Pre-mRNA splicing occurs in the spliceosome, a large RNA-protein complex that contains a pre-mRNA, four essential small nuclear ribonucleoprotein (snRNP) particles (U1, U2, U5, and U4/U6), and numerous non-snRNP splicing factors (1–3). Each snRNP particle consists of one (U1, U2, and U5) or two (U4/U6) snRNAs complexed with a set of Sm or Sm-like proteins and several particle-specific proteins (4–6). These snRNPs recognize conserved sequences of the pre-mRNA and assemble into a catalytically active spliceosome that catalyzes the two cleavage-ligation reactions of pre-mRNA splicing (1, 2, 7, 8). Spliceosome assembly follows an ordered pathway through the formation of several intermediate complexes (2).

Prior to the first catalytic step of splicing, important conformational rearrangements occur to create a catalytically active spliceosome. For example, U1 snRNA dissociates from the 5′-splice site, and the U4/U6 snRNA association is disrupted (14–16), leaving the U6 snRNA free to base pair with both the U2 snRNA and the 5′-splice site (17). The U2 and U6 snRNAs, together with the pre-mRNA, form the catalytic core of the spliceosome, whereas the U5 snRNP interacts with exonic sequences adjacent to both splice sites, possibly aligning the exons during splicing (18). After a round of splicing, the snRNPs are recycled to participate in further splicing, whereas the intron RNA is degraded (19–21).

The assembly of U4/U6 snRNP, its recruitment into the pre-spliceosome, and numerous conformational changes of its snRNA components are poorly understood processes. Proteins associated with U4 and U6 snRNAs are of particular interest because they could mediate the conformational changes that occur between U4 and U6 snRNAs (4, 22) and the recruitment of the U4/U6 snRNP to the pre-spliceosome (23). U4/U6 snRNP contains at least five specific proteins, viz. the 15.5-kDa protein (Smu13p in yeast) that binds the 5′-stem-loop of U4 snRNA (24–27), the 61-kDa protein (Prp31p in yeast) (4), and the heterotrimer cyclophilin H-Hprp4p-Hprp3p complex (26–29). We previously identified, along with others, Hprp3p and Hprp4p as homologs of the yeast U4/U6 snRNP-specific factors Prp3p and Prp4p, respectively (28, 30, 31). Hprp3p and Hprp4p can be isolated from HeLa cell nuclear lysates. By co-immunoprecipitation and isothermal titration calorimetry, we demonstrated that purified Hprp3p and its mutants containing the central region, but lacking either the N-terminal 194 amino acids or the C-terminal 240 amino acids, were able to interact with Hprp4p. Conversely, Hprp3p mutants containing only the N- or C-terminal region did not interact with Hprp4p. In addition, by co-immunoprecipitation, we showed that intact Hprp3p and its mutants containing the central region interacted with Hprp4p in HeLa cell nuclear extracts. Primer extension analysis illustrated that the central region of Hprp3p is required to maintain the association of Hprp3p-Hprp4p with U4/U6 small nuclear RNAs, suggesting that this Hprp3p-Hprp4p interaction allows the recruitment of Hprp4p, and perhaps other protein(s), to the U4/U6 small nuclear ribonucleoprotein particle.
two-hybrid system (30). Therefore, whether these two human splicing factors directly interact with each other and how they interact with the U4/U6 snRNP remains unclear. Understanding these interactions within the spliceosome is of particular importance because it will help to elucidate the molecular mechanisms of an essential genetic process, splicing. Here, we show that Hprp3p directly interacts with Hprp4p. Furthermore, we have identified the domain of Hprp3p involved in this interaction as being within the central region of this molecule. Our results suggest a possible role for Hprp3p in the recruitment of Hprp4p for the U4/U6 snRNP assembly.

MATERIALS AND METHODS

Preparation of HA-tagged Hprp3p Mutant Constructs for Expression in Mammalian and Escherichia coli Systems—The pcDNA3–3xHA-HPRP3 construct containing the 2.2-kb coding region of Hprp3p (31) was used as a template to create Hprp3p deletion mutants. The coding region of the mutants was amplified by PCR using the following primers: N1, 5′-aagggctgagctcagctgtctgggggtgctt-3′; N2, 5′-aa-aaggggctgagctcagctgtctgggggtgcgg-3′; N3, 5′-aagggcgggcaagaaggggcaagaagggg-3′; C1, 5′-aagggctgagctcagctgtctgggggtggg-3′; C2, 5′-aaggggctgagctcagctgtctgggggtggg-3′; and C3, 5′-agggagggcgggcaagaaggggcaagaagggg-3′. All mutagenic primers were designed with a T7 promoter sequence upstream of the HPRP3 coding sequence. The design of the Hprp3p mutant constructs is illustrated in Fig. 2. The 5′-primer N1 and either the 3′-primer C1 or C2 were used to generate deletion mutant fragments I and II, respectively. Either the 5′-primer N2 or N3 in combination with the 3′-primer C3 were used to generate deletion mutant fragments III and IV, respectively. To build mammalian expression constructs, all those from pcDNA3-HPRP3 were subcloned individually into pET28a (Novagen, Madison, WI) bearing a N-terminal His tag for expression in E. coli system. pET28a–HPRP4 was previously constructed (31). DNA sequencing with an automated DNA sequencing facility. The mouse monoclonal anti-HA antibody was purchased from BABC.

Isothermal Titration Microcalorimetry—The ITC data were generated using Microcal Origin (version 5.5). Titrations were carried out at 25 °C and 800 rpm carefully degassed before titration. Each titration experiment consisted of 28 injections of 10 μl of Hprp3p (20 μM) or Hprp3p mutant (50 μM) into a cell containing 1.6 ml of 16 μM Hprp4p. Titrations were conducted in 100 mM potassium phosphate, pH 6.0, 500 mM KCl, and 10% glycerol. Phosphate buffer was chosen by virtue of its small ionization enthalpy change. To correct for dilution and mixing effects, a series of control injections was carried out in which the heat of dilution was measured in blank titrations by injecting the protein into the buffer and then subtracted from the binding heat.

Transfection and Immunostaining—HeLa cells (ATCC CCL2) were cultured in α-minimal essential medium with 10% (v/v) fetal bovine serum at 37 °C. Cells for immunostaining were seeded in six-well plates with glass coverslips and transfected at 40–60% confluency with 1 μg of DNA and 12 μl of Lipofectamine (Invitrogen) for each well under serum-free conditions as recommended by the manufacturer. Cells for nuclear extract preparation were seeded in 10-cm dishes and transfected at 40–60% confluency with 6 μg of DNA and 72 μl of LipofectAMINE for each dish under serum-free conditions. Immunostaining was performed at room temperature 24 h post-transfection. The transfected cells grown on coverslips were washed three times with PBS and fixed for 20 min with 4% (w/v) paraformaldehyde. Fluorescence micrographs were recorded using a 100× objective. All micrographs are representative of experiments repeated at least three times.

Nuclear Extract Preparation—The HeLa nuclear extracts were prepared as described previously (34, 35). HeLa cells in suspension culture were harvested in early mid-log phase, and the pellet were washed with 5× packed cell volume and resuspended in 2× packed cell volume of buffer C (10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, and 0.5 mM dithiothreitol, pH 7.9). The cells were lysed by 30 strokes with a Dounce homogenizer (A-type pestle). The nuclei were pelleted by centrifugation at 750 × g for 5 min at 4 °C. The nuclear pellet was resuspended in buffer D (20 mM HEPES, 10% glycerol, 1.5 mM MgCl2, 0.42 M KCl, 0.5 mM dithiothreitol, 0.2 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.9). The nuclei were lysed by 30 strokes with a B-type pestle. The resulting lysate was incubated at 4 °C for 30 min with gentle agitation and then centrifuged at 25,100 × g for 30 min at 4 °C. The resulting supernatant was dialyzed against 3 liters (1 liter × 3 changes) of buffer E (20 mM HEPES, 10% glycerol, 1.5 mM MgCl2, 0.1 mM EDTA, and 0.5 mM dithiothreitol, 0.2 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.9) for 3.5 h. The nuclear extracts from HeLa cells transfected with pcDNA-HA-HPRP3 or its deletion mutants were prepared in a similar manner. The nuclear extracts were quick-frozen in liquid nitrogen and stored at −80 °C.

Primer Extension—The presence of U4 and U6 snRNAs in immunocomplexes precipitated with anti-HA antibodies was determined by precipitation.
**RESULTS**

Interaction of Hprp3p and Hprp4p Expressed in E. coli—Hprp3p and Hprp4p are known to be present in the same

primer extension. RNA extractions from immunocomplexes and primer extension were performed as described (31, 36). Two primers (U4-82, 5'-ggattggaaaaaggttcacag-3'; and U6-91, 5'-tacgatgtgggtctatcctgcc-3') that yield extension products specific to human U4 (82 nucleotides) and U6 (91 nucleotides) snRNAs, respectively, were used in the experiment. Primers were labeled at the 5'-end with [γ-32P]ATP using T4 polynucleotide kinase (MBI Fermentas Inc., Burlington, Ontario, Canada). To anneal the end-labeled primers to their target snRNAs, the

U4 and U6 primers (~5 pmol each or ~15,000 cpm) were added, individually or together as indicated in the legend to Fig. 5C, to RNA samples in a solution containing 50 mM Tris-HCl, pH 8.3, and 25 mM KCl. The mixtures were incubated at 90 °C for 2 min and then slowly cooled to 30 °C. Extension reactions (20 μl) were carried out at 37 °C for 1 h in buffer containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM dithiothreitol, 10 mM MgCl2, and 200 μM each dNTP plus 1 unit of Omniscript reverse transcriptase (QIAGEN Inc.). The extended products were phenol-extracted, ethanol-precipitated, and resuspended in 5 μl of loading buffer (85% formamide, 20 mM EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol). They were then resolved on 5% acrylamide gels containing 8 M urea and Tris borate/EDTA, and their migration positions were visualized by autoradiography. For primer extension controls, total nuclear RNAs (0.1 μg) from HeLa cells were used.
U4/U6 snRNP particle (28, 30, 31). However, their role in U4/U6 snRNP assembly and the nature of their interaction are not clear. Because the splicing machinery is absent in bacterial cells, we first studied the interaction of Hprp3p and Hprp4p using soluble cell lysates of *E. coli* cells expressing these proteins. The cDNAs encoding Hprp3p and Hprp4p were cloned into pET28a and expressed in *E. coli*. The expression conditions were optimized for the yield of soluble proteins. More than 60% of Hprp3p and Hprp4p are soluble when cells are induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside at room temperature for 6–8 h. After cell lysis under native conditions (see "Materials and Methods"), the soluble cell lysates containing Hprp3p or Hprp4p were used for co-immunoprecipitation analysis with polyclonal antibodies against Hprp3p or Hprp4p in buffer containing 150 mM KCl. As shown in Fig. 1A, Hprp3p was detected in the complex immunoprecipitated with anti-Hprp4p antibodies (lane 1), but was not present in the sample immunoprecipitated with preimmune sera (lane 4). As expected, anti-Hprp4p antibodies failed to pull-down Hprp3p in the absence of Hprp4p (Fig. 1A, lane 2), indicating that there is no cross-reaction with Hprp3p and antibodies against Hprp4p. Likewise, anti-HA antibodies did not recognize Hprp4p (Fig. 1A, lane 3), suggesting there is no cross-reaction between Hprp4p and the anti-HA antibodies used in this experiment. Reciprocal experiments using anti-Hprp3p antibodies to precipitate the complex and anti-Hprp4p antibodies to detect the presence of Hprp3p yielded the same conclusion, i.e. Hprp3p and Hprp4p are present in the same protein complex (Fig. 1B, lane 1).

Hprp3p and Hprp4p together with the 20-kDa cyclophilin H can be purified from HeLa cells as a heterotrimer protein complex in the presence of >500 mM NaCl (28). To examine whether the Hprp3p-Hprp4p complex formed in bacterial cell lysates can withstand similar conditions, co-immunoprecipitation experiments were performed with increasing salt concentrations (up to 500 mM KCl). As shown in Fig. 1C (lanes 1–3), the Hprp3p-Hprp4p complex was not disrupted even at 500 mM KCl. These results support the notion that Hprp3p and Hprp4p interact with each other. However, because these experiments were performed with soluble cell lysates, the possibility of a third partner in the system nonspecifically mediating the interaction of the two proteins cannot be discarded. For example, the interaction may proceed through another protein or even RNA that contacts both Hprp3p and Hprp4p. To exclude this possibility, His-tagged HA-Hprp3p and Hprp4p were produced and purified to >90% homogeneity using Ni²⁺-nitrilotriacetic acid affinity chromatography (Supplemental Fig. 1). Equal amounts of the two highly purified proteins were incubated and added to protein A-agarose beads pre-absorbed with antisera against Hprp4p. The presence of Hprp3p in the complex precipitated by antisera against Hprp4p was confirmed by immunoblotting using antibodies against the HA tag of Hprp3p (Fig. 2A, upper panel, lane 5). Because highly purified proteins were used in this experiment, it is unlikely that molecules in *E. coli* cell lysates would play a role in mediating the Hprp3p/Hprp4p interaction.

Central Region of Hprp3p Mediates the Hprp3p/Hprp4p Interaction—Currently, the domain structure and function of Hprp3p are virtually unknown. Because we demonstrated above that Hprp4p is likely to interact with Hprp3p, the first step toward unraveling Hprp3p domain structure and function is to determine the regions responsible for such an interaction. Therefore, we generated four HA-tagged Hprp3p deletion mutants (Hprp3p mutants I–IV) (Fig. 2B). Each mutant was cloned into pET28a for the expression of His-tagged HA-Hprp3p in *E. coli*. As with the wild-type protein, each purified
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Hprp3p mutant was incubated with purified Hprp4p, and the complex was co-immunoprecipitated with anti-Hprp4p antibodies. Similar to full-length Hprp3p, mutants II and III formed a complex with Hprp4p (Fig. 2A, upper panel, lanes 2 and 3, respectively), but mutants I and IV did not (lanes 1 and 4, respectively). This result indicates the formation of an Hprp3p-Hprp4p complex, in particular through the central region of Hprp3p, and suggests that neither the N- nor C-terminal region of Hprp3p is necessary for the interaction.

Characterization of Hprp3p/Hprp4p Interaction by ITC—ITC is an increasingly used technique for the detection of macromolecular interactions. It enables unmodified native forms of proteins to be characterized in solution phase (37, 38). The ITC experiment usually consists of injections of one binding partner into the other, with both solutions contained in exactly the same buffer. The instrument measures the amount of energy as heat required to maintain a constant temperature with respect to a reference cell. As the binding sites are saturated, each injection peak becomes smaller until the heat reflects only the dilution of the titrant molecule. This feedback power is the base-line level in the absence of any reaction, which is provided in an independent experiment with only buffer in the cells.

In this experiment, the interactions of Hprp3p and its mutants with Hprp4p were measured by ITC. Before analyzing these interactions, blank titrations of the titrators (i.e., Hprp3p and its mutants as well as Hprp4p) into a buffer were performed (see “Materials and Methods”). Surprisingly, a large endothermic enthalpy signal was observed in the blank titration of Hprp3p (9 mg/ml) (Supplemental Fig. 2). The enthalpy signal dropped as the Hprp3p concentration increased in the buffer, thus suggesting a possible dissociation reaction of oligomeric assemblies of Hprp3p in the syringe. The enthalpy signal was dependent on the concentration of Hprp3p. When a lower concentration of Hprp3p (1.8 mg/ml) was titrated into the buffer, the heat of dilution was negligible (Supplemental Fig. 2), further supporting the notion that Hprp3p, at a high concentration, may form oligomers. Interestingly, titration of Hprp3p mutant I at a high concentration (10 mg/ml) into buffer showed a much smaller signal (~1 μcal/s) (data not shown). The heat of dilution of other mutants (II–IV) was slightly higher than that of mutant I (data not shown).

To avoid the problem of oligomerization, Hprp3p and its mutants at relatively low concentrations (as indicated in the legend to Fig. 3) were chosen for the binding experiments with Hprp4p (1.2 mg/ml), and the signals from blank titrations were subtracted from the binding heat after integration of each injection peak. As shown by the representative data in Fig. 3, full-length Hprp3p as well as mutants II and III interacted with Hprp4p, whereas neither mutant I nor IV showed measurable interactions with Hprp4p, consistent with results from the co-immunoprecipitation experiment. We were unable to obtain dissociation constants (Kd) and binding stoichiometry with high confidence from these data for the following reasons. (i) For Hprp3p, we had to use a low concentration (1.8 mg/ml) for the binding experiment to avoid the problem of oligomerization; thus, the binding reaction is not saturable (Fig. 3A). (ii) For mutant II or III, the titration curves were irregular, which is not uncommon for a binding reaction involving two large proteins and may reflect the complexity of the nature of the interaction (39). Nevertheless, the large enthalpy signals obtained when Hprp3p or mutant II or III was titrated into Hprp4p and the decrease of this signal as the concentration of injected protein increased clearly demonstrated a direct interaction. The fact that the ITC results are in agreement with data obtained from the co-immunoprecipitation experiment strongly supports the conclusion that Hprp3p interacts with Hprp4p directly and that the central domain (amino acids 195–443) of Hprp3p is responsible for this interaction.

Hprp3p/Hprp4p Interaction in HeLa Nuclear Extracts—So far, we have provided in vitro evidence that the human splicing factor Hprp3p interacts with Hprp4p, and we have further shown that the central domain of Hprp3p is responsible for this interaction. To examine whether such an interaction can be observed in human cells, full-length Hprp3p and mutants I–IV were cloned into the pcDNA3 expression vector and expressed in HeLa cells. Hprp3p is normally localized in the nucleus, but whether deletion at either end affects its cellular distribution or function is unknown. Before examining their interaction with Hprp4p, we performed immunohistochemistry analysis to examine the expression and cellular localization of the mutant
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proteins (pcDNA3–3xHA-HPRP3 mutants I–IV) in mammalian cells. The expression plasmids were introduced into HeLa cells through transient transfection and immunostained with anti-HA antibody 48 h post-transfection. Full-length Hprp3p and the empty vector were employed as positive and negative controls, respectively. An FITC-conjugated secondary antibody was used to visualize the location of protein expression, and 4,6-diamidino-2-phenylindole staining was used to mark the cell nuclei by fluorescence microscopy (Fig. 4). Full-length Hprp3p, as expected, and its mutants II and III were localized in the nucleus (Fig. 4, j, d, and f, respectively). On the other hand, Hprp3p mutants I and IV were distributed both in the cytoplasm and in the nucleus, as shown by immunostaining (Fig. 4, h and b, respectively) and immunoblot analysis (data not shown). It is possible that the deleted regions of these mutant proteins are necessary for Hprp3p nuclear translocation. Also, because mutants I and IV are small, ~26 and 35 kDa in size, respectively, they could enter and leave the nucleus by diffusion if lacking the ability to bind to Hprp4p, which is exclusively localized in the nucleus.2

To examine whether the Hprp3p mutants interact with Hprp4p in HeLa cells, nuclear extracts from the transfected cells were used for immunoprecipitation with anti-HA antibodies. The immunocomplexes of different mutants were analyzed for the presence of Hprp4p by immunoblotting using anti-Hprp4p antibodies. As shown in Fig. 5A (upper panel), mutants II and III (lanes 2 and 3, respectively), but not mutants I and IV (lanes 1 and 4, respectively), were able to interact with endogenous Hprp4p, suggesting that the central region of Hprp3p is indeed required for the interaction. Under these experimental conditions, the Hprp3p mutants I and IV were distributed both in the cytoplasm and in the nucleus, as shown by immunostaining (Fig. 4, h and b, respectively) and immunoblot analysis (data not shown). It is possible that the deleted regions of these mutant proteins are necessary for Hprp3p nuclear translocation. Also, because mutants I and IV are small, ~26 and 35 kDa in size, respectively, they could enter and leave the nucleus by diffusion if lacking the ability to bind to Hprp4p, which is exclusively localized in the nucleus.2

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\(^{2}\) C. Ushida and Y. Tomabechi, unpublished data.

**Fig. 5.** Co-immunoprecipitation of Hprp3p and its deletion mutants with endogenous Hprp4p. A: **upper panel,** HeLa cells were transfected with the pcDNA3 vectors expressing different N- or C-terminal Hprp3p deletion mutants. Nuclear extracts from cells transfected with Hprp3p mutants I (lane 1), II (lane 2), III (lane 3), and IV (lane 4) were immunoprecipitated (I.P.) with anti-HA antibody, and the immunocomplexes were fractionated by 10% SDS-PAGE. The presence of endogenous Hprp4p in a complex with Hprp3p mutants was detected by immunoblotting (I.B.) using the polyclonal antibody directed against Hprp4p (diluted 1:4000) (lanes 1–4). Nuclear extracts from non-transfected cells (HeLaNE) and from cells transfected with the empty vector were used as controls (lanes 5 and 6, respectively). The migration positions of molecular mass markers (in kilodaltons) run in size from 26 to 35 kDa are given. **Middle panel,** the nuclear extracts from these cells were analyzed by immunoblotting with monoclonal anti-HA antibodies (diluted 1:1000) as shown in lanes 1–4. Nuclear extracts from non-transfected cells and from cells transfected with the empty vector were used as controls (lanes 5 and 6, respectively). The migration positions of molecular mass markers (in kilodaltons) run in size from 26 to 35 kDa are given. **Lower panel,** the nuclear extracts from these cells were analyzed by immunoblotting with monoclonal anti-HA antibodies (diluted 1:1000) as shown in lanes 1–4. Nuclear extracts from non-transfected cells and from cells transfected with the empty vector were used as controls (lanes 5 and 6, respectively). The migration positions of molecular mass markers (in kilodaltons) run in size from 26 to 35 kDa are given.
levels were undetectable in immunocomplexes of Hprp3p mutant I (lane 7) and mutant IV (lane 10), which does not contain the Hprp3p central region. The absence of U4 and U6 snRNAs in the immunocomplexes of Hprp3p mutant II (Fig. 5C, lane 8), which retains the central region, was due to the lack of the Hprp3p C-terminal region required for RNA binding (data not shown). As a negative control, immunoprecipitation was performed with extracts from HeLa cells transfected with the pcDNA3 cloning vector, and the amount of U4 and U6 snRNAs present in the immunoprecipitates was determined by the same primer extension analysis. There was no nonspecific precipitation of U4 or U6 snRNA by the anti-HA antibodies (Fig. 5C, lane 5). These results suggest the importance of the Hprp3p central region for maintaining the association of Hprp3p-Hprp4p with U4/U6 snRNAs. Because the Hprp3p central region was demonstrated to be essential for interaction with Hprp4p and because Hprp4p does not bind to RNA (data not shown), we interpret that this interaction is important for the recruitment of Hprp4p to the U4/U6 snRNP.

**DISCUSSION**

Hprp3p and Hprp4p, two integral components of the human U4/U6 snRNP, are required for the assembly and activation of the spliceosome (41–44), although their specific involvement in this process is still not clear. Nevertheless, a large body of indirect evidence suggests that these proteins play a major role during spliceosome assembly through their interactions with each other and other components of the splicing machinery. Overexpression of yeast Prp3p suppresses temperature-sensitive alleles of the yeast PRP4 gene (40, 41) and the prp4-1 prp3-1 double mutant exhibits synthetic lethality (45). These observations indicate a genetic interaction between the two proteins. In addition, it has been reported that the WD domain of yeast Prp4p interacts with Prp3p in the yeast two-hybrid system (46). Hprp3p and Hprp4p can be copurified with cyclophilin H in a stable complex from HeLa cells (28), also suggesting an Hprp3p/Hprp4p interaction. On the other hand, the observation that there is no interaction between human Hprp3p and Hprp4p in the yeast two-hybrid system (30) has been confirmed by our group (data not shown). This result may simply reflect the limitation of the yeast two-hybrid system in the analysis of protein/protein interactions. Proteins might not be able to fold into the correct conformation and retain activity as fusion proteins in yeast. False negatives can also be caused by the failure of binding domain X (X, Hprp3p or Hprp4p) and/or activation domain Y (Y, Hprp4p or Hprp3p) to localize to the yeast nucleus. It has been estimated that the number of false negatives in the two-hybrid system is ~45% (47, 48).

We have demonstrated the Hprp3p/Hprp4p interaction without the presence of cyclophilin H by using co-immunoprecipitation assays with proteins expressed in bacteria. We have also shown that these proteins interact with each other in HeLa nuclear extracts, where Hprp3p and Hprp4p are present in a similar concentration range as found in the cell. Similarly, because the proteins are in their natural state of post-translational modification, interactions that require phosphorylation or dephosphorylation are more realistically assessed. Considering that other factors could be present in the immunoprecipitation system and that the proteins in question might be part of a larger complex, it cannot be concluded using only these assays that Hprp3p and Hprp4p directly interact. Consequently, we used another approach (ITC) to further investigate whether the Hprp3p/Hprp4p interaction is direct. The ITC experiments combined with the study of these proteins expressed in HeLa cells suggest that direct interaction between these splicing factors may also occur in vivo. Furthermore, our analyses of the interactions between Hprp3p mutants and Hprp4p indicate
that the first 194 amino acids of Hprp3p (mutant I) are not required for the interaction. By amino acid sequence comparison, it has been noted that this region is poorly conserved in Prp3p, the Saccharomyces cerevisiae homolog (31). This difference may reflect the complexity of the splicing system of higher eukaryotes in comparison with yeast. We have also shown that the last 240 amino acids of Hprp3p (mutant IV) are not required for its interaction with Hprp4p. Because this region is highly conserved from human to yeast, it is likely that the C terminus plays an important role in RNA splicing through its interaction with other conserved components of the splicing machinery. This assumption is consistent with the finding that this region of Hprp3p (amino acids 554–626) shares a 40% similarity with the double-stranded binding domain of RNase III (amino acids 142–222), pointing to the possibility that Hprp3p could be a U4/U6 snRNA-binding protein (30, 49).

Results from our group suggest that Hprp3p (and, in particular, its C-terminal region (mutant IV)) is involved in RNA binding. As demonstrated in our primer extension analysis (Fig. 5C), both U4 and U6 snRNAs could be coprecipitated with Hprp3p or its mutant III, but not mutants I, II, and IV. Mutant II, which lacks the C-terminal region of Hprp3p, failed to yield U4 and U6 primer extension products, suggesting that it is required for RNA binding. Because this mutant could be co-immunoprecipitated with Hprp4p (Figs. 2 and 5), the association of Hprp4p with U4 and U6 snRNAs (31) is likely mediated through Hprp3p. If U4 or U6 snRNA could not be co-immunoprecipitated with mutant IV (Fig. 5C), indicating that the C-terminal region alone is not sufficient to form a stable RNA-protein complex. Our data indicate that the Hprp3p middle region (amino acids 195–442), present in mutants II and III, contains the domain required for interaction with Hprp4p because deletion of this region from either end abolishes its interaction with Hprp4p. This interaction is critical for the recruitment of Hprp4p to the U4/U6 complex because Hprp4p does not directly interact with RNA (data not shown).

Based on our results and those of other groups (23, 28, 30, 31, 40, 46, 50, 51), we propose the following model to explain the role of Hprp3p in spliceosome assembly (Fig. 6). In our model, Hprp3p interacts with both proteins and U4/U6 snRNAs. The Hprp3p central region directly contacts Hprp4p, whereas its C-terminal region interacts with U4/U6 snRNAs. Because yeast genetic analyses suggest that the WD domain (the seven β-transducin repeats) of Prp4p is involved in the association with Prp3p, it is likely that the central region of human Hprp3p interacts with the WD domain of Hprp4p (40). In addition, we suggest that Hprp3p directly interacts with the stem II region of U4/U6 snRNAs; and thereby, the other proteins associated with Hprp3p interact with the U4/U6 snRNAs. Hprp3p does not have any known RNA-binding motif; however, it is the most positively charged (pI 9.90) protein in a heterotrimer protein of U4/U6 snRNAs; and thereby, the other proteins associated with Hprp3p directly interact with the stem II region of human Hprp3p. It is likely that the central region of human Hprp3p interacts with the stem II region because Hprp3p contains the domain required for interaction with Hprp4p. This interaction is critical for the recruitment of Hprp4p to the U4/U6 complex because Hprp4p does not directly interact with RNA (data not shown).

Further supporting our model is the finding that SPF30, a U2 snRNP-associated protein, may dock the U4/U6/U5 tri-snRNP to the pre-spliceosome by preferentially binding Hprp3p (23, 51). Because neither the N-terminal region of Hprp3p nor SPF30 is conserved in S. cerevisiae, we speculate that both SPF30 and the Hprp3p N terminus have evolved to serve higher eukaryotic splicing functions. In our model, the N-terminal region of Hprp3p is proposed to interact with SPF30 to dock the whole U4/U6/U5 tri-snRNP to the pre-spliceosome, a crucial step that triggers conformational rearrangements to activate the spliceosome. Recently, a protein domain (the PWI motif) has been identified in Hprp3p (50). The PWI motif is present in the mammalian (mouse and human) splicing factor Prp3p, but not in Caenorhabditis elegans and yeast Prp3p. The function of the PWI motif is not known, but its presence in two splicing factors (SRm160 and mammalian Prp3p) and in proteins related to splicing (MAL3P4.20 and W04D2) suggests that it may be important for pre-mRNA splicing (50). Furthermore, because the N terminus of Hprp3p contains the PWI motif, a likely protein/protein-binding domain (50), it can be speculated that SPF30 might interact with Hprp3p throughout this motif. Interestingly, the biological significance of Hprp3p has been highlighted by a very recent report indicating that mutations in HPRP3 lead to autosomal dominant retinitis pigmentosa (53). The authors speculated that the U4/U6/U5 tri-snRNP recruitment to the pre-mRNA could be rate-limiting step in splicing and that its deficiency could be detrimental to a highly metabolically active tissue such as the retina. In conclusion, we propose that Hprp3p plays an important role in recruiting other splicing factors such as Hprp4p to the U4/U6 snRNPs and docking the U4/U6/U5 tri-snRNP to the pre-spliceosome.

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REFERENCES

1. Moore, M. J., and Sharp, P. A. (1983) Nature 365, 364–368
2. Kramer, A. (1986) Annu. Rev. Biochem. 55, 887–909
3. Staley, J. P., and Guthrie, C. (1998) Cell 92, 315–326
4. Will, C. L., and Luhrmann, R. (2001) Curr. Opin. Cell Biol. 13, 290–301
5. Wu, J. A., and Manley, J. L. (1991) Nature 352, 818–821
6. Datta, B., and Weiner, A. M. (1991) Nature 352, 821–824
7. Datta, M., and Sharp, P. A. (1998) Cell 46, 845–855
8. Datta, K., and Sharp, P. A. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5459–5462
9. Jones, M. H., Frank, D. N., and Guthrie, C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9687–9691
10. Teigelkamp, S., Whitaker, E., and Beggs, J. D. (1995) Nucleic Acids Res. 23, 320–326
11. Lamm, G. M., Blencowe, B. J., Sprott, B. S., Iribarren, A. M., Ryder, U., and Lamond, A. I. (1991) Nucleic Acids Res. 19, 3193–3198
12. Maroney, P. A., Ronfo, C. M., and Nilsen, T. W. (2000) Mol. Cell 6, 317–328
13. Johnson, T. L., and Abelson, J. (2001) Genes Dev. 15, 1957–1970
14. Siatok, J. H., Love, J. L., and Konarska, M. M. (1999) Genes Dev. 13, 1983–1995
15. Collins, C. A., and Guthrie, C. (1999) Genes Dev. 13, 790–1982
16. Schwer, B., and Gross, C. H. (1998) EMBIO J. 17, 2096–2094
17. Frank, D., Patterson, B., and Guthrie, C. (1990) Mol. Cell. Biol. 12, 5197–5205
18. Ast, G., Paveltz, T., and Weiner, A. M. (2001) Nucleic Acids Res. 29, 1741–1749
19. Vijayraghavan, U., Company, M., and Abelson, J. (1989) Genes Dev. 3, 1206–1216
20. Horowitz, D. S., and Abelson, J. (1993) Mol. Cell. Biol. 13, 2959–2970
21. Umen, J. G., and Guthrie, C. (1995) RNA 1, 869–885
22. Will, C. L., and Luhrmann, R. (1997) Curr. Opin. Cell Biol. 9, 320–328
23. Rappaport, J., Ajlu, P., Lambon, A. I., and Mann, M. (2001) J. Biol. Chem. 276, 31142–31150
24. Watkins, N. J., Segall, V., Charpentier, B., Nottrott, S., Fabrizio, P., Bachi, A., Wilm, M., Rosbach, M., Brabant, C., and Luhrmann, R. (2000) Cell 103, 457–466
25. Vidovic, I., Nottrott, S., Hartmann, K., Luhrmann, R., and Fischer, R. (2000) Mol. Cell 6, 1311–1324
26. Nottrott, S., Hartmann, K., Fabrizio, P., Urlaub, H., Vidovic, I., Fischer, R., and Luhrmann, R. (1999) EMBIO J. 18, 6119–6133
27. Nottrott, S., Urlaub, H., and Luhrmann, R. (2001) Sixth Annual Meeting of the
Molecular Interaction of Human Hprp3p and Hprp4p

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RNA Society, May 29 to June 3, Banff, Alberta, Canada, p. 5

28. Horowitz, D. S., Kobayashi, R., and Krainer, A. R. (1997) RNA 3, 1374–1387

29. Reitz, U., Reuter, K., Achoel, T., Ingelfinger, D., Lahrmann, R., and Picner, R. (2000) J. Biol. Chem. 275, 7439–7442

30. Lauber, J., Plessel, G., Prehn, S., Will, C. L., Fabrizio, P., Groning, K., Lane, W. S., and Lahrmann, R. (1997) RNA 3, 928–941

31. Wang, A., Forman-Kay, J., Luo, Y., Luo, M., Chow, Y. H., Plumb, J., Friesen, J. D., Tsui, L. C., Heng, H. H., Woolford, J. L., Jr., and Hu, J. (1997) Hum. Mol. Genet. 6, 2117–2126

32. Teigelkamp, S., Achoel, T., Mundt, C., Gothel, S. F., Cronshagen, U., Lane, W. S., Marahiel, M., and Lahrmann, R. (1998) RNA 4, 127–141

33. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254

34. Blencowe, B. J., and Lamond, A. I. (1999) Methods Mol. Biol. 118, 275–287

35. Kramer, A., and Keller, W. (1990) Methods Enzymol. 181, 3–19

36. Hu, J., Xu, D., Schappert, K., Xu, Y., and Friesen, J. D. (1995) Mol. Cell. Biol. 15, 1274–1285

37. Pierce, M. M., Raman, C. S., and Nall, B. T. (1999) Methods 19, 213–221

38. Holdgate, G. A. (2001) BioTechniques 31, 164–168

39. Kim, M., Sun, Z. Y., Byron, O., Campbell, G., Wagner, G., Wang, J., and Reinherz, E. L. (2001) J. Mol. Biol. 312, 711–720

40. Hu, J., Xu, Y., Schappert, K., Harrington, T., Wang, A., Braga, R., Mogridge, J., and Friesen, J. D. (1994) Nucleic Acids Res. 22, 1724–1734

41. Last, R. L., Maddock, J. R., and Woolford, J. L., Jr. (1987) Genetics 117, 619–631

42. Lustig, A. J., Lin, R. J., and Abelson, J. (1986) Cell 47, 953–963

43. Ayadi, A., Miller, M., and Banroques, J. (1997) RNA 3, 197–209

44. Banroques, J., and Abelson, J. N. (1989) Mol. Cell. Biol. 9, 3710–3719

45. Ruby, S. W., Chang, T. H., and Abelson, J. (1993) Genes Dev. 7, 1909–1925

46. Ayadi, L., Callebaut, I., Saguez, C., Villa, T., Mornon, J. P., and Banroques, J. (1998) J. Mol. Biol. 284, 673–687

47. Walhout, A. J., Boulton, S. J., and Vidal, M. (2000) Yeast 17, 88–94

48. Walhout, A. J., Sordella, R., Lu, X., Hartley, J. L., Temple, G. F., Brasch, M. A., Thierry-Mieg, N., and Vidal, M. (2000) Science 287, 116–122

49. Burd, C. G., and Dreyfuss, G. (1994) Science 265, 615–621

50. Blencowe, B. J., and Ouzounis, C. A. (1999) Trends Biochem. Sci. 24, 179–180

51. Meister, G., Hannus, S., Plotz, O., Baars, T., Hartmann, E., Fakan, S., Laggerbauer, B., and Fischer, U. (2001) EMBO J. 20, 2304–2314

52. Xu, Y., Petersen-Bjorn, S., and Friesen, J. D. (1990) Mol. Cell. Biol. 10, 1217–1225

53. Chakarova, C. F., Hima, M. M., Bolz, H., Abu, S. L., Patel, R. J., Papaioannou, M. G., Inglehearn, C. F., Keen, T. J., Willis, C., Moore, A. T., Rosenberg, T., Webster, A. R., Bird, A. C., Gal, A., Hunt, D., Vithana, E. N., and Bhattacharya, S. S. (2002) Hum. Mol. Genet. 11, 87–92