CA5-coupled Sepharose column pre-equilibrated with buffer DK. After recycling four times, the column was washed with 80 column-volumes of buffer DK containing 0.1% Nonidet P-40 (NP-40). These procedures were performed at 4°C. The column was then brought to room temperature, and further washed with 10 column volumes of buffer DK containing 0.1% NP-40. The bound materials were then eluted with 0.6 mM 12CA5 peptide diluted in buffer DK at room temperature.

Sedimentation analysis

Sedimentation analysis of the PRP19-associated complex was performed with 10–30% glycerol gradient in gradient buffer (20 mM HEPES, pH 7.9, 0.2 mM EDTA and 50 mM NaCl). The PRP19-associated complex isolated from the antibody column from 30 liters of yeast culture was first dialyzed against gradient buffer at 4°C for 2 h, and then concentrated with Centricon-30 (Amicon Inc.). The concentrated material was then applied to a 10–30% glycerol gradient in a Beckman SW60 tube, and centrifuged at 55 000 r.p.m. at 4°C for 1.5 h. Fractions of 0.25 ml were collected for further analysis.

In vitro transcription and translation

In vitro transcription was performed in 50 μl of the reaction mixture containing 40 mM Tris–HCl, pH 7.6, 10 mM NaCl, 12 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 2 mM each of ATP, CTP and UTP, 0.1 mM GTP, 1 mM of ^{7}mGpppG, 200 U of RNasin, 5 μg of template DNA and 50 U of SP6 RNA polymerase, at 40°C for 2 h. The transcripts were precipitated by addition of Tris–HCl, pH 8.0, to 0.5 M and 2 volumes of ethanol. Ethanol precipitation was repeated three times to remove unincorporated nucleotides. To monitor RNA synthesis, 5 μl of the reaction mixture were removed and supplemented with 5 μCi of [α-^{32}P]UTP, and the synthesized RNAs were analyzed by electrophoresis on a 5% polyacrylamide/8 M urea gel.

In vitro translation was performed according to the instructions of the manufacturer (Promega Biotec). A typical 50 μl of the reaction mixture contained 0.1–0.3 pmol of the transcript, 35 μl of reticulocyte lysates, 1 nmol of amino acid mixture, 40 U of RNasin, 80 pmol of [^{35}S]methionine (80 μCi), and was incubated at 30°C for 2 h. One microliter of the reaction mixture was analyzed by SDS–PAGE to quantify the amount of synthesis. When used as probes, unincorporated labels were removed by chromatography on NAP columns (Pharmacia).

Far Western blot analysis

To analyze protein interaction by far Western blot, 60 μl of the affinity-purified proteins were fractionated on 10% SDS–PAGE and then transferred onto PVDF membranes (Bio-Rad) in a buffer containing 192 mM glycine and 25 mM Tris-base. The blot was washed with TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl and 0.05% Tween 20) for 10 min and then incubated with denaturation buffer (7 M guanidine hydrochloride, 50 mM Tris-base, 50 mM DTT and 2 mM EDTA) for 1 h at room temperature. After a brief rinse with TBST, the blot was then incubated with renaturation buffer (10 mM Tris–HCl, pH 7.4, 140 mM NaCl, 2 mM DTT, 2 mM EDTA, 1% BSA and 0.1% NP-40) at 4°C overnight, followed by incubation with TBST containing 4% BSA and 1% low-fat milk, at room temperature for 30 min. The blot was washed with TBST for another 5 min, and then incubated with 5×10^5 to 1×10^6 c.p.m./ml of ^{35}S-labeled PRP19 or its derivatives in TBST containing 1% BSA, at room temperature for 2 h. The blot was then washed with TBST four times to remove unbound proteins and then autoradiographed.

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