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Biochemistry

A mammalian protein of 220 kDa binds pre-mRNAs in the spliceosome: A potential homologue of the yeast PRP8 protein

(pre-mRNA splicing/UV-crosslinking)

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ABSTRACT **A mammalian protein of approximately 220 kDa (p220) was UV-crosslinked to precursor mRNAs (pre-mRNAs) under splicing conditions. The kinetics and biochemical requirements of the UV-crosslinking of p220 corresponded to the kinetics and biochemical requirements of spliceosome formation. On Western blots, antibodies against the yeast splicing factor PRP8 recognized a doublet of proteins, the faster migrating of which comigrated with p220. Furthermore, UV-crosslinked p220 was immunoprecipitated with anti-PRP8 antisera. These results suggest structural conservation of the splicing factor PRP8 from yeast to mammals and show that this protein is in close proximity to the pre-mRNA in the spliceosome.**

Splicing of precursor mRNAs (pre-mRNAs) requires the U1, U2, U4, U5, and U6 small nuclear ribonucleoprotein particles (snRNPs) and several protein factors (1–8). These assemble to form a splicing body, the spliceosome (9, 10). The U-small nuclear RNAs (7) and the spliceosome formation pathway (11–14) have been conserved from yeast to man. In yeast and mammalian systems, this pathway can be divided into distinct steps by the identification of intermediates in the formation of the spliceosome (3, 9, 12–14). U1 and U2 snRNPs bind pre-mRNAs to form pre-spliceosome complexes (4, 9, 12, 13). Subsequently, U4, U5, and U6 snRNPs associate in the absence of exogenously added pre-mRNA to form a U4,5,6 multi-snRNP complex (12), and this complex binds the prespliceosome complexes to form spliceosomes (12–14).

Genetic and biochemical analyses predict that a large number of gene products are required for pre-mRNA splicing. The analysis of pre-mRNA splicing conditional mutants in *Saccharomyces cerevisiae* has led to the identification and characterization of more than 20 gene products essential for splicing (15–19). One of these, PRP8, is a U5 snRNP-associated 280-kDa protein, shown to be required for pre-mRNA splicing *in vivo* and *in vitro* (20). Under splicing conditions PRP8 associates with the U4,5,6 multi-snRNP complex and with pre-mRNA (19). Fractionation of *in vitro* splicing systems has revealed the existence of several proteins that collaborate with snRNPs in splicing pre-mRNAs (3, 5). It remains to be determined whether some of these act directly on the pre-mRNA or have a role in the assembly of the spliceosome.

To identify proteins that interact directly with pre-mRNAs during splicing reactions, we have used UV-crosslinking of RNA to protein (21). We have identified a 220-kDa protein (p220) in HeLa cell nuclear extracts that binds pre-mRNAs under splicing conditions. p220 binds pre-mRNAs with specificity; point mutants in the splice sites that are impaired in splicing do not bind to p220. We investigated the relationship between HeLa p220 and the yeast splicing factor PRP8. Rabbit antibodies against yeast PRP8 protein (19) detect a protein that comigrates in SDS/PAGE with p220 and immunoprecipitate UV-crosslinked p220, strongly suggesting these two proteins are related.

MATERIALS AND METHODS

RNAs, Nuclear Extracts, and *in Vitro* Splicing Reactions. pBSAd10 (22) and pBS (Stratagene) were used as templates for the T7 RNA polymerase-directed *in vitro* synthesis of uniformly labeled Ad10 pre-mRNA and pBS RNA, respectively (23). The RNAs were labeled with [α-^{32}P]UTP (NEN) at high specific activity (23) and purified by gel electrophoresis as described (24). HeLa nuclear extracts were obtained using the procedure of Dignam *et al.* (25). Splicing reactions (24 μl) contained 8 μl of nuclear extract and had the following final composition: labeled RNA (1×10^5 cpm), 20 mM Hepes·KOH (pH 7.9), 62 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 5 mM creatine phosphate, 150 μM dithiothreitol, and 6% (vol/vol) glycerol. Incubations were at 30°C for 45 min, unless otherwise stated.

UV-Crosslinking and RNP Gels. Splicing reactions were quenched on ice and the mixture was divided. The largest portion (15 μl) of these reaction mixtures was irradiated on ice for 10 min with a 254-nm UV lamp (10 mW/min at the surface; Ultraviolet Products, San Gabriel, CA) at 4.5 cm from the surface of the lamp. Subsequently 1.5 μl of RNase A (Pharmacia) at 10 mg/ml was added and the sample was incubated at 30°C for an additional 15 min. Proteins were denatured by the addition of 1 vol of 2× SDS/PAGE sample buffer followed by boiling (22, 26). A second portion (5 μl) of each splicing reaction mixture was loaded onto a nondenaturing polyacrylamide gel to analyze the formation of splicing complexes (12). A third portion (4 μl) was used to analyze the labeled RNA on denaturing polyacrylamide gels (24).

To enhance the efficiency of crosslinking for immunoprecipitations and Western blots, labeled Ad10 RNA was synthesized *in vitro* as described above, except that all of the unlabeled UTP was replaced with 15 μM 5-bromouridine 5′-triphosphate (Sigma). This RNA (2×10^7 cpm) was subjected to a preparative splicing reaction (400 μl of nuclear extract in a final reaction volume of 1200 μl) after which the samples were divided in 50-μl aliquots into 96-well plates. A 254-nm lamp was placed on the 96-well plate that was kept on wet ice, and the aliquots were irradiated for 20 min. The irradiation at the bottom of the well was approximately 8 mW/min. After irradiation, the samples were pooled, mixed well, and treated with RNase A (1 mg/ml). This mixture (200 μl) was loaded on one lane of a 7.5% polyacrylamide gel containing SDS, which was electrophoresed until a 50-kDa

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Abbreviation: snRNP, small nuclear ribonucleoprotein particle.

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prestained molecular mass marker migrated off the bottom of the gel.

Western Blot Analysis. After electrophoresis the gel was soaked in transfer buffer (25 mM Tris/25 mM glycine, pH ≈ 8.6) at room temperature for 15 min and electroblotted onto Immobilon P (treated as recommended by the manufacturer, Millipore) in transfer buffer at 200 mA, 4°C for 14 hr. The Immobilon P membrane was blocked with BLOTTO [5% (wt/vol) nonfat dry milk in phosphate-buffered saline], incubated with either a 1:5 dilution of an affinity-purified antibody (8.2 in ref. 27) against a PRP8 protein or a 1:200 dilution of Y12 anti-Sm monoclonal antibody (28). Subsequently, the membrane was blocked with BLOTTO again and incubated with an alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin antiserum (Promega Biotec) to detect PRP8 protein. To detect the Y12 mouse anti-Sm monoclonal antibody (28), an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin antiserum was used (Promega Biotec). The incubation with the primary antibodies was at room temperature for 1 hr and with the secondary antibody was at room temperature for 30 min. The Immobilon P membrane was air-dried and exposed to XAR-5 film without intensifying screens. ^{14}C-labeled ink was used to mark the Immobilon P membrane to allow precise alignment of the immunostained blot with the autoradiograph.

Immunoprecipitations. Immunoprecipitations were performed as follows: Dry protein A-Sepharose 4B beads (Pharmacia) were resuspended in IP-1 buffer (10 mM Tris·HCl, pH 7.5/200 mM NaCl/2.5 mM $MgCl_2$/0.5% Nonidet P-40), washed extensively, and resuspended in a 50% suspension (vol/vol) of IP-1 buffer. Antibodies or antisera were incubated with the beads under continuous rotation at 4°C for 1 hr. The beads were washed three times with 10 vol of IP-1 buffer and twice with IP-2 buffer (10 mM Tris·HCl, pH 7.5/200 mM NaCl/2.5 mM $MgCl_2$/0.1% Nonidet P-40). Antibody-bound beads were incubated with crosslinked products under continuous rotation at 4°C for 5 hr. Subsequently, the beads were washed three times with IP-2 buffer and three times with IP-1 buffer. The washed beads were resuspended in 2× Phorcast loading buffer (Amersham), boiled for 5 min, and loaded onto a precast gradient Phorcast gel (Amersham).

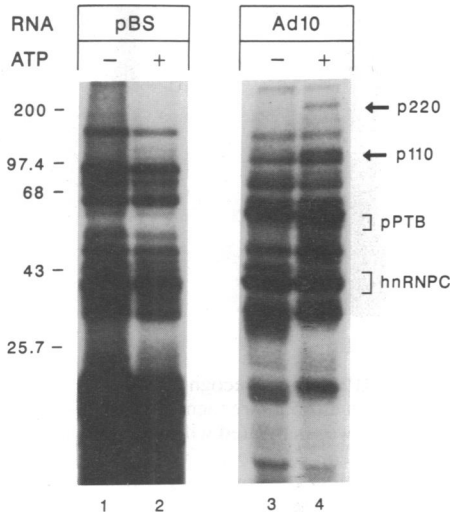

FIG. 1. A 220-kDa protein (p220) is UV-crosslinked to Ad10 pre-mRNA during splicing in HeLa cell nuclear extracts. Labeled Ad10 pre-mRNA was incubated with HeLa nuclear extracts under splicing conditions, except where ATP and creatine phosphate were not added, as indicated (–). The RNase A-trimmed UV-crosslinked products were resolved by SDS/PAGE. Molecular mass markers (BRL) are myosin (200 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and α-chymotrypsinogen (25.7 kDa), p220, p110, pPTB, and heterogeneous nuclear RNP C (hnRNPC) proteins are indicated. +, 1 mM ATP/5 mM creatine phosphate.

RESULTS

Identification of a 220-kDa Protein That Binds Pre-mRNAs Specifically. A subset of the proteins that bind pre-mRNAs during splicing reactions has been identified by 254-nm UV-crosslinking of RNA to protein (22). A protein of approximately 220 kDa (p220) was UV-crosslinked to Ad10 RNA, a pre-mRNA derived from the adenovirus 2 major late transcription unit (Fig. 1). This protein was detected by UV-crosslinking only when the reaction mixtures contained exogenously added ATP (Fig. 1, compare lanes 3 and 4). In contrast, many other proteins were crosslinked to Ad10 RNA independent of the addition of ATP; for example, see the 62-kDa polypyrimidine-tract-binding protein, pPTB, and the heterogeneous nuclear RNP C proteins (Fig. 1, lanes 3 and 4) (22). A protein of 110 kDa (p110 in Fig. 1) was also crosslinked preferentially in the presence of ATP. It is possible that p110 is related to the intron binding proteins described by Tazi *et al.* (29) and Gerke and Steitz (30).

p220 was not UV-crosslinked to RNAs that are not splicing precursors (for example, pBS RNA in Fig. 1, lanes 1 and 2). The specificity of the UV-crosslinking of p220 was confirmed by competition experiments. The crosslinking of p220 to uniformly labeled Ad10 RNA could be competed by the addition of excess unlabeled Ad10 RNA to the reactions (Fig. 2*A*, lanes 2–4), but not by addition of excess unlabeled pBS RNA (Fig. 2*A*, lanes 5–7). The extent of competition for the binding of p220 correlated with the degree of competition for the formation of splicing complexes (Fig. 2*B*).

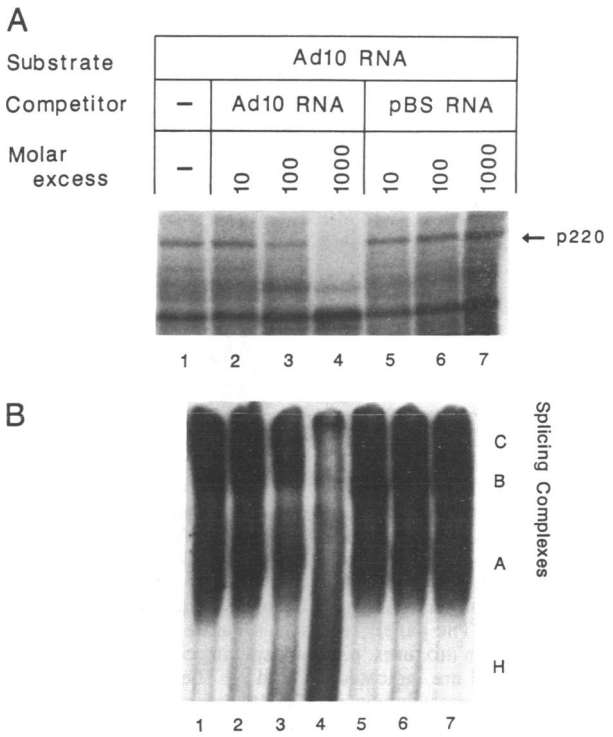

FIG. 2. p220 binds pre-mRNAs specifically. Labeled Ad10 RNA was incubated with HeLa nuclear extracts under splicing conditions, except that unlabeled (Ad10 or pBS) RNA was added to the reaction mixtures. The RNase A-trimmed UV-crosslinked products were resolved by SDS/PAGE (*A*). The pattern of splicing complex formation is shown (*B*). *A* and *B* show separate analyses of the same splicing reaction mixtures. p220 and splicing complexes A, B, and C and the nonspecific heterogeneous complex (H) are indicated. –, No competitor RNA.

A pre-mRNA derived from the second intron of the rabbit β-globin gene (31) was also crosslinked to p220 under splicing conditions (data not shown). p220 did not crosslink to a rabbit β-globin pre-mRNA containing a point mutation in the 3′ splice site (AG → UG) under splicing conditions (data not shown). This mutant β-globin pre-mRNA is defective for splicing and for the formation of splicing complexes *in vitro* (31). Pre-mRNAs containing multiple point mutations in the polypyrimidine tract of an adenovirus intron did not crosslink to p220, whereas a pre-mRNA with a wild-type polypyrimidine tract did (data not shown). These pre-mRNAs with mutant polypyrimidine tracts are defective for splicing *in vitro* (22).

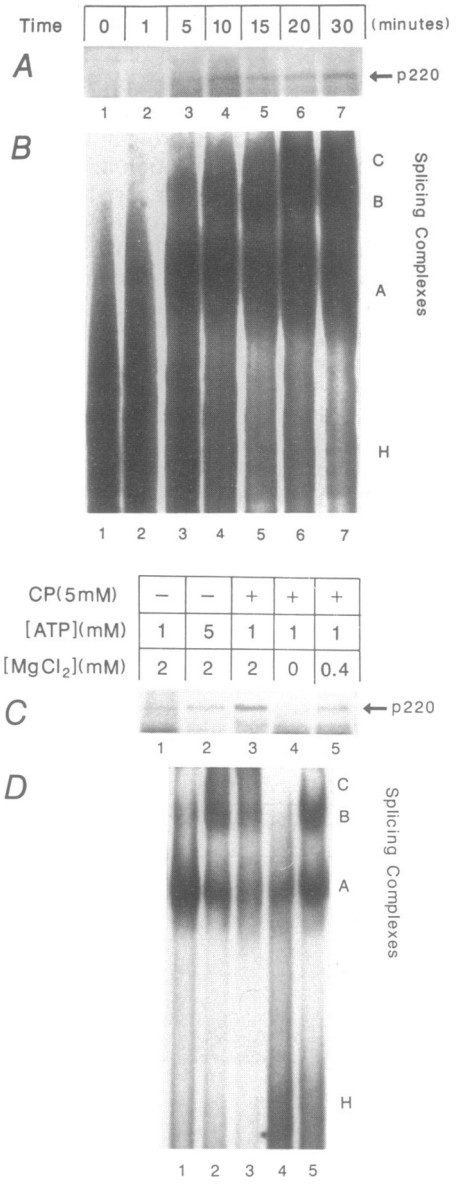

FIG. 3. p220 binds to Ad10 pre-mRNA in the spliceosome. (*A* and *B*) The time course of appearance of p220 correlates with the time course of spliceosome formation. Labeled Ad10 pre-mRNA was incubated with HeLa nuclear extracts under splicing conditions, except that the time of incubation at 30°C was varied. The crosslinking to p220 is shown in *A*. The pattern of splicing complex formation is shown in *B*. The panels represent separate analyses of the same splicing reaction mixtures. p220 and splicing complexes A, B, and C and complex H are indicated. (*C* and *D*) The biochemical requirements for UV-crosslinking of p220 to pre-mRNA correlate with requirements for spliceosome formation. Labeled Ad10 pre-mRNA was incubated with HeLa nuclear extracts under splicing conditions, except that the concentration of reagents was varied as indicated. The standard splicing conditions are those in lane 3. Lanes 1 and 2 show the effects of omitting (−) creatine phosphate (CP) and varying the ATP concentration. Lanes 4 and 5 show the effects of omitting $MgCl_2$ or of addition of 0.4 mM $MgCl_2$. The crosslinking to p220 is shown in *C*. The pattern of splicing complex formation is shown in *D*. *C* and *D* show separate analyses of the same splicing reaction mixtures. p220 and splicing complexes A, B, and C and the nonspecific heterogeneous complex (H) are indicated.

p220 Binds Pre-mRNAs in the Spliceosome. U2 snRNP forms a stable complex with pre-mRNA (splicing complex A) detectable in native Tris/glycine gels (12). Subsequently, the U4,5,6 multi-snRNP complex binds the pre-mRNA-U2 snRNP complex to form complex B, and finally complex C, lacking U4 snRNA, is generated (32). Crosslinking of p220 to Ad10 RNA was not detectable before 5 min of incubation under splicing conditions (Fig. 3*A*, lane 3) but became clearly detectable after 10 min of incubation (lane 4). The level of crosslinked p220 remained constant from 20 min to as long as 60 min of incubation (lanes 6 and 7; data not shown). The appearance of a crosslinked p220 was paralleled by the kinetics of formation of splicing complex B (Fig. 3*B*).

In the absence of added creatine phosphate, included to regenerate ATP during *in vitro* splicing reactions, the addition of 1 mM ATP resulted in the efficient formation of complex A and in the formation of low levels of complex B (Fig. 3*D*, lane 1). When 5 mM ATP was added (lane 2) or when creatine phosphate and 1 mM ATP were present (lane 3), all three complexes were generated. UV-crosslinking of Ad10 RNA to p220 was low with 1 mM ATP but was clearly observed under the latter two conditions (Fig. 3*C*, lanes 1–3). Under other conditions where the pathway was arrested at complex A (for

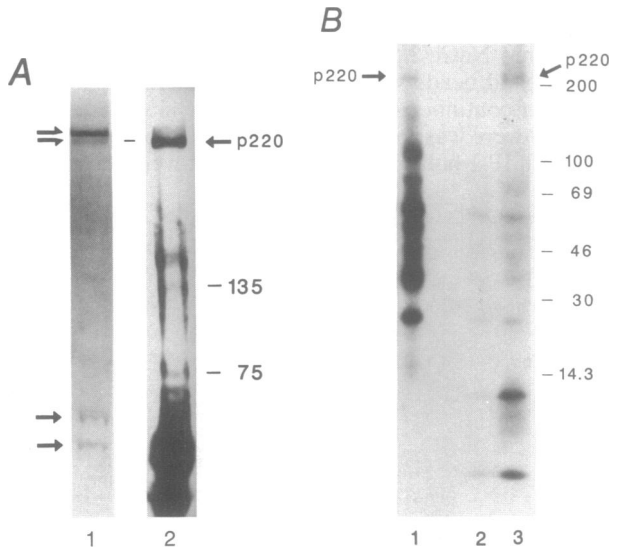

FIG. 4. Anti-PRP8 antisera recognize proteins in HeLa cells. (*A*) p220 comigrates with a protein recognized by anti-PRP8 antibodies. Labeled Ad10 RNA was incubated with HeLa nuclear extract under splicing conditions. The UV-crosslinked proteins were resolved on a 7.5% polyacrylamide gel containing SDS, electroblotted onto Immobilon P, and immunostained with affinity-purified anti-PRP8 antibodies (lane 1). When the same immunostained filter was exposed on film the UV-crosslinked proteins were visualized (lane 2). Immunostained proteins are indicated with arrows; p220 is indicated. Molecular mass markers shown are prestained markers (Bio-Rad): phosphorylase *b* (135 kDa), bovine serum albumin (75 kDa) (the prestaining procedure alters the molecular mass). (*B*) p220 is immunoprecipitated by anti-PRP8 antisera. Lanes: 1, total UV-crosslinked reaction mixture; 2, UV-crosslinked products precipitated by preimmune sera; 3, products precipitated by immune sera. Molecular mass markers (Amersham) were myosin (200 kDa), phosphorylase *b* (100 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (14.3 kDa).

example, in the absence of exogenously added Mg^{2+}), the crosslinking of p220 to pre-mRNA was not detected (Fig. 3 *C* and *D*, lanes 4). When 0.4 mM Mg^{2+} was added, complexes with mobilities corresponding to complexes A and B were detected and UV-crosslinking of p220 to pre-mRNA was observed (lane 5). The data shown in Fig. 3 suggest that p220 is associated with the pre-mRNAs after formation of complex B.

p220 Is a Potential Homologue of PRP8. To investigate the relationship between p220 and the U5 snRNP-associated yeast PRP8 protein, rabbit antisera to PRP8 were used in Western blots (33). A preparative splicing reaction mixture containing Ad10 RNA in which uridines were replaced with bromouridines (34) was incubated for 45 min and UV-crosslinked. The crosslinked products were digested with RNase, resolved by SDS/PAGE, and transferred onto Immobilon P membrane. The membrane was probed with affinity-purified antibodies against PRP8 protein. Antibodies were raised against PRP8–β-galactosidase fusion proteins and then affinity-purified against PRP8–TrpE fusion proteins, resulting in reagents with high specificity for PRP8 (27). The Western blot procedure detected four proteins, two of which had apparent molecular masses greater than 200 kDa (Fig. 4*A*, lane 1). Subsequent autoradiography of the filter revealed that the UV-crosslinked p220 comigrated with the faster migrating of the two large (>200 kDa) proteins (Fig. 4*A*, lanes 1 and 2). Lanes 1 and 2 in Fig. 4*A* represent the same lane in the gel. The two large proteins recognized by anti-PRP8 antibodies were also detected with extracts that were not UV-irradiated. These two proteins were also weakly recognized by the Y12 anti-Sm monoclonal antibody (data not shown).

Crosslinked p220 was selectively immunoprecipitated with anti-PRP8 antisera (Fig. 4*B*). Rabbit anti-PRP8 antisera 8.2, 8.3, and 8.4 (see ref. 27) were pooled, as were the respective preimmune sera, and these pooled antisera were used to precipitate UV-crosslinked proteins (compare lanes 2 and 3). The enrichment for p220 in the pellet of the immunoprecipitation with immune sera was greater than 10-fold that for the typical background bands. In contrast, immunoprecipitations with preimmune or normal rabbit sera did not yield selective enrichment of p220. Small (less than 14.3 kDa) proteins were also selectively precipitated in this experiment; these may represent degradation fragments of p220. These small peptides, however, were not consistently observed in all immunoprecipitation experiments.

DISCUSSION

A protein of approximately 220 kDa (p220) was shown to bind pre-mRNAs under *in vitro* splicing conditions in HeLa cell nuclear extracts. UV-crosslinking was used to detect binding of this protein to pre-mRNA. p220 was shown to specifically bind to pre-mRNAs that are functional in splicing. The kinetics of this binding were the same as the kinetics of formation of the spliceosome complex B. Moreover, the biochemical requirements for the binding of p220 were again the same as those for formation of the spliceosome. p220 was shown to be antigenically related to the yeast splicing factor PRP8. Antibodies to the yeast protein PRP8 detected two large mammalian proteins (approximately 220 kDa), one of which exactly comigrated with p220. Furthermore, antisera to the yeast protein PRP8 immunoprecipitated UV-crosslinked p220.

The data presented above strongly suggest that p220 is related to the PRP8 protein and may be its functional human homologue. While these studies were in preparation for publication the following related observations were reported. Two proteins of approximately 200 kDa present in purified HeLa U5 snRNP preparations were shown to crossreact with affinity-purified antibodies against PRP8 (27, 35). These two polypeptides undoubtedly correspond to the doublet detected with similar antisera in our study (Fig. 4*A*). It is interesting that the p220 protein that crosslinks specifically to the pre-mRNA only comigrates with the most rapidly migrating species in this doublet. The fact that the larger of the

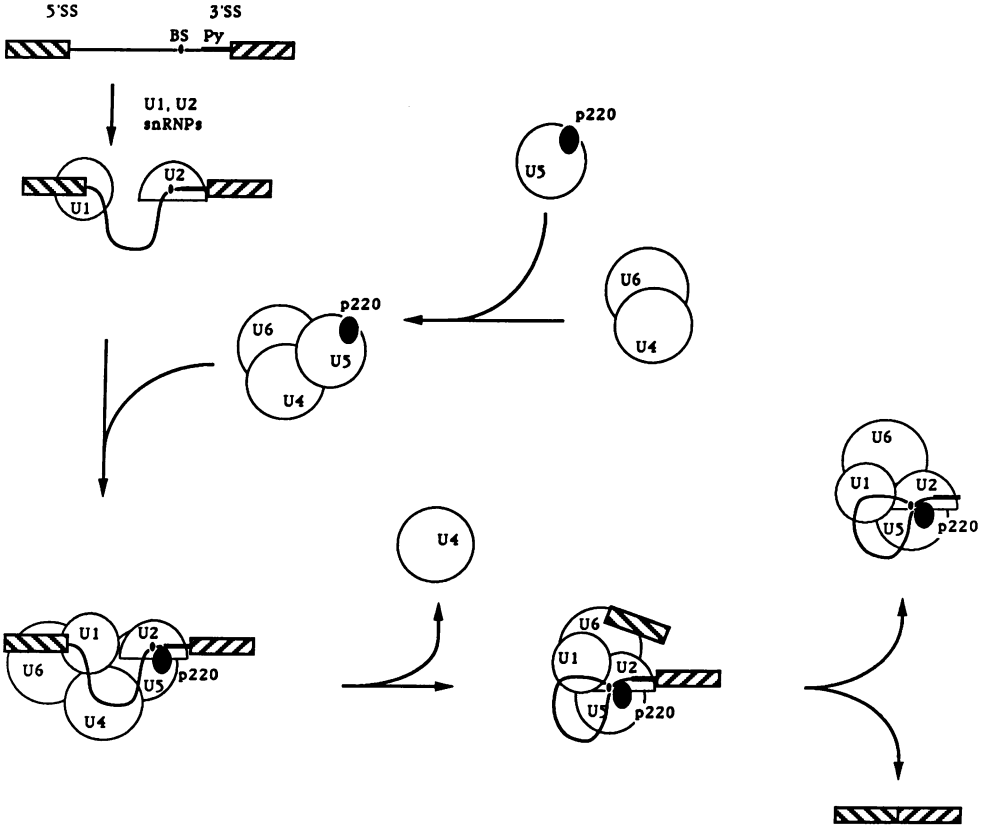

FIG. 5. Model for the interactions of p220 in the pathway of spliceosome assembly. A schematic of the pathway of spliceosome assembly is shown to illustrate the association of snRNPs. Several proteins that bind to the pre-mRNAs to facilitate the binding of snRNPs are not indicated (3, 5, 22). U4 snRNP is shown as being released from the spliceosome (32). Evidence suggests it may remain associated but in an altered conformation (37). The p220 is shown associated with the intron lariat as was suggested (19).

immunostained proteins did not crosslink to Ad10 RNA implies that it does not interact directly with this RNA. An intriguing possibility is that the larger protein is an inactive precursor of the p220 and the transition to the p220 form represents a regulatory step. We cannot exclude, however, the possibility that these are distinct proteins that share immunological epitopes.

The yeast PRP8 protein is associated with yeast U5 snRNP and with the U4,5,6 multi-snRNP complex (19). Again using antisera to yeast PRP8, a crossreacting protein larger than 200 kDa was shown to be associated with human U5 snRNP, with the U4,5,6 multi-snRNP complex, and with spliceosomes (36). Since the binding of the U4,5,6 multi-snRNP complex probably converts the prespliceosome complex A to complex B, this observation is consistent with the specific crosslinking of p220 only after formation of the spliceosome.

Formation of the reasonably efficient crosslink between p220 and pre-mRNAs shows that these two components are at intermolecular distances typical of a covalent bond length (21). This proximity suggests that a portion of p220 may recognize the substrate pre-mRNA directly in a sequence-specific fashion. If so, part of the conservation of PRP8/p220 between yeast and man may reflect recognition of sequences conserved in the pre-mRNAs of yeast and man. The 5′ splice site and the branch site, two sets of conserved sequences, are recognized by U1 snRNP and U2 snRNP, respectively, during the early stages of the pathway of spliceosome assembly (4, 12) (Fig. 5). The U4,5,6 multi-snRNP complex is thought to bind after U1 and U2 snRNP and the p220 protein is probably incorporated into the spliceosome at this stage (12, 31). The third set of conserved sequences, the 3′ splice site, is important for the binding of U2 snRNP and U4,5,6 multi snRNP complex and may be the site of binding of p220. Alternatively, p220 might recognize sequences at the 5′ splice site and branch site in conjunction with U1 and U2 snRNPs. Since p220 is closely associated with precursor RNA, it probably remains in the spliceosome and is released from the intron-containing complex along with U5 snRNP.

This study presents evidence that PRP8/p220 directly binds the pre-mRNA in the spliceosome. It is interesting to speculate that a portion of this protein may be an active component of the catalytic pocket of the spliceosome.

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