Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 2216–2219, March 1990
Biochemistry

Affinity purification of spliceosomes reveals that the precursor RNA processing protein PRP8, a protein in the U5 small nuclear ribonucleoprotein particle, is a component of yeast spliceosomes

(yeast splicing/poly(A)-binding protein)

ERICA WHITTAKER, MARIE LOSSKY*, AND JEAN D. BEGGS

Department of Molecular Biology, University of Edinburgh, King's Buildings, Mayfield Road, Edinburgh EH9 3JR, Scotland

Communicated by Joan A. Steitz, December 29, 1989 (received for review September 26, 1989)

ABSTRACT **Nuclear pre-mRNA splicing in *Saccharomyces cerevisiae*, as in higher eukaryotes, occurs in large RNA–protein complexes called spliceosomes. The small nuclear RNA components, U1, U2, U4, U5, and U6, have been extensively studied; however, very little is known about the protein components of yeast spliceosomes. Here we use antibodies against the precursor RNA processing protein PRP8, a protein component of the U5 small nuclear ribonucleoprotein particle, to detect its association with spliceosomes throughout the splicing reaction and in a post-splicing complex containing the excised intron. In addition, an indirect immunological approach has been developed that confirms the presence of precursor RNA processing protein PRP8 in isolated spliceosomes. This method has possible general application for the analysis of ribonucleoprotein particle complexes.**

PRP8 (*p*recursor *R*NA *p*rocessing 8, the protein product of the *PRP8* gene; formerly called RNA8; ref. 1) of *Saccharomyces cerevisiae* is essential for nuclear pre-mRNA splicing *in vivo* and *in vitro*. The mutation *prp8-1* (2) causes temperature-sensitive growth with accumulation of unspliced pre-mRNA at the nonpermissive temperature (1). *In vitro* the splicing activity of a cell extract prepared from the *prp8-1* strain displays increased temperature sensitivity (3), and an extract from wild-type cells loses RNA splicing activity after immunological depletion of PRP8 (1). PRP8 is exceptionally large, as estimated by denaturing gel electrophoresis (1), and is predicted from the sequence of the cloned *PRP8* gene to be 280 kDa (S. P. Jackson and J.D.B., unpublished results). By using an immunological approach, it was demonstrated that PRP8 is specifically associated with U5 small nuclear ribonucleoprotein particles (snRNPs) and with U4/U5/U6 snRNP complexes that form when yeast splicing extracts are incubated in the presence of ATP (4). It has been proposed (4, 5) that these snRNP complexes may facilitate efficient spliceosome assembly since the U4, U5, and U6 small nuclear RNAs (snRNAs) appear to enter spliceosomal complexes simultaneously; however, a precursor–product relationship has not been demonstrated.

The RNA components of yeast spliceosomes have been extensively characterized (6), but very little is known about the protein content. Only PRP11, a 30-kDa nuclear protein, has been shown conclusively to be a yeast spliceosomal protein. When *in vitro*-synthesized and radiolabeled PRP11 was used to complement the splicing defect of a *prp11* heat-inactivated extract, PRP11 was demonstrated to enter 40S spliceosomal complexes (7).

In our studies of the role of PRP8 and of its interaction with other splicing factors, an important question is whether this protein remains associated with U5 snRNPs as they assemble into spliceosomes and, if so, whether PRP8 is present in spliceosomes at all stages of the RNA splicing reaction. The experiments described here demonstrate the presence of PRP8 in spliceosomes throughout the splicing reaction and in a post-splicing complex containing excised intron. We describe a method for purification of spliceosomes based on indirect immuno-affinity selection through antibodies against poly(A)-binding protein that associates with a poly(A) sequence attached to the 3′ end of the substrate RNA.

MATERIALS AND METHODS

***In Vitro* Splicing Reactions.** Precursor RNAs were transcribed *in vitro* using bacteriophage SP6 or T7 RNA polymerase (8). Actin pre-mRNA was transcribed from pSPT19-actin/alu DNA (1), and plasmids pSPrp51A, pSPrp51A[5′-0], or pSPrp51A[Δ3b] (9) were used as templates to produce the polyadenylylated precursor RNAs. Yeast whole cell extracts were prepared and *in vitro* splicing reactions were performed as described (1, 10). The splicing extract used for the affinity selection of spliceosomes was a mix of 3 parts whole cell extract (10) to 2 parts 35% saturated ammonium sulfate precipitate of whole cell extract (11). For the affinity selection of spliceosomes, 0.01 ml, 0.04 ml, and 0.2 ml of splicing reaction mixture (for the analysis of reaction products and snRNAs and for immunoblotting, respectively) were incubated at 25°C for 10 min and then quenched on ice.

Immunoprecipitations. Antisera to PRP8–β-galactosidase fusion proteins were raised as described (1, 4). Splicing reactions were quenched on ice, adjusted to 150 mM Na^+/K^+, 10 mM Hepes·KOH (pH 7.0), 2.5 mM $MgCl_2$, 0.1% Nonidet P-40, and then immunoprecipitated with antibodies to FP8.4 or with purified anti-trimethylguanosine antibodies as described (4). For antigen competition, 2 μg of purified fusion protein was added during binding of antibodies to protein A-Sepharose (PAS). In the PAS control, no antibodies were bound to the PAS. For the time-course experiment, at each incubation time, 9 μl of a 10-μl splicing reaction mixture was immunoprecipitated and RNA was extracted from the remaining 1 μl without further treatment.

Affinity Selection of Spliceosomes. Rabbit antiserum to poly(A)-binding protein (PAB) was raised as described in ref. 12. PAB-specific antibodies were affinity-purified against pure PAB and concentrated on a PAS column (13). Antibodies were bound to PAS in NTN buffer (150 mM NaCl/50 mM Tris·HCl, pH 7.5/0.05% Nonidet P-40) and washed three times with the same buffer. For analysis of the protein components of affinity-selected spliceosomes, PAB antibod-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PAS, protein A-Sepharose; PAB, poly(A)-binding protein; PRP, *p*recursor *R*NA *p*rocessing; PRP8, protein product of the *PRP8* gene; snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein particle.

*Current address: Department of Biochemistry, Brandeis University, Waltham, MA 02254.

ies were covalently coupled to the PAS with dimethylpimelimidate (13). Splicing reaction mixtures were adjusted to contain 200 mM K^+, 2 mM Mg^{2+}, 11 mM EDTA, and 9 mM Tris·HCl (pH 8.0), and *Escherichia coli* tRNA was added to 0.6 mg/ml. The samples were incubated at 4°C with PAS-bound antibodies for 1 hr with mixing. The antibody complexes were washed twice with NTN buffer and once with NT buffer (150 mM NaCl/50 mM Tris·HCl, pH 7.5).

Immunoblot Analysis of Spliceosomal Proteins. The antibody-bound complexes were denatured by boiling, the PAS beads were removed by centrifugation, and the supernatant was fractionated by SDS/PAGE. The gel was electroblotted to nitrocellulose in the absence of methanol and probed with PRP8 antiserum to FP8.2 as described (4).

RNA Analyses. Precursor RNA, splicing intermediates, and products were recovered from splicing reaction mixtures by treatment with proteinase K and extraction with phenol and separated by electrophoresis through a 6% polyacrylamide/7 M urea gel. Detection of snRNAs by Northern blot analysis was performed as described (4). The oligodeoxynucleotides used as probes for the snRNAs were as follows (5′ to 3′): U1 (CTTAAGGTAAGTAT); U2 (CTACACTTGATCTAAGCCAAAAGGC); U4 (CCGTGCATAAGGAT); U5 (AATATGGCAAGCCC); and U6 (TCATCTCTGTATTG). Detection of snRNAs by pCp labeling was carried out by standard procedures (14) using T4 RNA ligase kindly provided by M. Gate (Medical Research Council Laboratory for Molecular Biology, Cambridge, U.K.). Quantitative determination of the efficiency of immunoprecipitation and of the enrichment of each RNA species was performed by densitometric scanning of autoradiographs with a Shimadzu Dual-Wavelength Chromato Scanner, model CS-930 (Howe, London).

RESULTS

Antibodies that recognize the trimethylguanosine cap structure of snRNAs precipitated spliceosomal complexes from an *in vitro* splicing reaction mixture due to their snRNA content (Fig. 1*A*, lane 5). The precipitation of precursor RNA, splicing intermediates (exon 1 and lariat intron-exon 2), the excised intron, and spliced exons indicates precipitation of splicing complexes participating in all stages of the splicing reaction. Similarly, antibodies to PRP8 precipitated splicing precursor, intermediates, and products (Fig. 1*A*, lane 1), suggesting that PRP8 interacts with spliceosomes. Preimmune serum (lane 2) did not precipitate any RNA species above the level of the PAS control (lane 6). Antigen competition experiments demonstrated the specificity of the PRP8 immunoprecipitation. Presaturation of the antigen-binding sites with purified β-galactosidase–PRP8 fusion protein 8.4 (lane 3), against which the PRP8 antiserum was raised (4), eliminated immunoprecipitation of the RNA species, whereas competition with fusion protein 8.1, which contains another region of PRP8 and saturates only the β-galactosidase antibodies, had no effect (lane 4).

The time course in Fig. 1*B* shows that the precipitation of splicing intermediates and excised intron by PRP8 antibodies correlates with the appearance of these RNA species in the splicing reaction. PRP8 antibodies did not precipitate RNA out of mock splicing reaction mixtures with no added ATP or using a mutant substrate RNA that has the 5′ splice site deleted (data not presented), indicating that PRP8 only associates with RNA present in spliceosomal complexes.

In the experiment shown in Fig. 1*B*, PRP8 antibodies immunoprecipitated ≈3% of the total amount of exon 1 and of intron product, 1% of lariat intron-exon 2, and only 0.3% of spliced exon 1-exon 2 RNA. In other experiments, the efficiency of immunoprecipitation of the splicing intermediates and intron product varied up to a maximum yield of 15%, depending on the pre-mRNA used as substrate and on the

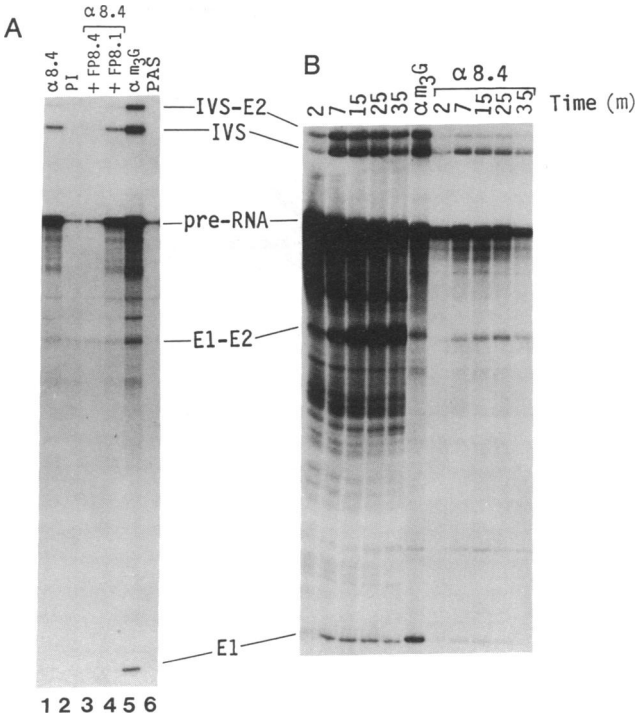

FIG. 1. Immunoprecipitation of complexes from *in vitro* splicing reactions with PRP8 antibodies. (*A*) Splicing reactions containing pre-RNA, intermediates exon 1 (E1), and intron-exon 2 (IVS-E2), spliced exons (E1-E2), and intron product (IVS) were subjected to immunoprecipitation with antibodies to PRP8 fusion protein 8.4 (α8.4) (FP8.4; lane 1), antibodies from preimmune serum (PI; lane 2), antibodies to FP8.4 presaturated with FP8.4 (lane 3), antibodies to FP8.4 presaturated with FP8.1 (lane 4), anti-trimethylguanosine (αm_3G) antibodies (lane 5), or PAS only (lane 6). (*B*) A time course of a splicing reaction is shown in the left half with lane labels in min, and on the right are the corresponding immunoprecipitations with antibodies to FP8.4.

conditions of incubation with the PRP8 antibodies. Immunoprecipitation in the presence of EDTA (1–11 mM) resulted in increased efficiency of precipitation of both lariat RNA species (intron-exon 2 and intron product), although in these conditions exon 1, the other intermediate RNA species, was underrepresented (data not shown). These data indicate that EDTA causes conformational changes in spliceosomal complexes, resulting in some dissociation of the two intermediate RNA species but making certain PRP8 epitopes more available to the antibodies. EDTA has been reported to cause changes in the conformation, composition, or both of spliceosomal complexes formed in mammalian cell extracts *in vitro* (15). Under all conditions tested with PRP8 antibodies, the spliced exon product was precipitated with at least 10 times lower efficiency than the intron product RNA, suggesting that the spliced exon RNA dissociates away from a post-splicing complex containing PRP8 protein and the excised intron. This indicates that PRP8 is associated with spliceosomes throughout the splicing reaction and interacts with a post-splicing complex containing the excised intron product (5, 16).

To confirm the presence of PRP8 in spliceosomal complexes, we developed a method for the affinity purification of spliceosomes (Fig. 2*A*). An RNA substrate with a poly(A) tail was used to exploit the fact that PAB (17), endogenous to yeast splicing extracts, will associate with the poly(A) tail during the splicing reaction. Spliceosomes formed on the poly(A)-tailed substrate were immunoprecipitated using antibodies raised and affinity-purified against yeast PAB. Fig. 2*B* shows that antibodies to PAB precipitated precursor RNA, lariat intron-exon 2, and the spliced exons with ap-

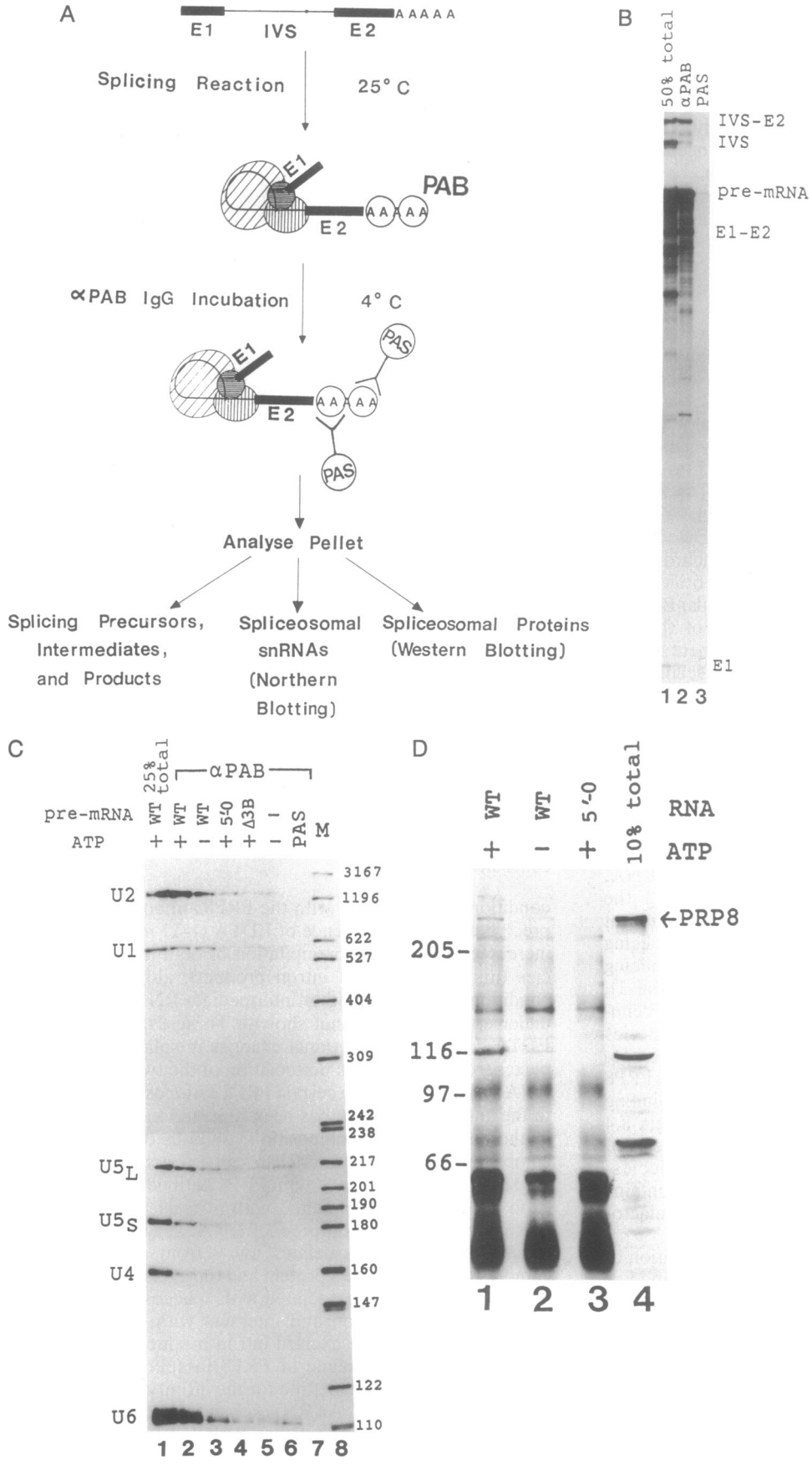

FIG. 2. (*A*) Strategy for the affinity purification of spliceosomes by immunoprecipitation with antibodies to PAB. PAB affinity-selected spliceosomes were analyzed for the presence of precursor RNA, splicing intermediates, and products, snRNAs, and PRP8, as shown in *B*, *C*, and *D*. (*B*) Splicing reaction mixtures containing ^{32}P-labeled rp51A precursor RNA (lane 1) were subjected to immunoprecipitation with PAS-bound antibodies to PAB (lane 2) or with PAS only (lane 3). (*C*) Anti-PAB precipitates were analyzed by Northern blot to visualize the snRNAs associated with affinity-purified spliceosomes from a standard splicing reaction mixture (lane 2) and from mock splicing reaction mixtures (lanes 3–6), in either the absence of ATP and with (lane 3) or without (lane 6) the addition of wild-type rp51A precursor RNA or the presence of ATP and with polyadenylylated mutant precursor RNA with the 5′ splice site deleted [5′-0] (lane 4) or the "TAC-TAAC" box deleted [Δ3b] (lane 5). Lane 1 shows 25% of the snRNAs present in an unfractionated splicing reaction mixture (this includes snRNAs from snRNPs not present in spliceosomes), lane 7 is a control with no antibodies bound to the PAS, and lane 8 contains end-labeled *Msp* I and *Ava* I–*Nhe* I digests of pBR322 DNA. (*D*) Western blot analysis was used to detect PRP8 in an unfractionated whole cell splicing extract (lane 4), in the protein fraction precipitated with PAB antibodies from a standard splicing reaction mixture (lane 1), or from mock splicing reaction mixtures without the addition of ATP (lane 2) or using a mutant substrate RNA with the 5′ splice site deleted [5′-0] (lane 3). Sizes of protein molecular mass standards are given in kDa. The background in the lower portion of the blot is due to antibodies present in the gel.

proximately 50% efficiency and exon 1 with 22% efficiency from an *in vitro* splicing reaction mixture. As with PRP8 antibodies, the presence of EDTA during immunoprecipitation resulted in optimal recovery of poly(A)-tailed RNAs but may have partially destabilized spliceosomes, giving reduced yields of exon 1. The excised intron was not precipitated due to the lack of a binding site for PAB and, presumably, because the spliced exon product [which has a poly(A) tail]

had dissociated from the post-splicing complex (5, 16).

Northern blots containing snRNAs associated with PAB affinity-selected spliceosomes were analyzed (Fig. 2*C*). The five snRNAs implicated in splicing (U1, U2, U4, both species of U5, and U6; ref. 6) were all enriched in the purified spliceosome fraction (lane 2) by 2- to 4-fold compared to a control reaction mixture to which no ATP was added (lane 3) and by 5- to 10-fold compared to reaction mixtures to which mutant RNAs (lanes 4 and 5) or no substrate RNAs (lane 6) were added. The levels of snRNAs affinity-selected on the wild-type poly(A)-tailed pre-mRNA without added ATP (e.g., lane 3) were reproducibly higher than the levels selected with mutant RNAs or with no pre-mRNA added (e.g., lanes 4–6). This is explained by low levels of endogenous ATP in splicing extracts, sufficient to permit some spliceosome assembly (assayed by nondenaturing gel electrophoresis; data not shown) but with no splicing activity. Low levels of all the spliceosomal snRNAs were always selected by PAB antibodies from extracts to which mutant RNAs or no pre-mRNA had been added. This presumably represents the affinity selection of spliceosomes formed on a low level of unspliced RNA endogenous to the yeast splicing extract. In addition to Northern blot analysis, 3′ end-labeling of the PAB-selected RNAs with [^{32}P]pCp and T4 RNA ligase confirmed that only the spliceosome-associated snRNAs copurified with PAB-affinity-selected spliceosomes (data not presented).

The presence of both splicing intermediates and all five snRNAs in PAB-selected complexes is evidence for the purification of intact spliceosomes. The presence of PRP8 in affinity-selected spliceosomes was investigated by immunoblotting, using antibodies to PRP8 fusion protein 8.2 (Fig. 2*D*). Lane 4 shows PRP8 present in unfractionated yeast splicing extract, which includes PRP8 in uncomplexed U5 snRNPs. The species at approximately 116 kDa may correspond to a cross-reacting protein or to a specific breakdown product of PRP8 that was sometimes observed (1). PRP8 was enriched in the spliceosomes purified from a standard splicing reaction mixture (lane 1) as compared to proteins immunoprecipitated from mock splicing reaction mixtures incubated in the absence of ATP or with a mutant substrate RNA (lanes 2 and 3), with the levels of enrichment in each case reflecting those of the spliceosomal snRNAs under similar conditions.

DISCUSSION

We have developed an approach for the affinity purification of spliceosomal complexes that conclusively demonstrates that the yeast U5 snRNP protein PRP8 is a component of spliceosomes. The PAB affinity-purification procedure may be of general use for the analysis of the snRNA and protein components of spliceosomes or for the isolation and characterization of other protein–RNA complexes by incorporation of a poly(A) sequence into an RNA component. In the experiment presented in Fig. 2*D*, although the PAB antibodies were covalently linked to the PAS beads, the subsequent processing of the samples for gel electrophoresis released some antibodies, resulting in a high background in the lower part of the blot. Elution of spliceosomes from the antibody-bound PAB with excess oligo(A) or with Cibacron blue 3gA (Sigma), both of which compete with poly(A)$^+$ RNA for binding to PAB could reduce this background, permitting the use of this procedure for the analysis of low molecular mass proteins or of native spliceosomes. In comparison to other methods that have been described for the isolation of yeast spliceosomes (9, 18), the PAB procedure is simple yet efficient. It selects approximately 50% of the spliceosomes from a splicing reaction, and it avoids the use of immobilized pre-mRNA that can decrease the efficiency of the splicing reaction (18).

Immunoprecipitations from *in vitro* splicing reactions using anti-PRP8 antibodies indicate that PRP8 is present in spliceosomal complexes during both steps of the RNA splicing reaction and in a post-splicing complex containing the excised intron. Although all four antisera that have been raised against different regions of PRP8 detect the protein efficiently by immunoblotting, only anti-8.2 and anti-8.4 antibodies immunoprecipitate U5 snRNPs (4) and only anti-8.4 antibodies precipitate spliceosomal complexes. This suggests that a large part of the protein is concealed in the U5 snRNP and that epitopes recognized by anti-8.2 antibodies, although exposed in the free snRNP, may be involved in interactions with other factors within the spliceosome.

A putative homologue of PRP8 has been discovered in HeLa U5 snRNPs (19), in HeLa cell U4/U5/U6 snRNP complexes, and in affinity-purified HeLa spliceosomes (20). There is also evidence that both PRP8 and the HeLa protein can be UV-crosslinked to the pre-mRNA in spliceosomes (E.W., unpublished results; M. Garcia-Blanco, G. J. Anderson, J.D.B., and P. A. Sharp, unpublished data). This suggests that PRP8 plays a sufficiently important role in splicing for several features to be conserved from yeast to man. The conservation of the exceptionally large size (the HeLa protein is larger than 200 kDa) may be significant in that this protein may act as a scaffold in the spliceosome with multiple domains conferring different functions or interactions with other splicing factors.

We are indebted to Alan Sachs for the generous provision of pure PAB and for advice regarding the PAB selection procedure. We are grateful to Claudio Pikielny for providing the plasmids encoding the polyadenylylated transcripts, to David Brow for the U6-specific oligo, to Debbie Fields for the U2-specific oligo, and to Reinhard Lührmann for the anti-trimethylguanosine antibodies. We thank M. Dalrymple for critical reading of the manuscript. This research was funded by the Cancer Research Campaign. E.W. and M.L. were funded by a British Marshall Scholarship and by a Cancer Research Campaign Studentship, respectively. J.D.B. holds a Royal Society EPA Cephalosporin Fund Senior Research Fellowship.

1. Jackson, S. P., Lossky, M. & Beggs, J. D. (1988) *Mol. Cell. Biol.* **8**, 1067–1075.
2. Hartwell, L. H., McLaughlin, C. S. & Warner, J. R. (1970) *Mol. Gen. Genet.* **109**, 42–56.
3. Lustig, A. J., Lin, R.-J. & Abelson, J. (1986) *Cell* **47**, 953–963.
4. Lossky, M., Anderson, G. J., Jackson, S. P. & Beggs, J. D. (1987) *Cell* **51**, 1019–1026.
5. Konarska, M. M. & Sharp, P. A. (1987) *Cell* **49**, 763–774.
6. Guthrie, C. & Patterson, B. (1988) *Annu. Rev. Genet.* **22**, 387–419.
7. Chang, T.-H., Clark, M. W., Lustig, A. J., Cusick, M. E. & Abelson, J. (1988) *Mol. Cell. Biol.* **8**, 2379–2393.
8. Melton, D. A., Kreig, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. & Green, M. R. (1984) *Nucleic Acids Res.* **12**, 7034–7056.
9. Pikielny, C. W. & Rosbash, M. (1986) *Cell* **45**, 869–877.
10. Lin, R.-J., Newman, A. J., Cheng, S.-C. & Abelson, J. (1985) *J. Biol. Chem.* **260**, 14780–14792.
11. Vijayraghavan, U., Parker, R., Tamm, J., Iimura, Y., Rossi, J., Abelson, J. & Guthrie, C. (1986) *EMBO J.* **5**, 1683–1695.
12. Sachs, A. B., Bond, M. W. & Kornberg, R. D. (1986) *Cell* **45**, 827–835.
13. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
14. England, T. E., Bruce, A. G. & Uhlenbeck, O. C. (1980) *Methods Enzymol.* **65**, 65–74.
15. Zillman, M., Zapp, M. L. & Berget, S. M. (1988) *Mol. Cell. Biol.* **8**, 814–821.
16. Cheng, S.-C. & Abelson, J. (1987) *Genes Dev.* **1**, 1014–1027.
17. Sachs, A. B., Davis, R. W. & Kornberg, R. D. (1987) *Mol. Cell. Biol.* **7**, 3268–3276.
18. Ruby, S. W. & Abelson, J. (1988) *Science* **242**, 1028–1035.
19. Anderson, G. A., Bach, M., Lührmann, R. & Beggs, J. D. (1989) *Nature (London)* **342**, 819–821.
20. Pinto, A. & Steitz, J. A. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 8742–8746.