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The human Prp8 protein is a component of both U2- and U12-dependent spliceosomes

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ABSTRACT

This study reports the cloning, sequencing, and development of antisera against the human U5 snRNP 220-kDa protein or hPrp8p. Prp8p is the most highly conserved large nuclear protein known to date, but it is not related to any other protein. Southern, Northern, and expressed sequence tag analyses indicate that hPrp8p is encoded by a single gene. Prp8p is a core component of U5 snRNP and the U4/U6.U5 tri-snRNP, and antibodies raised against it immunoprecipitate both the major, U2-dependent and minor, U12-dependent spliceosomes. These spliceosomes, which excise different classes of introns, contain distinct sets of snRNAs overlapping only with U5 snRNA. Other than the core Sm proteins, hPrp8p is the first splicing factor shown to be common to both spliceosomes.

Keywords: pre-mRNA splicing; Prp8p; spliceosome; U12-dependent spliceosome

INTRODUCTION

The removal of introns from precursors to messenger RNAs (pre-mRNAs) by the process of RNA splicing is an essential step in eukaryotic gene expression (Moore et al., 1993; Adams et al., 1996; Krämer, 1996; Will & Lührmann, 1997). In mammals, the vast majority of introns (more than 99%; see Sharp & Burge, 1997) are removed by the major, U2-dependent spliceosome, a 50–60S complex composed of four small nuclear ribonucleoprotein particles (snRNPs: U1, U2, U4/U6, and U5) and numerous associated proteins. Each snRNP is itself a multicomponent complex, containing one or two snRNAs, a common set of core proteins (the Sm proteins), and snRNP-specific polypeptides. In vitro, these snRNPs bind to the pre-mRNA in an ordered pathway to generate active spliceosomes, within which intron excision proceeds in two chemical steps: cleavage at the 5′ splice site coupled with lariat formation at the branch site, and then exon ligation at the 3′ splice site (Moore et al., 1993; Adams et al., 1996; Krämer, 1996; Will & Lührmann, 1997).

Amazingly, a second type of spliceosome was recently discovered—the minor or U12-dependent spliceosome that removes so-called U12-type introns (Hall & Padgett, 1996; Tarn & Steitz, 1996a, 1996b, 1997). U12-type introns, which comprise $<$1% of all introns in mammals, are thought to be evolutionary remnants of an endosymbiotic genetic merging event that produced the common ancestor of higher eukaryotes (Burge et al., 1998). Introns of this minor class have consensus sequences distinct from those of the major class and the spliceosome that removes them contains a different set of snRNAs: U11, U12, U4atac, U5, and U6atac (Hall & Padgett, 1996; Tarn & Steitz, 1996a, 1996b; Kolossova & Padgett, 1997). Note that only U5 snRNA is shared by both the major, U2-dependent spliceosome and minor, U12-dependent spliceosome.

Although the snRNA components in the minor spliceosome have been relatively well characterized, very little is currently known about its protein constituents. So far there is only evidence for two general classes of major spliceosomal proteins, Sm and SR proteins, being present in the minor spliceosome. Sm proteins are the core snRNP proteins that recognize a sequence (AUUUUUG) found in the major U1, U2, U5, and U4 and minor U11, U12, and U4atac snRNAs, and all of these RNAs can be immunoprecipitated with α-Sm antibodies (Baserga & Steitz, 1993; Tarn & Steitz, 1996b). SR proteins are also likely common to both spliceosomes because assembly of U12-dependent spliceosomes can be enhanced by the presence of a downstream 5′ splice site that binds U1 snRNP (Wu & Krainer, 1996). Such communication between individual spliceosomes across exons, known as “exon definition” (Berget, 1995), usually involves SR proteins

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(Manley & Tacke, 1996). Other than these protein families, however, which specific proteins are shared and which are different between the two spliceosomes has not been determined.

As noted above, U5 is the one snRNA common to both spliceosome types. Interestingly, of all snRNAs in the major spliceosome, U5 is the least phylogenetically conserved (Guthrie & Patterson, 1988), so why it should be the common denominator has been somewhat of a puzzle. In human cells, ten different forms of U5 snRNA (U5A, U5B1–4, U5C1–2, U5D, U5E, and U5F) are known. These isoforms vary in sequence at numerous internal positions (Sontheimer & Steitz, 1992), but as of this writing it is not known which, if any, predominate in the U2- and U12-dependent spliceosomes (Tarn & Steitz, 1997). Neither is it known whether all the isoforms bind exactly the same set of proteins, although U5 snRNP can be isolated by biochemical fractionation as an apparently homogeneous 20S complex (Bach et al., 1989). In addition to the Sm core proteins, 20S U5 snRNP contains nine specific polypeptides with molecular weights of 15, 40, 52, 100, 102, 110, 116, 200, and 220 kDa (Bach et al., 1989), the most of any snRNP in the major spliceosome (Krämer, 1996).

The largest protein in human U5 snRNP, p220, exhibits extensive sequence similarity to the *Saccharomyces cerevisiae* splicing factor Prp8p (Hodges et al., 1995). Yeast Prp8p is encoded by an essential gene (Jackson et al., 1988) first identified in a screen for mutants having conditional lethal phenotypes because of defects in pre-mRNA processing (Vijayraghavan et al., 1989; Ruby & Abelson, 1991). This protein is a core component of U5 snRNP, the U4/U6.U5 tri-snRNP and fully assembled spliceosomes (Whittaker et al., 1990; Brown & Beggs, 1992). In the absence of Prp8p, the levels of U4, U5, and U6 snRNAs decline dramatically in vivo, suggesting a role for Prp8p in the stability of these snRNAs (Brown & Beggs, 1992). Crosslinking studies have revealed that yeast Prp8p makes direct contact with both the 5′ and 3′ splice sites (Whittaker & Beggs, 1991; Teigelkamp et al., 1995a, 1995b; Umen & Guthrie, 1995a, 1995b), and similarly, human U5 p220 can be crosslinked to the 5′ splice, branch and 3′ splice sites, as well as nearby exonic nucleotides (Wyatt et al., 1992; MacMillan et al., 1994; Chiara et al., 1996, 1997). Moreover, mutational studies have implicated Prp8p in both poly-pyrimidine tract and 3′ splice site recognition prior to exon ligation (Umen & Guthrie, 1995a, 1996). Taken together, these data suggest that Prp8p has crucial functions at several steps in spliceosome assembly and may additionally contribute active site groups for both transesterification reactions.

Given that Prp8p is such a central player in the major U2-dependent spliceosome, it was of therefore of great interest to determine if it is also found in U12-dependent spliceosomes. In this article, we report cloning and sequencing of the full-length cDNA for human U5 p220 or hPrp8p. We developed an antiserum against the C-terminal 50 kDa of hPrp8p and demonstrated that it immunoprecipitates splicing intermediates and products of both U2- and U12-type introns. Thus hPrp8p is common to both spliceosomes. Southern blotting indicated that hPrp8p is encoded by a single gene that yields one predominant mRNA as detected by Northern and expressed sequence tag (EST) analyses.

RESULTS

The complete cDNA sequence for hPRP8

When we began this work the complete cDNA sequence for human PRP8 had not been reported. We therefore determined that sequence (see Materials and Methods) from five overlapping partial cDNAs (GenBank accession #AF092565). Sequences of two independent 5′-RACE products indicated that the 5′-UTR extends only 38–49 nt beyond the first ATG, with no upstream stop codons. Likewise the 3′-UTR is relatively short (190 nt) with no obvious special sequence elements, as expected for a housekeeping gene. During the course of these studies, the complete cDNA sequence of hPRP8 was also deposited in GenBank by Shimada et al. (Accession #B007510); the sequence they determined matches ours exactly.

Analysis of new sequence alignments

Previous alignment of the full-length Prp8p sequences from budding yeast (ScPrp8p) and worms (CePrp8p), plus a number of partial EST fragments from other organisms, had revealed that Prp8p is strikingly conserved (Hodges et al., 1995). Since then, full-length sequences have also become available for fission yeast (SpPrp8p; Assession #U48733), trypanosomes (TbPrp8p; Assession #Y12638; Lucke et al., 1997), and humans (hPrp8p) (this work). The incredible conservation of this protein becomes only more remarkable when all five full-length sequences are now compared (Fig. 1).

The hPRP8 cDNA contains a single 7,005 nt open reading frame (ORF) encoding a 2,335 amino acid protein with predicted molecular weight of 274 kD and isoelectric point of 9.48. Overall, the human protein is 86% identical and 91% similar to the nematode protein, and 62% identical and 71% similar to *S. cerevisiae* Prp8p. The *Trypanosoma brucei* protein (Lucke et al., 1997) exhibits the least similarity to the other four. One of the most striking regions of conservation is a 290-amino acid stretch (comprising amino acids 1301–1590 in hPrp8p) where identity between the nematode, budding yeast, and human sequences together reaches 83%. The least conserved regions are located at the N-and C-termini and are roughly 400 amino acids each.

Whereas the *S. cerevisiae* protein contains a 78-amino acid proline-rich region at its N-terminus, the other Prp8ps do not retain this feature (Hodges et al., 1995).

Surprisingly, in spite of its length and amazingly high level of conservation, hPrp8p contains no recognizable protein sequence motifs of any kind (Hodges et al., 1995; Lucke et al., 1997). However, some clues as to functional domains have come from mutational and crosslink mapping studies, and it is of interest to note which of these positions are conserved and which are not (Fig. 1). Mutagenesis of ScPrp8p identified specific amino acids involved in poly-pyrimidine tract recognition and 3′ splice site fidelity (Umen & Guthrie, 1996). The poly-pyrimidine tract recognition mutations mapped to two positions in ScPrp8p: F1834 and E1960. Interestingly, whereas E1960 is well conserved across species (E1960 is replaced by a similar amino acid only in *T. brucei*), F1834 is not. Mutations in five other positions in ScPrp8p can suppress point mutations in the PyAG motif at the 3′ splice site (Umen & Guthrie, 1996). Three of those positions (*prp8-121*, *prp8-4*, and *prp8-125*) are identical across all sequences, whereas the others (*prp8-2* and *prp8-3*) are conserved between all the sequences except *T. brucei*. Another yeast PRP8 mutation, *prp8-1*, can be suppressed by a mutation in SPP81/DED1 (a DEAD-box protein) (Jamieson et al., 1991); this mutation maps to yeast G2347 (Hodges et al., 1995), a position that is identical across all species (Fig. 1). Finally, the region of hPrp8p that crosslinks to the 5′ splice site GU dinucleotide has been mapped to amino acids 1894–1898 (QACLK) (Reyes et al., 1999), but this peptide stretch is not so highly conserved. Clearly many more such analyses will be required to map fully the functional domain structure of this remarkably conserved protein.

Characterization of α-hPrp8p antiserum

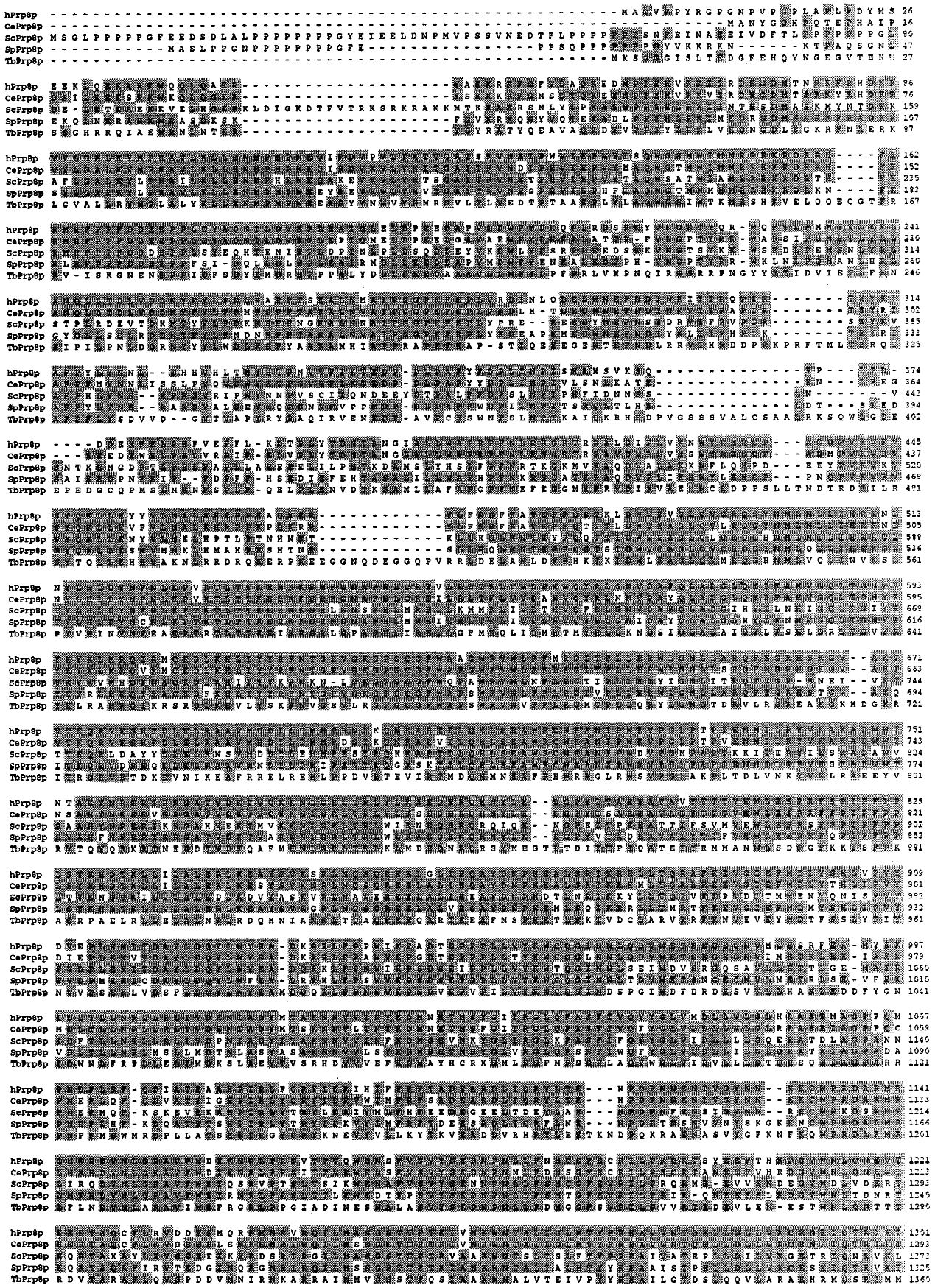
Although antisera raised against ScPrp8p have been available for some time (Anderson et al., 1989), they were not sufficiently crossreactive with the human U5 p220 to permit efficient immunoprecipitation. Thus with the hPRP8 cDNA in hand, we attempted to raise antisera against several different regions of the encoded protein. Of five different His_6-tagged fusion proteins injected into mice, chickens, and/or rabbits, only two yielded useful antisera: a 60-kDa fragment corresponding to hPrp8p amino acids 1391–1902 produced cross-reacting antibodies in chickens and a 50-kDa fragment comprising the C-terminal 460 amino acids of hPrp8p proved immunoreactive in rabbits. Both antisera recognized the same 220-kDa band in HeLa nuclear extracts, but the rabbit antiserum had both a higher titer and a higher specificity for this 220-kDa protein (Fig. 2A). For these reasons, the rabbit serum was used exclusively in the experiments below.

That the 220-kDa protein recognized by the rabbit antiserum was hPrp8p was demonstrated in two ways. First, the snRNP association of p220 was analyzed by Northern blotting the snRNAs immunoprecipitated from HeLa nuclear extract (Fig. 2B). Under low-salt conditions, the α-hPrp8p serum specifically precipitated U4, U5, and U6 snRNAs, consistent with the formation of U4/U6.U5 tri-snRNPs under these conditions (Konarska & Sharp, 1987; Behrens & Lührmann, 1991). When the beads were subjected to higher-salt washes, where tri-snRNPs tend to dissociate (Konarska & Sharp, 1987; Bach et al., 1989), only U5 snRNA was specifically brought down. These results are entirely consistent with p220 being an integral U5 snRNP and U4/U6.U5 tri-snRNP protein.

We also used the α-hPrp8p serum to immunoprecipitate proteins from U2-type splicing reactions subjected to UV-crosslinking (Fig. 3). One human splicing substrate that had previously yielded particularly efficient crosslinks to U5 p220 is a derivative of the Adenovirus Major Late (AdML) pre-mRNA containing a single benzophenone-derivatized adenosine and associated ^{32}P-tag adjacent to the 5′ splice site (MacMillan et al., 1994). Figure 3 shows a timecourse of protein crosslinking to this modified substrate upon incubation in HeLa cell nuclear extract under splicing conditions. Crosslinking to p220 developed as splicing occurred (Fig. 3, lanes 1–7), whereas no p220 crosslinking was observed when ATP was omitted (data not shown), because spliceosomes cannot assemble without ATP. When immunoprecipitations of the crosslinked proteins were carried out after protein denaturation, the sole labeled protein precipitated from these reactions was the 220-kDa band (Fig. 3, lane 9). Control reactions with preimmune (Fig. 3, lane 8) or beads alone (Fig. 3, lane 10) failed to immunoprecipitate any crosslinked protein. Additionally, the 220-kDa protein that crosslinks to the GU dinucleotide of a short 5′ splice site RNA in a first step *trans*-splicing assay was also immunoprecipitable with this antiserum (Reyes et al., 1996, 1999). Thus the 220-kDa protein recognized by this antiserum is unequivocally the human Prp8 protein, since Prp8p is known to be associated with U5 snRNA and to interact with the 5′ splice site in the major spliceosome (see Introduction).

Anti-hPrp8p serum immunoprecipitates both U2- and U12-dependent spliceosomes

We next asked whether the α-hPrp8p antiserum could immunoprecipitate pre-mRNA splicing intermediates and products contained within either spliceosome. A modified AdML pre-mRNA transcript (Gozani et al., 1994) was used as a template for U2-dependent spliceosome formation, and a splicing substrate derived from the human P120 gene (Tarn & Steitz, 1996b) served to


hPrp8p
CePrp8p
ScPrp8p
SpPrp8p
TbPrp8p

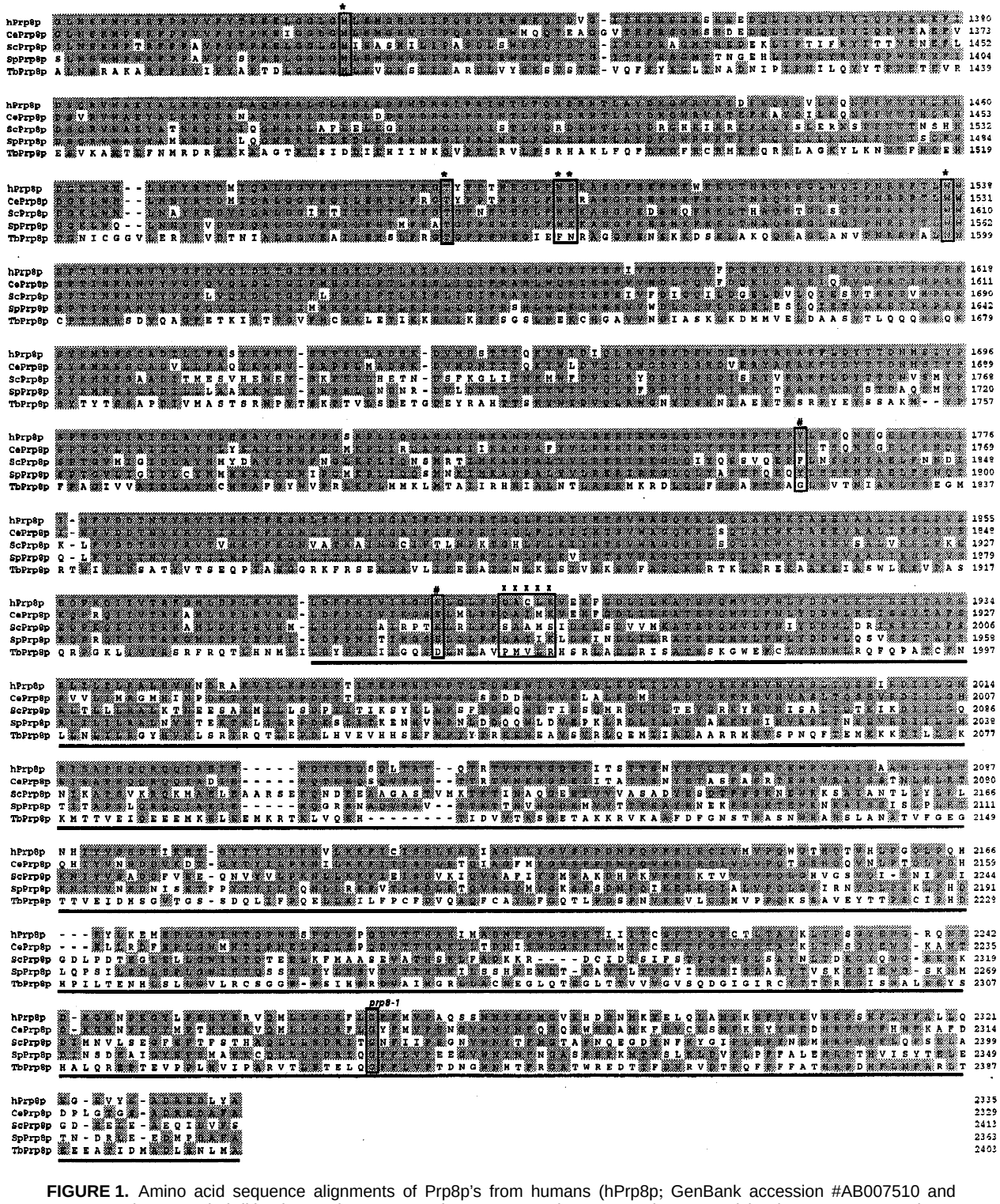


FIGURE 1. Amino acid sequence alignments of Prp8p's from humans (hPrp8p; GenBank accession #AB007510 and #AF092565), *Caenorhabditis elegans* (CePrp8p; Swiss-Prot accession #P34369), *S. cerevisiae* (ScPrp8p; GenBank accession #U00027), *Schizosaccharomyces pombe* (SpPrp8p; GenBank accession #U48733) and *T. brucei* (TbPrp8p; EMBO accession #Y12638). Sequences were aligned using DNAstar software. The portion of hPrp8p used to generate the antiserum used in this article is demarcated by a solid line. The *S. cerevisiae prp8-1* mutation (Hodges et al., 1995) and the human sequence that can be crosslinked to the GU dinucleotide at the 5′ splice site (QACLK; Reyes et al., 1999) are indicated. Positions where mutations in ScPrp8p affect 3′ splice site fidelity and uridine tract recognition (Umen & Guthrie, 1996) are represented by * and #, respectively.

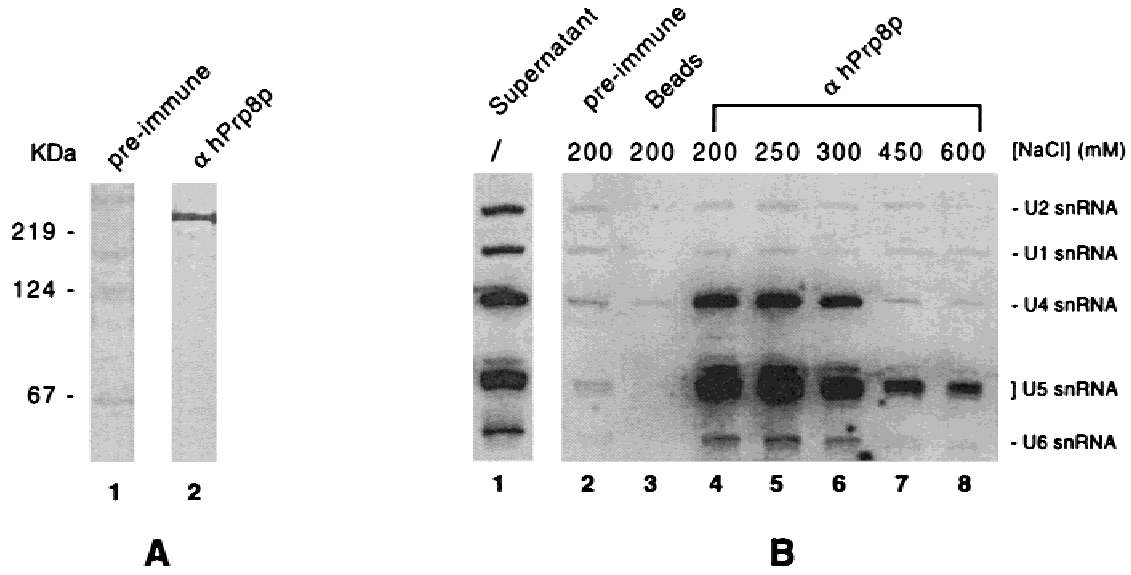


FIGURE 2. **A**: Western blot of nuclear extract proteins with preimmune (lane 1) or α-hPrp8p serum (lane 2). **B**: Northern blot of major spliceosomal snRNAs immunoprecipitated α-hPrp8p serum. Lane 1: Supernatant of immunoprecipitation in lane 4; lane 2: preimmune pellet; lane 3: beads alone pellet; lanes 4–8: pellets from α-hPrp8p serum under NaCl concentrations shown. The positions of individual snRNAs are indicated.

assemble U12-dependent spliceosomes. To ensure that only the appropriate spliceosome type was formed on each substrate, an antisense oligo directed against the branch site binding region of either U2 or U12 snRNA was included to prevent formation of the other spliceosome. U2 snRNA was targeted with a 2′-*O*-methyl oligo to physically block its binding to the pre-mRNA branch site, whereas U12 snRNA was targeted with a 2′-deoxy oligo that most likely mediated RNase H degradation of this snRNA. The effectiveness of these oligos is demonstrated in Figures 4A and 5A. Whereas splicing of AdML pre-mRNA was completely abolished by the addition of 2.4 μM anti-U2 oligo (Fig. 4A, lane 7), the anti-U12 oligo had no effect on the major spliceosome even at a much higher concentration (10 μM) (Fig. 4A, lanes 1–4). Similarly, splicing of the P120 pre-mRNA was abolished by inclusion of 1.2 μM anti-U12 oligo (Fig. 5A, lane 3), but 2.5 μM of the anti-U2 oligo did not disrupt function of the minor spliceosome (Fig. 5A, lane 2). This result is entirely consistent with the previous finding that P120 splicing is mediated by the U12-dependent, not the U2-dependent, spliceosome (Tarn & Steitz, 1996b).

When U2-dependent spliceosomes were allowed to form on the AdML pre-mRNA, the α-hPrp8p antiserum coimmunoprecipitated the AdML pre-mRNA and its splicing intermediates and products (Fig. 4B, lane 5). Although addition of the anti-U12 oligo had no effect on the immunoprecipitation pattern (Fig. 4B, lane 11), when spliceosome assembly was inhibited by either omitting ATP (Fig. 4B, lanes 14–16) or including the anti-U2 oligo (Fig. 4B, lanes 8–10), no labeled RNA species were brought down above background. Therefore, as

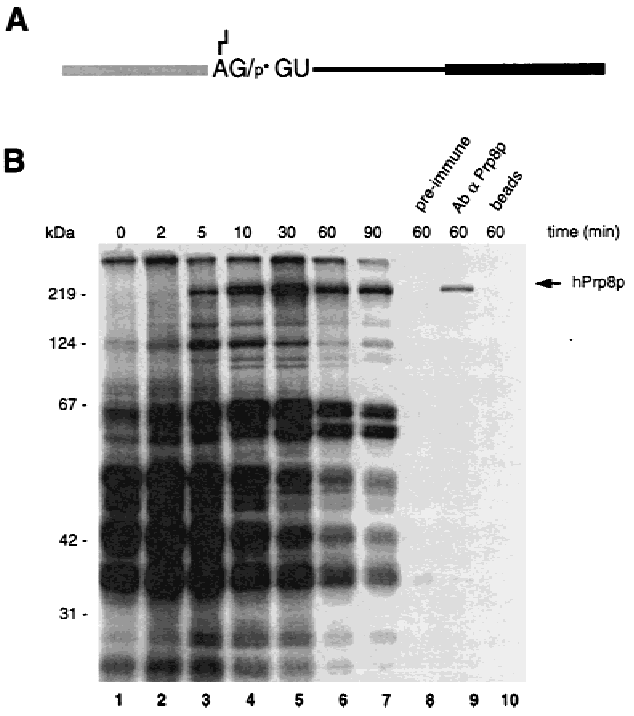


FIGURE 3. Immunoprecipitation of crosslinked p220. **A**: The photocrosslinkable PIP85.B pre-mRNA splicing template. This pre-mRNA was identical to that used in MacMillan et al. (1994). It was made by three way ligation (see Materials and Methods) and contained a site-specific benzophenone group (⌐) on the penultimate nucleotide of the first exon plus a single ^{32}P label (*) at the 5′ splice site. **B:** Lanes 1–7: This pre-mRNA was incubated under U2-dependent splicing conditions for the times indicated. Following UV irradiation and RNase treatment, the crosslinked protein species were analyzed on a 10% SDS-PAGE gel. Lanes 8–10: Immunoprecipitation of crosslinked proteins from 60 min splicing reactions. Immunoprecipitations were carried out under denaturing conditions with preimmune serum (lane 8), α-hPrp8p serum (lane 9), or beads alone (lane 10).

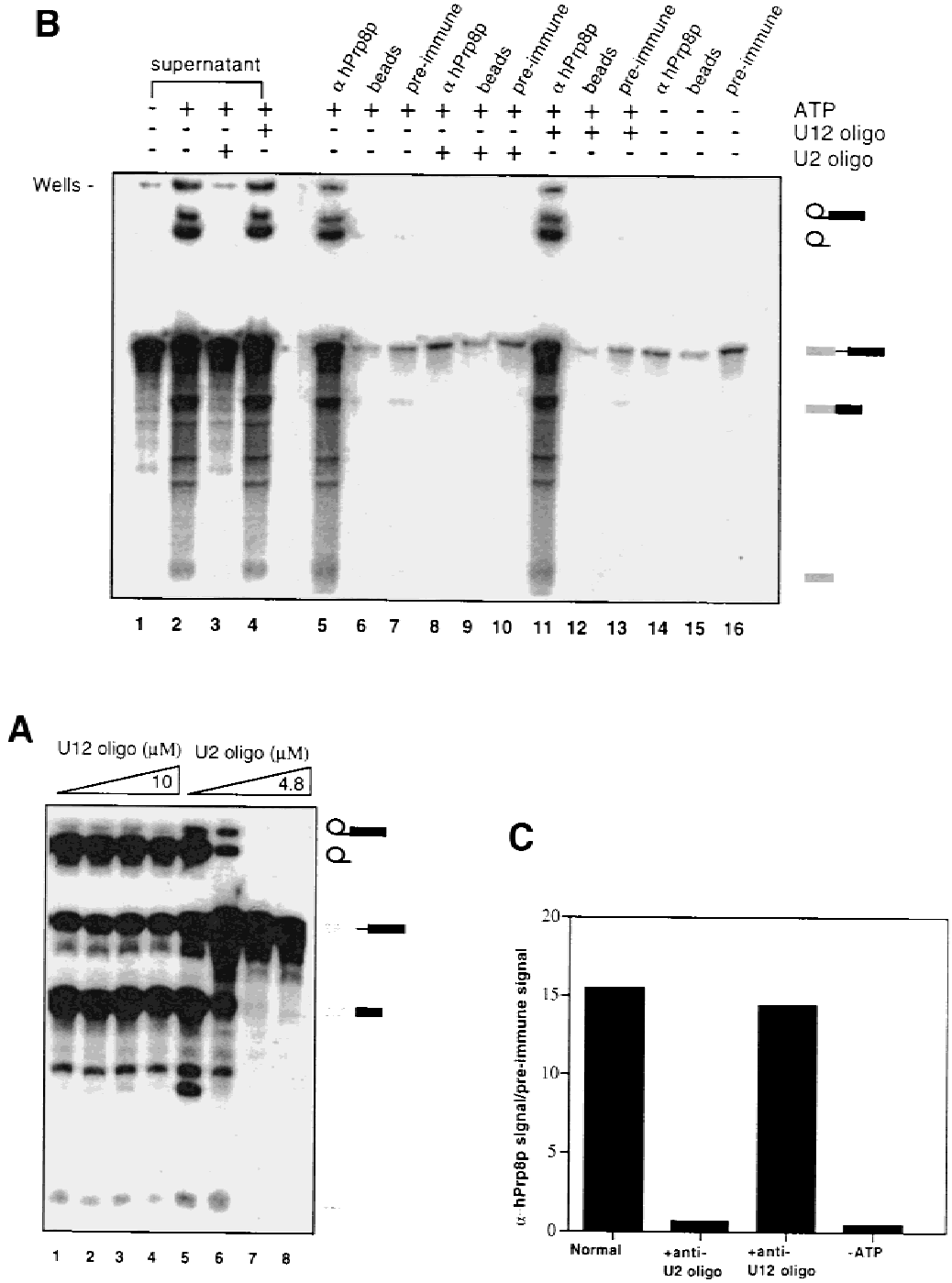


FIGURE 4. Immunoprecipitation of the major, U2-dependent spliceosome with α-hPrp8p serum. **A**: Effect of anti-U2 and anti-U12 oligos on splicing of the AdML pre-mRNA transcript. Anti-U12 oligo concentrations were 2.5 μM (lane 2), 5 μM (lane 3), and 10 μM (lane 4); anti-U2 oligo concentrations were 0.6 μM (lane 5), 1.2 μM (lane 6), 2.4 μM (lane 7), and 4.8 μM (lane 8). Lane 1 contained no oligo. After preincubation of oligos with nuclear extract for 10 min, pre-mRNA was added and incubated under splicing conditions for 1 h. RNAs were separated on a 10% denaturing polyacrylamide gel. The positions of the pre-mRNA substrate, splicing intermediates and products are indicated to the right. **B**: Immunoprecipitations with α-hPrp8p serum (lanes 5, 8, 11, and 14), beads alone (lanes 6, 9, 12, and 15) or preimmune serum (lanes 7, 10, 13, and 16). Lanes 1–4 are the supernatants of immunoprecipitations in lanes 14, 5, 8, and 11, respectively. Splicing reactions contained no oligos (lanes 5–7), 2.5 μM anti-U2 oligo (lanes 8–10), 2.5 μM anti-U12 oligo (lanes 11–13), or no ATP (lanes 14–16). **C**: Ratios of total labeled AdML RNA immunoprecipitated with α-hPrp8p serum versus preimmune serum under conditions indicated.

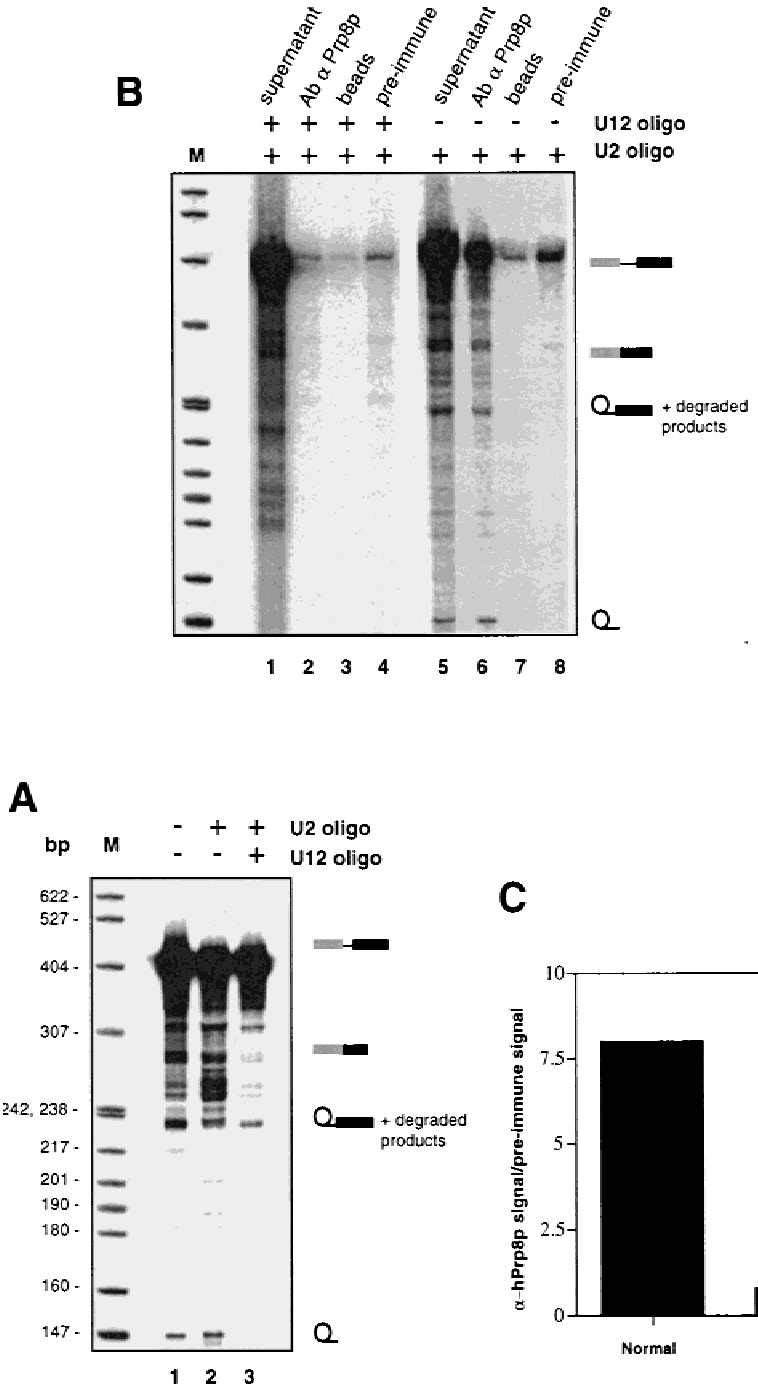


FIGURE 5. Immunoprecipitation of the minor, U12-dependent spliceosome with α-hPrp8p serum. **A**: Effect of anti-U2 and anti-U12 oligos on splicing of P120 pre-mRNA. Oligo concentrations were none (lane 1); 2.5 μM anti-U2 oligo and no anti-U12 oligo (lane 2), or 2.5 μM anti-U2 oligo and 1.2 μM anti-U12 oligo (lane 3). After preincubation of oligos with nuclear extract for 10 min, pre-mRNA was added and incubated under splicing conditions for 4 h. RNAs were separated on a 8% denaturing polyacrylamide gel. Positions of the pre-mRNA, splicing intermediates and products are indicated to the right. The cleaved 5′ exon could also be observed, but is not shown here. Size markers were γ-P^{32}-ATP kinased pBR322 DNA-Msp1 fragments (New England BioLabs). **B**: Immunoprecipitations with α-hPrp8p serum (lanes 2 and 6), beads alone (lanes 3 and 7) and preimmune serum (lanes 4 and 8). Lanes 1 and 5 are the supernatants of immunoprecipitations in lanes 2 and 6, respectively. Splicing reactions contained 2.5 μM anti-U12 oligo (lanes 1–4) or no anti-U12 oligo (lanes 5–8). All reactions contained 2.5 μM anti-U2 oligo. **C**: Ratios of total labeled P120 RNA immunoprecipitated with α-hPrp8p serum versus preimmune serum under conditions indicated.

expected, the α-hPrp8p antiserum immunoprecipitated the U2-dependent spliceosome. The precipitation of pre-mRNA in addition to splicing intermediates and products is entirely consistent with hPrp8p joining the spliceosome prior to the first chemical step and remaining associated with the complex throughout both splicing reactions.

Similar results were obtained for the P120 substrate. Under conditions where only U12-dependent spliceosomes could form (i.e., in the presence of the anti-U2 oligo), the α-hPrp8p antibodies coimmunoprecipitated the P120 pre-mRNA, lariat intermediate, lariat product, and ligated exons (Fig. 5, lane 6). However, when the anti-U12 oligo was included in the reaction to block U12 snRNA activity, no labeled RNA species were brought down above background (Fig. 5, lanes 1–4). Normalization of the signals obtained from α-hPrp8p antiserum to the backgrounds produced by the preimmune serum (Figs. 4C and 5C) demonstrated that specific immunoprecipitation of the P120 species was somewhat less efficient than for AdML (eightfold vs. 16-fold over background, respectively). However, given that the P120 substrate is spliced very inefficiently compared to the AdML pre-mRNA even under the best in vitro conditions, this difference is hardly surprising. Since a smaller percentage of the P120 pre-mRNA is likely taken up into splicing complexes than AdML pre-mRNA, it follows that a smaller proportion of P120 RNAs should be immunoprecipitated.

There is a single PRP8 locus in the human genome

BLAST (Altschul et al., 1997) comparison of the hPRP8 cDNA sequence to the human EST database revealed more than 100 related ESTs. Without exception, taking into account obvious sequencing errors (e.g., unspecified "n"s or 1-nt frameshifts in the EST DNA sequences), all of the ESTs had identical nucleotide sequences to the hPRP8 cDNA sequence we determined (although one EST did have a 42-nt deletion; see below). The same set of closely related ESTs was found when either the protein or nucleotide sequence was used for the initial BLAST query (tblastn or blastn, respectively). Thus the EST database contained no evidence for more than one PRP8 gene in humans.

Similar comparison of the hPRP8 cDNA sequence with the human genomic HTGS database (Whitehead Institute/MIT Center for Genome Research) revealed that the locus encoding this cDNA is located in the 17p13.3 region of chromosome 17. The genomic sequence encodes a 34,470-nt pre-mRNA containing 42 introns. All of the introns are between 82 and 1,761 nt except one that spans 11,011 nt. The ORF begins in exon 2 and ends in exon 43. The 5′ and 3′ terminal exons are 27–37 and 330 nt long, respectively, whereas the internal exons range from 108 to 250 nt, consistent with the exon definition model (Berget, 1995). Although no other related sequences arose from this search, the current human genomic databases are far from complete.

To test directly the possibility that the human genome might contain more than one PRP8 gene, we performed a Southern blot of human genomic DNA. The probe used was derived from a highly conserved region of the hPRP8 cDNA encompassing amino acids 451–690 (see Fig. 1). Both *Bam*HI- and *Eco*RI-digested human genomic DNA fragments were analyzed, and only a single band was detected in each case (Fig. 6). The 6-kb band in Figure 6, lane 2, and the 4.1-kb band in Figure 6, lane 3, matched exactly the expected sizes of the digested fragments calculated from the human PRP8 genomic sequence on chromosome 17: 6,001 nt for *Eco*RI and 4,118 nt for *Bam*HI.

If there were more than one PRP8 gene in the human genome, it should have been detected by this experiment. Notably, whereas the human and *S. cerevisiae* PRP8 genes are only 60% identical at the nucleotide level in the region of the hPRP8 probe used here, the expected 5.9-kb band from the yeast PRP8 gene was readily detectable on the same blot (Fig. 6, lane 1). A second cross-reacting 8.3-kb band was also observed with yeast genomic DNA, and a BLAST search of the Stanford *S. cerevisiae* genome database (SGD) revealed this to represent a sequence having 58% nu-

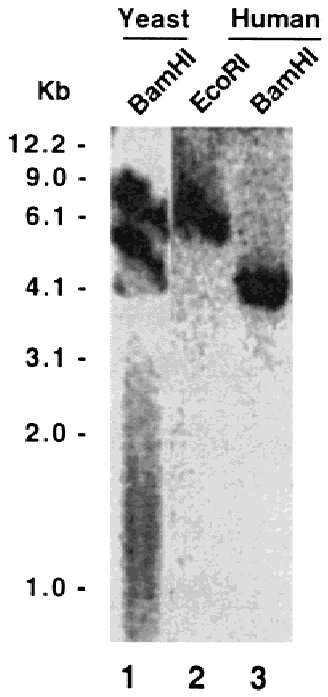


FIGURE 6. Southern blot of human and yeast genomic DNA with a cDNA probe against hPRP8. Lane 1: *Bam*HI-digested yeast genomic DNA; lane 2: *Eco*RI-digested human genomic DNA; lane 3: *Bam*HI-digested human genomic DNA. Migration positions of size markers (GIBCO 1 kb DNA Ladder) are indicated.

cleotide identity over 450 nt with the hPRP8 cDNA probe. (This sequence is from the noncoding strand of a predicted ORF in the YDR435C region and is unrelated to either yeast or human Prp8p at the amino acid level.) Therefore, given that even distantly related (58–60% identical) sequences in the yeast genome were readily detectable under the conditions used here, the detection of a single band in human genomic DNA can be reasonably interpreted to indicate that there is just one PRP8 gene in the human genome.

Analysis of hPRP8 mRNA

To examine the hPrp8p mRNA, we probed a multitissue human polyA-RNA blot with probes from two separate regions of the hPRP8 cDNA, as well as a probe against β-actin mRNA. Both probes against hPRP8 mRNA produced the same pattern: a single 7.8-kb band on both blots (Fig. 7B,C) consistent with the 7,244-nt cDNA sequence. Curiously, however, the level of the hPRP8 mRNA relative to that of β-actin varied significantly among different tissues. In Figure 7A, the 2.0-kb β-actin signals are the same across all lanes (although see note in figure legend). The highest levels of hPRP8 mRNA were observed in cardiac and skeletal muscle (Fig. 7A, lanes 3 and 8), where, based on the intensities of the signals and exposure time of the autoradiograms, the abundance of hPRP8 mRNA can be estimated to be 80–100 times lower than that of β-actin mRNA. In contrast, the observed level of hPRP8 mRNA was significantly lower in lung and kidney (Fig. 7A, lanes 2 and 5) and almost undetectable in liver (Fig. 7A, lane 4). One possible explanation for these observations could be that the quality of the polyA RNA preparations from different tissues varied, with high molecular weight mRNAs in the range of hPRP8 being more sensitive to limited degradation during blot preparation than species in the size range of β-actin. Arguing against this, however, was the absence of any lower molecular weight signals in the lanes where the 7.8-kb signal was absent or reduced, as well as the sharp intensity of the β-actin bands in these lanes (Fig. 7A). Alternatively, the level of hPRP8 mRNA in each tissue could reflect the relative transcriptional activity, and therefore RNA processing activity, of that tissue. Consistent with this latter possibility, similar tissue-specific variability in the levels of other splicing factors and hnRNP proteins have also been observed (Faura et al., 1995; Kamma et al., 1995; Arning et al., 1996; Hanamura et al., 1998).

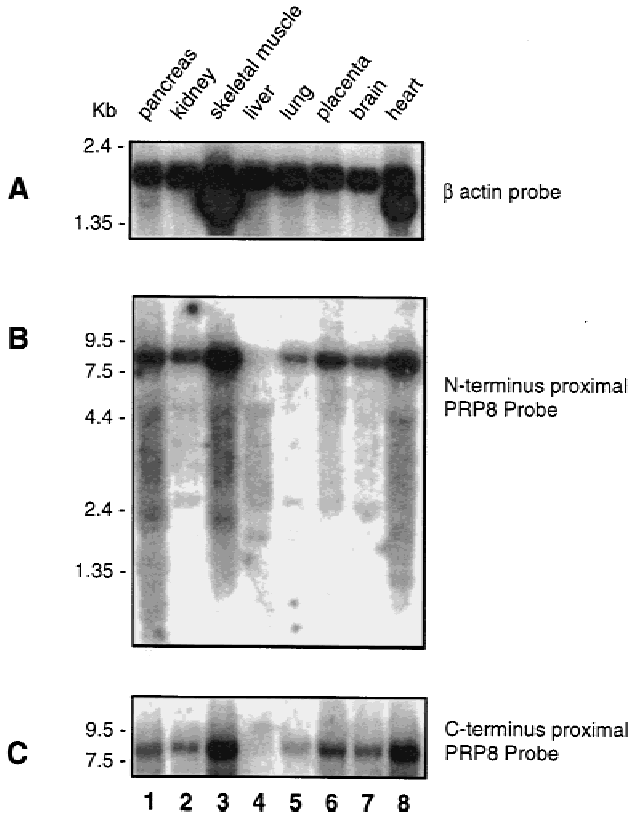


FIGURE 7. Northern blot. A single membrane, with approximately 2 μg per lane of poly-A+ RNA from multiple human tissues (Clontech), was probed successively with the probes indicated. **A**: β-actin specific probe. Note that heart and skeletal muscle contain two isoforms of β-actin (2.0 and 1.8 kb); the other tissues present only the longer species (Lamballe et al., 1991; Pari et al., 1991). **B**: N-terminus proximal hPRP8 probe. **C**: C-terminus proximal hPRP8 probe. Size markers are as indicated.

DISCUSSION

In this article, we show that a polyclonal antiserum raised against the C-terminal 50 kDa of the human Prp8 protein immunoprecipitated both the major (U2-dependent) and minor (U12-dependent) spliceosomes. These experiments minimally demonstrated the presence of a protein cross-reacting with the α-hPrp8p antibodies in each spliceosome. Western blotting indicated that our antiserum recognizes a single band of 220 kDa apparent molecular weight in HeLa nuclear extract (Fig. 2A). We confirmed that this 220-kDa protein was hPrp8p by showing that it is associated with both U5 snRNA (Fig. 2B) and the 5′ splice site region in the major spliceosome (Fig. 3). This is entirely consistent with previous reports indicating that human U5 p220 protein is identical to human Prp8p (Anderson et al., 1989; Pinto & Steitz, 1989; Garcia-Blanco et al., 1990). By all measures, therefore, the antiserum is highly specific for hPrp8p, giving no significant cross-reaction with unrelated proteins. Even so, a question that could not be addressed by immunoprecipitation was whether only one form of hPrp8p exists, or whether each spliceosome contains its own unique, but cross-reacting, version of this protein.

If the minor spliceosome contains a distinct form of hPrp8p, it must be either of very similar size or of much

lower abundance than the major p220 band. Since the snRNAs in the minor spliceosome are about 100-fold less abundant than their major counterparts (Tarn & Steitz, 1997), a low expression level might be expected for a minor hPrp8p. However, Southern blotting (Fig. 6) clearly indicated that there is just one PRP8 gene in the human genome. Given that yeast sequences only 58–60% identical to human PRP8 were readily observable on the same blot, it seems highly unlikely that a second human PRP8 gene would have escaped detection in that experiment. The existence of a single PRP8 gene is also in agreement with the EST database analysis, as well as the observation of a single-sized hPRP8 mRNA by Northern blotting (Fig. 7).

Although hPrp8p is encoded by a single gene, it is entirely possible that different forms of the protein are generated by alternative splicing of its pre-mRNA. Such alternative processing has been observed for several other mammalian splicing factors, including SF1 (Arning et al., 1996), U2AF65 (Ding et al., 1998), SRp20, SRp40, Srp55, ASF/SF2 (Jumaa & Nielsen, 1997). At present, we cannot definitively say whether the hPrp8p pre-mRNA is alternatively spliced or not. Even though a single-sized band was observed in both Northern or Western blots, very small migration differences that might result from exclusion of one exon, for example, would be all but impossible to distinguish for such large mRNA and protein species. Interestingly, as a result of our EST database comparisons, we did identify one hPRP8 EST out of 35 in the C-terminal region that clearly reflected use of an alternative 3′ splice site within exon 43. This caused the final 52 amino acids of hPrp8p to be replaced with 38 amino acids in another frame. However, it is difficult to know whether this EST represents an authentic alternative processing event or a splicing mistake that escaped nonsense-mediated decay because it was in the last exon (Nagy & Maquat, 1998).

Regardless of the possibility that the two spliceosomes contain different spliced forms of hPrp8p, our finding that there is just one genetic locus for this protein in humans has an important implication. Burge et al. (1998) recently argued that the present day existence of two spliceosomes most likely resulted from a primordial merging of genetic material from two separate, but homologous lineages to produce the progenitor of higher eukaryotes. In this view, by the time the merging of genetic materials occurred, the introns and snRNAs of the two lineages had diverged enough that removal of both intron classes in the descendant merged lineage necessitated maintenance of two separate spliceosomes. However, there would be no selective pressure for retaining multiple copies of any interchangeable parts of the two machines. Since the PRP8 gene is so highly conserved, it is entirely conceivable that both Prp8 proteins in the merged organism could readily function in either machine, so over evolutionary time only one was retained. Strong evidence to support this would be some demonstration that Prp8p does indeed perform parallel functions in both spliceosomes. Unfortunately, the antiserum we used in this study did not specifically inhibit splicing of AdML pre-mRNA, so it was not possible to initiate functional studies with that reagent. Alternatively, it would be particularly revealing to determine whether hPrp8p interacts with the analogous set of pre-mRNA sequence elements in the minor spliceosome as in the major spliceosome (i.e., the 5′ splice, branch, and 3′ splice sites). However, the disappointingly low in vitro efficiency of U12-dependent splicing has so far hampered all attempts to determine what proteins crosslink to the pre-mRNA in the U12-dependent spliceosome. Such experiments may remain technically unapproachable until more efficient conditions for U12-dependent splicing are identified.

Why is Prp8p so conserved?

The level of sequence conservation of Prp8p is truly remarkable. Not only is Prp8p the most highly conserved splicing factor known thus far, but according to the Proteome YPD database (www.proteome.com), Prp8p is also the most highly conserved large protein in the nucleus. Of the known proteins in *S. cerevisiae*, only 15 are both nucleus associated and larger than 190 kDa. Included in this set are the large subunits of both RNA polymerase II (Rpo21p) and DNA polymerase epsilon (Pol2p), which are 55% and 43% identical between *S. cerevisiae* and humans, compared to an overall 63% identity of Prp8p. In fact, after Prp8p, Rpo21p is the next most highly conserved, large nuclear protein.

Both Rpo21p and Pol2p are core components of complex multiprotein machines. Rpo21p is the largest of the 12 subunits of the yeast RNA polymerase II holoenzyme. It interacts directly with other holoenzyme subunits as well as transcription, splicing, and polyadenylation factors (Buratowski, 1994; Mortillaro et al., 1996; Orphanides et al., 1996; Hirose & Manley, 1998). Pol2p is one of the core polymerases of the DNA replisome; it communicates with at least six other polypeptides at the replication fork (Baker & Bell, 1998). Therefore, the high levels of conservation of Rpo21p and Pol2p result from a need to conserve enzymatic activity as well as maintain multiple other protein-protein and protein-nucleic acid interactions.

The observation that the sequence of Prp8p is even more constrained than Rpo21p or Pol2p indicates that both its internal structure and outer surface must be under intense selective pressure. This suggests that Prp8p might maintain even more intermolecular interactions than either Rpo21p or Pol2p. In fact, numerous interactions between Prp8p and both RNAs and protein factors are known. Besides interacting with the pre-mRNA at 5′ splice, branch, and 3′ splice sites (see

Introduction), Prp8p has known genetic or direct biochemical interactions with U2, U4, U5, and U6 snRNAs. A genetic interaction between U2 snRNA and Prp8p in yeast was found on the basis of synthetic lethality of a prp8 mutant with a U2 snRNA mutation that may perturb the U2/U6 helix II interaction (Xu et al., 1998). Another allele of yeast PRP8 was found to suppress a cold-sensitive mutant of U4 snRNA, indicating an genetic interaction between Prp8p and U4 snRNA (Kuhn et al., 1999). Furthermore, extensive direct interactions between U5 snRNA and yeast Prp8p were revealed by a U5 snRNA reconstitution experiment combined with site-specific photochemical crosslinking (Dix et al., 1998). A similar site-specific photocrosslinking analysis with reconstituted U6 snRNA indicated even more direct contacts to Prp8p (Vidal & Beggs, pers. comm.). Given Prp8p's presence in the minor spliceosome, it seems likely that it maintains similar interactions with U12, U4atac, U5, and U6atac snRNAs in that complex.

Numerous protein splicing factors are also known to interact with Prp8p. Genetic suppression analyses revealed an interaction between yeast Prp8p and Prp28p (Strauss & Guthrie, 1991). Prp28p is the yeast ortholog of the human U5-100 kDa protein, a putative RNA helicase (Teigelkamp et al., 1997). More recently, it was shown that human Prp8p can form a stable RNA-free complex with several U5-specific proteins including U5 p40, p102, p116, and p200. Among these, the most tightly associated are hPrp8p and p116 (Achsel et al., 1998). This is consistent with site-specific photocrosslinking experiments showing that both ScPrp8p and Snu114p (the yeast ortholog of human U5 p116) contact the 5′ internal loop 1 (IL1) region of U5 snRNA (Dix et al., 1998), indicating that these two proteins are closely juxtaposed in the yeast U5 snRNP. Furthermore, a yeast allele of PRP8, *prp8-101*, is synthetically lethal with four genes required for the second catalytic step of splicing, PRP16, PRP17, PRP18, and SLU7 (Umen & Guthrie, 1995b), indicating possible interactions between Prp8p and these second step factors.

In addition to components in both the major and minor spliceosomes, Prp8p may also interact with components of other biological machines. One mutant allele of PRP8 (also called DBF3) exhibits a cell cycle phenotype indicative of a defect in DNA synthesis. This suggests a possible interaction between Prp8p and cell cycle factors (Shea et al., 1994). Another genetic interaction was found between yeast Prp8p and a putative RNA helicase, SPP81/DED1, required for cytoplasmic translation (Jamieson et al., 1991; Chuang et al., 1997). Of course these interactions may reflect either direct or indirect effects of the PRP8 mutations.

What other proteins are likely shared?

Given that both human U5 snRNA and its largest associated polypeptide, p220/hPrp8p, are common to both spliceosomes, it seems probable that most of the other known U5 snRNP proteins will also be shared. Particularly good candidates are the 40-, 102-, 116-, and 200-kDa polypeptides found stably complexed with hPrp8p in the absence of U5 snRNA (Achsel et al., 1998). Notably U5 p200 (also known as Brr2p/Snu246p in yeast) is also one of the more highly conserved large nuclear proteins (44% identical between human and yeast); such a high level of conservation could well result from a need, like hPrp8p, to maintain multiple intermolecular interactions in both spliceosomes. On the other hand, given that the remaining snRNAs in the minor spliceosome have significantly diverged from their counterparts in the major spliceosome, it seems unlikely that all protein components will be shared. Thus, not only will it be fascinating to elucidate the complete protein sets for both spliceosomes, it will also be of great interest to determine how the various functions necessary for splicing are parsed out among the common versus distinct components, and how docking interactions between the two types of components are maintained.

What about U5 snRNA?

Unlike hPrp8p, human U5 snRNA is encoded by multiple genes having somewhat variable sequences (Sontheimer & Steitz, 1992). One possible explanation for the existence of multiple forms of U5 snRNA is that, like the other spliceosomal snRNAs, each type of spliceosome contains a distinct subpopulation of U5 snRNAs (Tarn & Steitz, 1996b). In this scenario, the snRNA components of the major and minor spliceosomes would actually not overlap as had been previously thought and the existence of 10 variants of U5 snRNA could presage the existence of even more types of spliceosomes than we currently know. Alternatively, and perhaps more likely, the existence of multiple forms of U5 snRNA may simply indicate that the exact sequence of this snRNA is not critical for spliceosome function. Consistent with this hypothesis, U5 snRNA is the least conserved of the major spliceosomal snRNAs across evolution. Also, it is the only snRNA in the major spliceosome that has not been shown to interact with either another snRNA or a conserved intronic sequence (see below). So, unlike U11, U12, U4atac, and U6atac, which diverged from U1, U2, U4, and U6, respectively, to match distinct sequences in their corresponding partner snRNAs and/or consensus sequences in U12-type introns, U5 was likely subject to no such selective pressure.

Although many variants of U5 snRNA exist in humans, all retain the same overall secondary structure and the CCUUUUA central loop sequence (Sontheimer & Steitz, 1992). Substantial evidence indicates that the CCUUUA loop interacts with the terminal nucleotides of both exons to help position them for exon ligation

(O'Keefe et al., 1996, and references therein). The remainder of the RNA is covered by proteins to the extent that only two short regions of the snRNA (the CCUU UUA central loop and AACUC in internal loop 2) are accessible to chemical modification (Black & Pinto, 1989). Recently, in vitro reconstitution of yeast U5 snRNP combined with photocrosslinking showed that at least five proteins (Prp8p, Snu114p, p30, p16, and p10) interact directly with the RNA (Dix et al., 1998). The reason U5 snRNA is shared by both spliceosomes may be that, in addition to promiscuous base pairing interactions of CCUUUUA loop with the ends of the exons in both spliceosomes, its other main function is to bring along its specific proteins.

MATERIALS AND METHODS

Cloning

Three human ESTs, HPL15D8U (a gift from Dr. Ali Yazdani), HFBEKO5, and HIBBH70R (from ATCC), were initially identified by similarity with the known *S. cerevisiae* and *C. elegans* PRP8 sequences (Hodges et al., 1995) using the NCBI BLAST program (Altschul et al., 1997). A fourth, spanning a gap between two of the EST clones, was identified by screening a λ-gt11 HeLa cDNA library (a gift from Dr. Ruibao Ren) with probes from either side of the gap. The 5′ end of the cDNA was isolated by 5′-RACE using the Marathon cDNA amplification kit (Clontech) and the included human placental Marathon-Ready cDNA. Nucleotide sequencing of all clones were performed using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Inc.) and read by an Applied Biosystems 373A DNA sequencer. Each DNA was sequenced at least once in each direction. Contig constructions and sequence analyses were performed using the GCG software package.

Fusion protein expression and production of polyclonal antisera

The HIBBH70R partial cDNA contained the C-terminal 50 kDa of hPrp8p, 3′ UTR, and poly-A tail. The *Sac*I/*Eco*RI fragment of this construct was subcloned in-frame with the N-terminal His_6-tag of *Escherichia coli* expression vector pV2b (Dyke et al., 1992) to create pV2b-70R. The fusion protein was overexpressed in XL1-Blue *E. coli* cells grown in LB amp+ medium by induction with 2 mM IPTG for 3 h after the culture had obtained an $OD_{600} = 0.5$. The overexpressed protein was almost entirely associated with insoluble cellular debris, so subsequent purification was carried out under denaturing conditions as described in the QIAexpressionist kit (QIAGEN). Following elution from the Ni-NTA affinity column with Buffer E (8 M urea, 0.1 M sodium phosphate, 10 mM Tris-HCl, pH 4.5), the protein preparation was dialyzed three times against 100 vol. of PBS; this treatment caused the protein to come out of solution as a white precipitate. SDS-PAGE analysis of this sample showed it to be >80% pure by Coomassie blue staining (data not shown). One liter of initial culture yielded 10 mg of purified protein. This preparation was used with Freund's adjuvant for subcutaneous injection into three female rabbits, of which two developed usable antisera over a six month period.

Western blots

Proteins from nuclear or S-100 extracts were separated by SDS-PAGE and then transferred to nitrocellulose using a semidry blotting apparatus and standard procedures. After a blocking with 5% dry milk in PBS, membranes were incubated with a 1:5,000 dilution of Protein A-purified α-hPrp8p antibodies. Immunoreactive proteins were visualized using a ProtoBlot II kit (Promega) according to the manufacturer's directions.

Immunoprecipitation and Northern blotting of snRNAs

Anti-hPrp8p antibodies were bound to Protein A-Sepharose (PAS) beads (CL-4B from Pharmacia) for 1 h in IP100 buffer (100 mM NaCl; 2 mM $MgCl_2$; 50 mM Tris-HCl, pH 7.6, 0.05% NP_{40} and 0.5 mM DTT) and then washed three times with the same buffer. Twenty microliters of a 50% slurry of PAS-bound antibodies were mixed with 100 μL NE diluted into 800 μL IP100 buffer. After rotating at 4 °C for 2 h, beads were washed three times as quickly as possible with either IP100 or the solutions indicated in Figure 2B (the same buffer as IP100 plus the indicated NaCl concentration). Bound RNAs were eluted by incubation of the beads at 65 °C for 1 h in elution buffer (1% SDS; 0.1 μg/mL glycogen; 100 mM NaCl; 0.3 M sodium acetate, pH 5.2, and 0.5 mg/mL Protease K). After separation on a denaturing polyacrylamide gel, the snRNAs were transferred to ICN nylon membrane using a semidry blotting apparatus. After UV crosslinking, the membrane was prehybridized for 2 h at 68 °C in hybridization buffer (6× SSC, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA, 0.1% SDS). Hybridizations were carried out using 5×10^6 cpm/mL anti-snRNA riboprobes (Konarska & Sharp, 1987) for 18 h at 42 °C in the same buffer. The membrane was then washed with 1× SSC, 0.1% SDS at room temperature once, and then three times at 68 °C with 0.2× SSC, 0.1% SDS.

Immunoprecipitation of UV-crosslinked proteins

A version of the PIP85.B pre-mRNA substrate containing a single benzophenone-derivatized adenosine and associated ^{32}P-tag adjacent to the 5′ splice site was prepared by splinted ligation as previously described (MacMillan et al., 1994). This substrate was incubated under U2-dependent splicing conditions (see below) for the times indicated. UV-crosslinking of 20-μL aliquots was performed on ice with a 302-nm lamp as described (MacMillan et al., 1994) except that prior to crosslinking, heparin was added to a final concentration of 0.5 mg/mL, followed by an additional 5 min incubation at 30 °C. After crosslinking, samples were denatured by adding SDS to 0.5% (w/v) and Triton X-100 to 1.5% (v/v) and boiling for 3 min. Denatured samples were diluted 10-fold with IP100 buffer and incubated at 4 °C with 20 μL 50% slurry of the PAS-bound α-hPrp8p antibodies (see above) for 2 h with gentle rocking. Beads were then washed twice with IP150-1 M urea

(150 mM NaCl; 2 mM $MgCl_2$; 50 mM Tris-HCl, pH 7.6; 0.05% NP_{40}; 0.5 mM DTT; and 1 M urea) and once with IP100. Immunoprecipitated proteins were eluted by boiling the beads in SDS-PAGE loading buffer and then separated on a 10% SDS-PAGE gel. Gels were analyzed by autoradiography.

Immunoprecipitation of U2- and U12-dependent splicing reactions

All U2-dependent splicing reactions were carried out using a modified AdML pre-mRNA transcript generated from the pHMS81 plasmid (Gozani et al., 1994). The U12-dependent splicing substrate was derived from the pP120 plasmid (generous gift from Yitao Yu and Joan Steitz) (Tarn & Steitz, 1996b) pHMS81 and pP120 and were linearized with *Bam*HI and *Hin*dIII, respectively, and transcribed with T7 RNA polymerase.

U2-dependent splicing reactions were carried out in 40% nuclear extract, 2 mM $MgCl_2$, 60 mM KCl, 1 mM ATP, 5 mM creatine phosphate, 0.05 mg/mL *E. coli* tRNA and 4 nM AdML RNA. U12-dependent splicing of the P120 pre-mRNA was performed as described by Tarn & Steitz (1996b). Each 20 μL splicing reaction contained 40 fmol of the appropriate pre-mRNA substrate. The 2′-*O*-methyl antisense oligonucleotide used to block U2-dependent splicing contained the sequence 5′-AGAUACUACACUUGAUC-3′, complementary to nt 27–41 of human U2 snRNA. U12-dependent splicing was inhibited by inclusion of an antisense 2′-deoxyoligonucleotide with sequence 5′-TCCTTACTCATAAG-3′ complementary to nt 11–24 of U12 snRNA. Oligonucleotides were added to splicing reactions at the indicated concentrations and incubated for 10 min at 30 °C to promote binding to or RNaseH degradation of the target snRNA prior to the initiation of splicing by addition of the appropriate pre-mRNA substrate.

Following incubation under splicing conditions for the times indicated, each reaction was supplemented with heparin to a final concentration of 0.5 mg/mL, followed by an additional 5-min incubation at 30 °C. Reactions were then incubated with PAS-bound antibodies (see above) for 2 h with rotation at 4 °C. Beads were washed with IP100 and RNAs eluted as for snRNAs, except that the RNA elution was carried out for only 1 h at 65 °C. Eluted RNAs were separated by denaturing PAGE and quantitated with a PhosphorImager.

Southern blot

Samples of human (15 μg) and yeast (9 μg) genomic DNA (Clontech) were digested with the indicated restriction enzyme and separated on a 0.8% agrose gel. DNAs were transferred onto a positively charged nylon membrane (Amersham) and blotted according a standard protocol provided by the company. The final wash was carried out in a solution with 1× SSPE and 0.1% (w/v) SDS at 65 °C. The hPRP8 probe was derived from a 717-bp *Sca*I/*Nsi*I fragment of the PRP8 cDNA encompassing hPrp8p amino acids 451–690. The probe was labeled using a Prime-It random primer labeling kit (Stratagene) to final specific activity $\sim 1 \times 10^9$ cpm/μg and used at 2×10^6 cpm/mL in the hybridization reactions.

Northern blotting of hPRP8 mRNA

A multi-tissue human polyA+ RNA blot was purchased from Clontech. DNA probes were labeled by random priming (Feinberg & Vogelstein, 1984) to final specific activity $\sim 4 \times 10^9$ cpm/μg and used at 2×10^6 cpm/mL in the hybridization reactions. hPRP8 probes 1 and 2 were derived from a 176-bp *Bam*HI fragment of HPL15D8U and a 164-bp PCR product from HFBEK05, encompassing hPrp8p amino acids 295–343 and 1385–1441, respectively. The β-actin control probe was supplied by Clontech. Prehybridizations (2 h) and hybridizations (16 h) were carried out at 42 °C in hybridization solution (1× Denhardt's, 50 mM Tris, pH 7.5, 1% SDS, 50% formamide, 1.0 M NaCl, 10 μg/mL sheared, denatured salmon sperm DNA). Following hybridization, three washes were performed at room temperature for 10 min each in 2× SSC, two for 30 min each at 65 °C in 2× SSC supplemented with 0.5% SDS, and two for 30 min each at room temperature in 0.1% SSC. Following autoradiography, the membrane was stripped by incubating with 1% SDS in ddH_2O at 90–100 °C for 3 to 5 h. Stripping was verified by overnight exposure to a PhosphorImager screen.

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