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The mammalian analogue of the yeast PRP8 splicing protein is present in the U4/5/6 small nuclear ribonucleoprotein particle and the spliceosome

(RNA processing/pre-mRNA/antibody)

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ABSTRACT **HeLa cell nuclear extracts contain a protein reactive with antibodies against PRP8, a polypeptide essential for pre-mRNA splicing in yeast and a specific component of the yeast U5 small nuclear ribonucleoprotein (snRNP) [Lossky, M., Anderson, G. J., Jackson, S. P. & Beggs, J. (1987) *Cell* 51, 1019–1026]. The mammalian protein appears as a doublet at ≈200 kDa, smaller than the 260-kDa yeast protein, and possesses an Sm epitope as determined by immunoblotting. Its association with a snRNP of the Sm class other than U1 or U2 is indicated by its immunoprecipitation by anti-Sm and anti-trimethylguanosine antibodies but not by anti-(U1) or anti-(U2) RNP sera. Gradient fractionation of splicing extracts demonstrates that the 200-kDa protein is a component of the U4/5/6 snRNP complex and of U5 snRNPs. It is also present in affinity-purified spliceosomes.**

Accurate excision of intron sequences is an essential process in the maturation of eukaryotic mRNA precursors and appears to be well conserved across eukaryotic species. This splicing reaction proceeds via a two-step mechanism: cleavage at the 5′ splice site and formation of a lariat intron–3′ exon intermediate is followed by 3′ splice-site cleavage and exon ligation, with loss of the intron in lariat form. The reaction requires the assembly of a spliceosome, a large (50–60S in HeLa cells) complex comprising the Sm small nuclear ribonucleoproteins (snRNPs) U1, U2, U5, and U4/U6 and non-snRNP-associated protein factors in addition to the pre-mRNA (1–3).

Mammalian snRNPs of the Sm class are composed of one or two small RNA molecules and a core of common polypeptides called B, B′, D, E, F, and G (4). Polypeptides unique to specific snRNPs have also been identified: U1 snRNPs contain the 70-kDa A and C proteins (5), whereas the A′ and B″ proteins reside exclusively in U2 snRNP particles (6). The lack of patient antisera specific for other snRNP particles has impeded the determination of their protein composition, although new affinity chromatographic approaches appear promising in this regard (7, 8).

Genetic dissection of the splicing reaction in the yeast *Saccharomyces cerevisiae* has provided insights into spliceosome composition and assembly. Yeast strains prp2-11 (9) and prp17-20 (10) are temperature-sensitive mutants defective in pre-mRNA processing at the nonpermissive temperature. Biochemical analyses of these mutants have led to the identification of PRP8 (11, 12), PRP4 (13, 14), and PRP16 (15) as yeast proteins associated with the U5, the U4/U6, and possibly the U2 snRNP particles, respectively. In contrast, PRP11 (16) is found in the spliceosome and PRP2 (17) is essential for spliceosome assembly, but neither appears to be tightly associated with any snRNP.

Immunoprecipitation with antibodies raised against PRP8 fusion proteins has shown that PRP8 is associated with the yeast U5 snRNP (11). Preincubation of yeast splicing extracts with ATP results in enhanced precipitation of U5 and the additional precipitation of U4 and U6 snRNAs. Anti-PRP8 antibodies inhibit splicing of an actin pre-mRNA in yeast extracts and also precipitate splicing intermediates (11, 12).

Using antibodies against yeast PRP8, we have identified the mammalian analogue of the 260-kDa yeast PRP8 protein. This 200-kDa protein has its own Sm epitope and is associated with the U5 and U4/5/6 particles as well as affinity-purified spliceosomes.

MATERIALS AND METHODS

Antisera and Immunoprecipitation. The monoclonal anti-Sm antibody Y12 (18) was prepared from ascites fluid as described. Patient sera with anti-Sm (JE), anti-(U1)RNP (AG), or anti-(U2)RNP (GA) specificities were provided by John Hardin and Joe Craft (Yale Univ. School of Medicine). The monoclonal anti-trimethylguanosine antibody K121 (19) was provided by Adrian Krainer (Cold Spring Harbor Laboratory). Antibodies against PRP8 fusion protein 8.2 (11) were generously provided by Jean Beggs (Univ. of Edinburgh). Nonimmune serum (ME) was obtained from a healthy volunteer from our laboratory.

Antibodies (2.5 μl) were prebound to protein A-Sepharose (2.5 mg; Pharmacia) for 1 hr and washed four times with 1 ml of IPP buffer (10 mM Tris Cl, pH 8.0/500 mM NaCl/0.1% Nonidet P-40/0.5 mM dithiothreitol). In the case of anti-trimethylguanosine antibodies, 50 μl and 5 μl of a second antibody (19) and NET-2 buffer (50 mM Tris Cl, pH 7.5/150 mM NaCl/0.05% Nonidet P-40/0.5 mM dithiothreitol) were used. The washed beads were then incubated with 15 μl of HeLa nuclear extract (20) in 300 μl of buffer or with 500-μl gradient fractions for 1–2 hr at 4°C. The beads were washed another four times in the appropriate buffer, and the pellets were resuspended in 20 μl of SDS sample buffer and heated to 90°C for 3 min to release immunoprecipitated proteins.

Immunoblots. Immunoprecipitated proteins were fractionated in SDS/6% polyacrylamide gels and electrophoretically transferred to nitrocellulose in 25 mM Tris/192 mM glycine, pH 8.0, containing 20% methanol and 0.1% SDS (21). Nitrocellulose strips were then blocked for 30 min in TTBS [0.1 M Tris Cl, pH 7.5/0.9% NaCl/0.1% (vol/vol) Tween 20] containing 1% bovine serum albumin, 0.5% gelatin, and 1% serum from the animal against which the second antibody was raised. Incubation with the primary antibody was for 30

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Abbreviations: RNP, ribonucleoprotein; snRNP, small nuclear RNP.

min, using a 1:1000 dilution of Y12 or 1:250 dilution of anti-PRP8.2 antibodies. Subsequent incubation with a biotinylated second antibody (Vector Laboratories) and development of the blots were performed according to the manufacturer's guidelines.

RNase H Experiments and Glycerol Gradients. RNase H digestion was performed as described (22) using oligodeoxynucleotides complementary to nucleotides 63–84 of U4 and 78–95 of U6. Gradients [10–30% (vol/vol) glycerol in 20 mM Hepes, pH 7.9/100 mM KCl/1 mM $MgCl_2$] were centrifuged at 35,000 rpm for 16 hr in a Beckman SW41 rotor. After fractionation, fractions either were immunoprecipitated as described above or were extracted with phenol, ethanol-precipitated, and electrophoresed in denaturing 10% polyacrylamide gels for RNA analysis by silver staining or Northern blotting as described (23).

Affinity Selection. Biotinylated adenovirus precursor mRNA was prepared according to Grabowski and Sharp (24). Spliceosomes were assembled in a 1.8-ml splicing reaction mixture containing uniformly ^{32}P-labeled, biotinylated precursor RNA (1 μg/ml) in the presence of 2.5 mM EDTA, and the mixture was applied to a Sephacryl S-500 (Pharmacia) gel filtration column as described by Abmayr *et al.* (25). Peak spliceosome fractions were identified by Cerenkov counting of 50-μl aliquots of alternate fractions and by fractionating RNA from these aliquots in polyacrylamide gels to identify those that contained the 5′ cutoff exon. Spliceosomes were then affinity-selected with streptavidin-agarose (24) and analyzed by immunoblotting as described above or by Northern hybridization.

RESULTS

A 200-kDa protein that reacted with anti-PRP8 antibodies upon immunoblotting was efficiently immunoprecipitated from HeLa cell nuclear extract under stringent washing conditions (0.5 M NaCl/0.1% Nonidet P-40) by patient and monoclonal anti-Sm antibodies (Fig. 1*A*). It was also present in immunoprecipitates obtained with anti-trimethylguanosine antibodies, indicating its association with a snRNA of the Sm class. This snRNA was not U1 or U2, as the protein was not among those precipitated by sera with anti-(U1) or anti-(U2) RNP specificities. Probing of a parallel set of immunoprecipitates with the anti-Sm monoclonal antibody Y12 (Fig. 1*B*) revealed an anti-Sm-reactive protein with identical electrophoretic mobility to that detected with anti-PRP8. In this gel system, the protein migrated as a closely spaced doublet, both bands of which bound anti-PRP8.2 (Fig. 1*A*, see also Fig. 2*A*) and anti-Sm antibodies. A comparable doublet was readily visualized in a Y12 immunoprecipitate of a ^{35}S-labeled HeLa nuclear extract (Fig. 1*C*).

To eliminate the possibility that the anti-PRP8- and anti-Sm-reactive 200-kDa proteins are unrelated but accidentally comigrate in a one-dimensional gel, nuclear extracts were fractionated by velocity sedimentation. Extracts incubated for 30 min in the presence of 1 mM ATP and 3.2 mM $MgCl_2$ were centrifuged in 10–30% glycerol gradients to separate the various snRNP particles. Each fraction was immunoprecipitated with the Y12 monoclonal antibody, and the concentrated snRNP-associated proteins were subsequently separated in SDS/6% polyacrylamide gels and transferred to nitrocellulose. Immunoblots of alternate fractions of gradients run under identical conditions were probed with anti-PRP8 or Y12 (Fig. 2 *A* and *B*, respectively). In both blots, the 200-kDa protein peaked in fractions 6–8 (≈25 S), strongly arguing for the identity between the polypeptides detected by the two antibodies and indicating incorporation of this protein into higher-order snRNP complexes. No reactivity at 200 kDa was observed in either blot near the top of the gradient, suggesting either that the protein does not exist in a free form or that the Sm epitope is masked when the protein is in its nondenatured form.

A silver-stained gel of RNAs extracted from gradient fractions (Fig. 2*C*) showed that the majority of U5 RNA is found in a previously characterized U4/5/6 snRNP complex (23, 26) and that this complex peaks in the same region of the gradient as the 200-kDa protein. These results are in good agreement with the observation that the PRP8 protein is associated with the yeast U4/5/6 snRNP complex (11).

A faint set of bands corresponding to ≈130 kDa and ≈85 kDa was also visible in the immunoblots of the gradient fractions (Fig. 2 *A* and *B*) and of the unfractionated extract (Fig. 2*A*, total lane). Since these bands were recognized by both antibodies, it seems likely that they represent degradation products of the 200-kDa protein. When immunoprecipitates were washed with IPP buffer (500 mM NaCl) instead of NET-2 buffer (150 mM NaCl), these presumptive proteo-

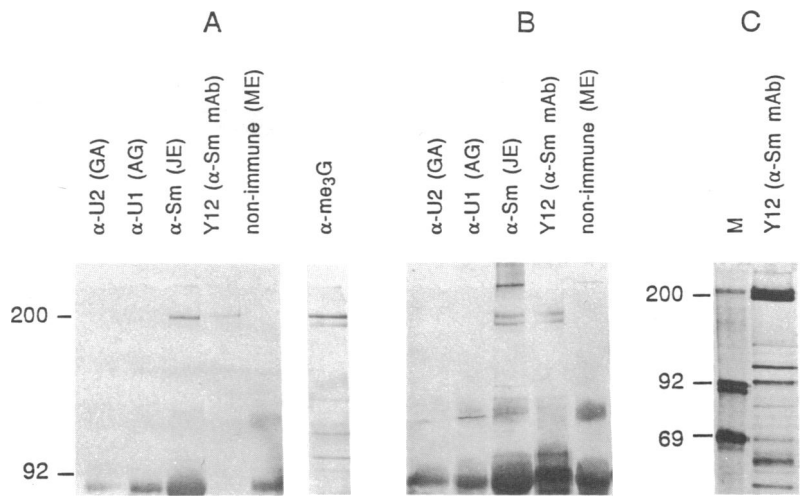

FIG. 1. A 200-kDa protein reactive with anti-Sm and anti-PRP8 antibodies is present in anti-Sm and anti-trimethylguanosine, but not anti-(U1) or anti-(U2)RNP, immunoprecipitates. (*A* and *B*) Proteins were immunoprecipitated from HeLa nuclear extracts, gel-fractionated, and transferred to nitrocellulose as described in *Materials and Methods*. Blots in *A* and *B* were probed with anti-PRP8.2 (1:250 dilution) and Y12 (1:1000) antibodies, respectively. Some nonspecific staining of serum proteins is observed as large diffuse bands near the bottoms of the blots. α, Anti; mAb, monoclonal antibody. (*C*) Autoradiograph of anti-Sm immunoprecipitate from a ^{35}S-labeled HeLa nuclear extract (6, 20). Lane M, ^{14}C-labeled size markers (Amersham).

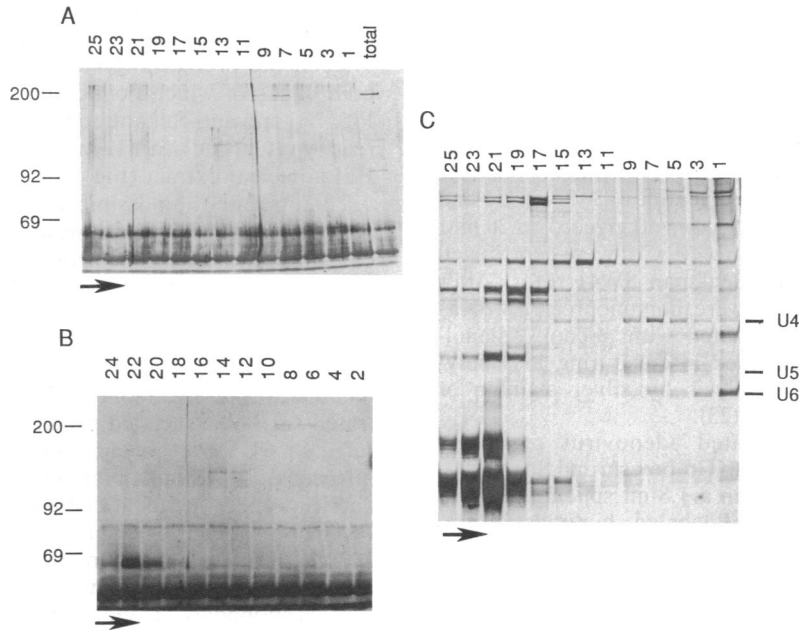

FIG. 2. The 200-kDa protein comigrates with the U4/5/6 snRNP in glycerol gradients. (*A* and *B*) Y12 immunoprecipitates (washed with NET-2 buffer) of glycerol gradient fractions, blotted with anti-PRP8.2 or Y12, respectively. Direction of sedimentation is indicated by the arrows. The 200-kDa protein peaks in fractions 6–9. The heavy, diffuse staining observed at the bottom of each blot represents nonspecific staining of the antibody heavy chain; in *B*, another nonspecific band, at ≈85 kDa, is also serum-derived. The positions of molecular size markers (200, 92, and 69 kDa) are indicated. Lane "total," Y12 immunoprecipitate of unfractionated extract. (*C*) Silver-stained gel of glycerol gradient fractions, showing the position of the major U RNAs in the gradient. The U4/5/6 complex peaks in fractions 5–9.

lytic fragments disappeared, suggesting that their association with snRNPs is weaker than that of the parent protein.

We next wished to ascertain whether the 200-kDa protein is associated with free U5 snRNPs or only the U4/5/6 complex. We therefore disrupted the U4/5/6 complex by RNase H digestion with oligonucleotides complementary to U4 and U6 (22, 23) and assayed gradient fractions by both immunoblotting with anti-PRP8 and Northern blotting for U1, U2, U4, and U5 RNAs (Fig. 3). In the undigested extract, the 200-kDa protein cosedimented with the U4/5/6 complex (Fig. 3 *Upper*; peaking in fraction 7 in the immunoblot and fraction 6 in the Northern blot). After complete degradation of U4 and U6 snRNAs, the 200-kDa protein still appeared in the heavy region of the gradient, comigrating with the U5 RNA, which was slightly shifted to lower *s* values (Fig. 3 *Lower*; peaking in fraction 9 in the immunoblot and fractions 8–12 in the Northern blot). This result argues that the 200-kDa protein remains associated with the released U5 snRNP, which sediments more heterogeneously than other snRNPs upon gradient fractionation (23), even after purification (7).

Finally, we investigated the association of the 200-kDa protein with active spliceosomes. Immunoblots of splicing reaction mixtures separated in glycerol gradients revealed that the 200-kDa protein was present not only in the region of the U4/5/6 complex, but also in the 50–60S spliceosome

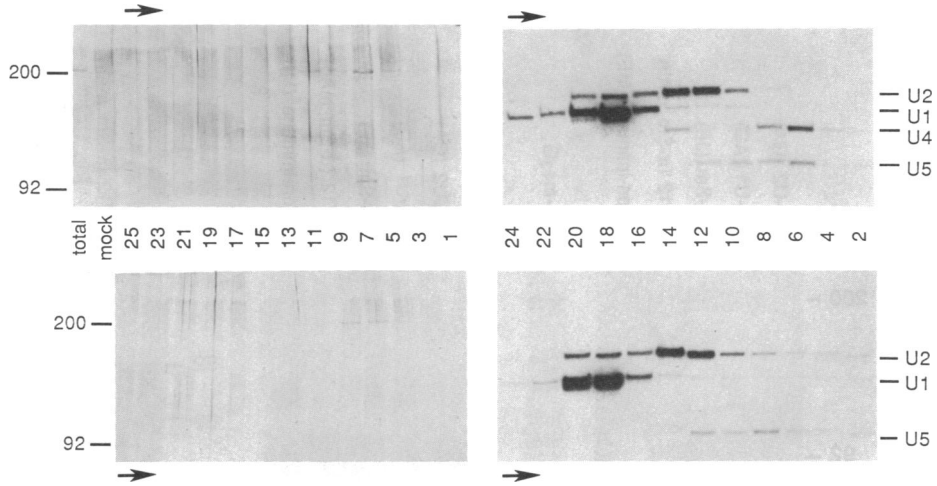

FIG. 3. Digestion of the U4/5/6 snRNP complex by RNase H in the presence of oligonucleotides complementary to U4 and U6 snRNAs. Nuclear extracts were digested with RNase H and fractionated in glycerol gradients. Fractions from undigested control extract (*Upper*) and from digested extract (*Lower*) were immunoprecipitated with monoclonal anti-Sm antibody for immunoblot analysis with anti-PRP8 (*Left*) and were analyzed by Northern blotting for U1, U2, U4, and U5 RNAs (*Right*). Lane "total," Y12 immunoprecipitate from 15 μl of HeLa nuclear extract; lane "mock," an immunoprecipitation reaction with no extract added. The direction of sedimentation is indicated by the arrows. Positions of molecular size markers (200 and 92 kDa) are indicated.

region (data not shown). Northern blots of the same gradients, however, showed that although splicing precursor and intermediates peaked in the 50–60S region, all the major snRNP particles were distributed throughout the entire gradient, so that it was impossible to distinguish between those in true splicing complexes and those that were simply in aggregates. A two-step procedure utilizing size fractionation (29) followed by affinity selection based on the presence of pre-mRNA (24) was therefore used to generate pure spliceosomes to analyze for the presence of the 200-kDa protein.

Splicing complexes (29) were assembled on a biotinylated adenovirus precursor mRNA (24) in the presence of 2.5 mM EDTA (25). Under these conditions spliceosomes form but do not promote pre-mRNA cleavage, thereby leading to their accumulation. The restriction is somewhat leaky, however, and the first step of the splicing reaction often does occur with variable low efficiency. EDTA also serves to protect the precursor mRNA from Mg^{2+}-dependent ribonucleases. Reaction mixtures were then size-fractionated on Sephacryl S-500 (25, 29) and spliceosomes were affinity-purified from peak fractions by precipitation with streptavidin-agarose (24). A second reaction mixture, identical except for the omission of precursor RNA, was carried through the same procedure for comparison. Northern blotting of fractions from the spliceosome region of the gel filtration columns showed that the relative abundances of the splicing snRNAs U1, U2, U4, U5, and U6 were unchanged regardless of the presence of precursor (Fig. 4*A*, lanes labeled "unsel"). Precipitation with streptavidin-agarose, however, selected only those snRNPs that had bound pre-mRNA and were thus present in true splicing complexes (Fig. 4*A*, lanes "sel"). Immunoblots of this streptavidin-selected material with the anti-PRP8 antibody showed that the 200-kDa protein is indeed a component of the spliceosome, as it was found in affinity-selected fractions only from the reaction mixture containing precursor RNA (Fig. 4*B*). Two proteins of lower molecular mass were visible in both lanes of the immunoblot (Fig. 4*B*). As they appeared in both reaction mixtures, and as the streptavidin-agarose beads were washed only with low-salt (100 mM KCl) buffer to keep spliceosomes intact, they most likely represent the nonspecific association of proteolytic fragments of the 200-kDa protein, as discussed above.

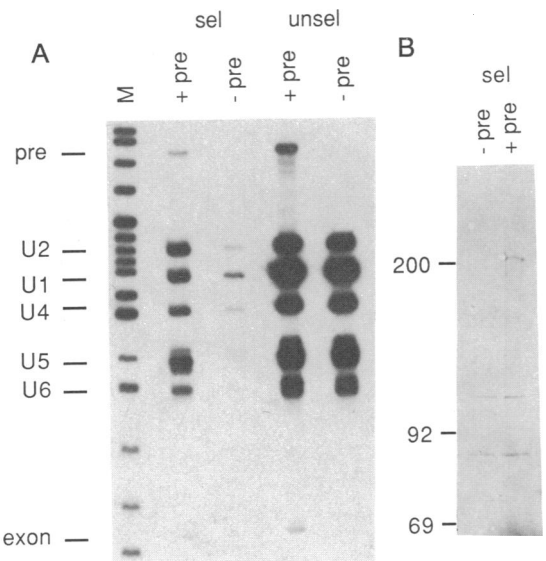

FIG. 4. The 200-kDa protein is found in affinity-purified spliceosomes. (*A*) Northern blot of RNAs from the spliceosome region of gel filtration column runs with or without precursor (+ or − pre). Lanes "sel," RNA that had been affinity-selected (from 0.6 ml of column fractions) with streptavidin-agarose; lanes "unsel," total RNA from 0.1 ml of these fractions. Positions of major U RNAs, pre-mRNA (pre), and 5′ cut-off exon (5′ exon) are marked. Lane M, a ^{32}P-labeled *Msp* I digest of pBR322 DNA used as size markers. (*B*) Immunoblot with the anti-PRP8.2 antibody of streptavidin-agarose-selected material from 1.2 ml of fractions from the spliceosome region of gel filtration columns with or without precursor (+ or − pre). Positions of molecular size markers (200, 92, and 69 kDa) are indicated.

DISCUSSION

Here we report the identification of the mammalian analogue of the yeast PRP8 protein first characterized by Lossky and coworkers (11, 12). Like its yeast counterpart, this large polypeptide (200 kDa in HeLa cells, 260 kDa in yeast) is associated with the U4/5/6 snRNP complex in HeLa nuclear extracts. The mammalian protein not only reacts with antisera against yeast PRP8, but has its own Sm epitope as determined by immunoblot experiments. A 200-kDa protein that reacts with anti-PRP8 antibodies and weakly with the Y12 monoclonal anti-Sm antibody was also reported in purified preparations of U5 snRNPs by Bach *et al.* (7). Our immunoblots of proteins from size-fractionated, affinity-purified splicing complexes demonstrate that the 200-kDa protein is an integral part of the mammalian spliceosome, consistent with the observation that anti-PRP8 antibodies can precipitate intermediates from yeast splicing reactions (11). Also in the HeLa system, M. Garcia-Blanco and P. A. Sharp (personal communication) have observed UV-crosslinking of an ≈220-kDa protein to competent splicing substrates in the presence of ATP and Mg^{2+}, with kinetics that parallel spliceosome formation; this crosslinked protein comigrates with an anti-PRP8-reactive band on immunoblots and is weakly immunoprecipitable with anti-PRP8 antibodies.

Bach *et al.* (7) reported that purified U5 snRNPs contain, in addition to the common core polypeptides, several unique proteins of 200, 116, 102/100, 52, and 40 kDa. The 100-kDa U5-specific protein reacts strongly with anti-Sm antibodies and is likely to be the intron-binding protein (IBP) previously identified by Tazi *et al.* (27) and Gerke and Steitz (28). The size of IBP reported by the two groups differed (100 vs. 70 kDa), but there are many indications (V. Gerke, personal communication) that the 70-kDa molecule may be a product of limited proteolysis of the larger polypeptide. We therefore expected that a 100-kDa or 70-kDa Sm-reactive protein associated with the U5 and/or the U4/5/6 snRNP would be apparent in our analyses. Yet immunoblots of nuclear extracts fractionated on glycerol gradients (Fig. 2*B*) did not reveal any bands of these sizes in the U5 or U4/5/6 regions. We have, however, repeatedly observed a strongly Sm-reactive protein of ≈65 kDa in our top gradient fractions (the region in which uncomplexed proteins would be expected to sediment). We believe this may be the aforementioned 70-kDa fragment of the U5-specific 100-kDa protein and IBP for several reasons. First, both its size and Sm antigenicity are consistent with previous reports. [It should be noted that the U1-specific 70-kDa protein does not react with the Y12 monoclonal anti-Sm antibody (5), nor does U1 RNA comigrate in glycerol gradients with the ≈65-kDa polypeptide observed in our gradients (Fig. 2*C*).] Second, it has been reported (7) that the majority of U5 snRNPs (80–85%) purified by anti-trimethylguanosine affinity chromatography from splicing extracts do not contain any U5-specific proteins. Thus it might be anticipated that the bulk of any of these proteins would separate from the core U5 snRNP upon gradient fractionation. Alternatively, it is possible that degradation of the 100-kDa protein to 70 kDa results in loss of interaction with the U5 snRNP, while the Sm epitope is retained. Further studies are required to clarify this situation. That we do not observe any 200-kDa protein at the top of our gradients suggests that it, in contrast, is significantly more stably associated with the U5 particle and its higher aggre-

gates, although the possibility that free 200-kDa protein is not efficiently immunoprecipitated despite its Sm epitope cannot be excluded.

The observation that the 200-kDa protein is present in the U4/U5/U6 snRNP complex and the spliceosome raises intriguing questions regarding its role in splicing. Its essentiality in yeast and its strong conservation from yeast to humans indicate some important function. Since the U4/5/6 complex is a precursor to the spliceosome (26), possibilities include recognition of the 3′ splice site in either the first or second step of the splicing reaction or mediation of snRNP–snRNP interactions during spliceosome assembly or disassembly.

Note Added in Proof. Yeast PRP8 protein has recently been identified in affinity-purified yeast spliceosomes (E. Whittaker, M. Lossky, and J. Beggs, personal communication), and its mammalian counterpart has been shown to be an RNA-binding protein (G. Anderson, M. Bach, R. Luhrmann, and J. Beggs, personal communication). A doublet at ≈205 kDa has also been observed in affinity-purified, ^{125}I-labeled spliceosomal proteins from HeLa nuclear extracts (R. Reed, personal communication).

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