Interactions of PRP2 protein with pre-mRNA splicing complexes in *Saccharomyces cerevisiae*

David S.King and Jean D.Beggs*
Institute of Cell and Molecular Biology, University of Edinburgh, King's Buildings, Mayfield Road, Edinburgh EH9 3JR, UK

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ABSTRACT

PRP2 protein of *Saccharomyces cerevisiae* is required for the pre-mRNA splicing reaction but not for the early stages of spliceosome assembly. Using anti-PRP2 antibodies we demonstrate that PRP2 protein is associated with spliceosomes prior to, and throughout step 1 of the splicing reaction. Heat-inactivated prp2 protein, by contrast, does not seem to associate with spliceosomes. By elution of electrophoretically distinct spliceosomal complexes from non-denaturing gels we identify the specific complex with which PRP2 initially interacts in the pathway of spliceosome assembly.

INTRODUCTION

RNA splicing is an essential step in the processing of many precursor messenger RNAs to their mature forms (for reviews see 1–3). The development of *in vitro* systems has allowed the elucidation of a two step pathway of RNA splicing. The first step consists of cleavage of the pre-mRNA at the 5′ splice site and the formation of a 2′–5′ phosphodiester bond between the first nucleotide of the intron and an adenosine residue near the 3′ end of the intron (the branchpoint residue), creating a branched, or lariat structure. In the second step of splicing the pre-mRNA is cleaved at the 3′ splice site and the two exons are joined. The intron is released in the lariat form.

Sequence studies of genes, from a variety of different organisms, have revealed consensus sequences at the 5′ and 3′ splice sites (4). In pre-mRNA introns in *Saccharomyces cerevisiae* (henceforth referred to as yeast), there exists a highly conserved sequence, UACUAAC, at the position of branchpoint formation (5) while a less stringently conserved version of this sequence is present in most metazoan introns (6). A large number of studies have shown that changes introduced into the conserved sequences affect RNA splicing (1,2).

Using *in vitro* systems it has been demonstrated for both metazoa (7,8) and yeast (9) that splicing occurs in a large complex with a sedimentation coefficient of approximately 40 to 60S. This complex, termed the spliceosome, contains, in addition to various protein factors, the small nuclear RNAs (snRNAs) U1, U2, U4, U5 and U6 (10–15). Using glycerol gradient fractionation and native gel electrophoresis, it has been possible to distinguish different forms of the yeast spliceosome, and to define a pathway of assembly. Cheng and Abelson (16) distinguished four complexes, which appeared in the order B, A2-1, A1, A2-2. A2-2 contained splicing intermediates and was thus defined as the active spliceosome. Another study, by Pikielny *et al* (17), defined three forms of the spliceosome, denoted I, II, III in order of increasing electrophoretic mobility. These appeared in the kinetic order III, I, II, with II being the active spliceosome.

In addition to the identification of splicing factors by biochemical fractionation, a number of genetic studies have identified other transacting factors in RNA splicing. In yeast the isolation of temperature sensitive pre RNA processing (*prp*) mutants (18,19) has permitted the characterisation of several protein splicing factors (reviewed in 20, 21). Splicing extracts prepared from *prp 2, 3, 4, 5, 7, 8* and *11* strains are temperature sensitive for splicing *in vitro*, indicating that the products of these genes are directly required for the splicing process (22).

Antibodies have been used to identify the *PRP2* gene product as a 100kd protein (23) with nuclear localisation (24). PRP2 protein appears to be distinct from other PRP gene products in that it is not required for the early stages of spliceosome assembly. Lin *et al* (25) showed that the 40S spliceosomes which formed in prp2 temperature sensitive extracts following heat treatment (prp2Δ spliceosomes) could be complemented *in vitro* by adding heat-treated *prp11* temperature sensitive extract plus ATP, indicating that the prp2Δ spliceosomes were precursors of active spliceosomes. Cheng and Abelson (16) showed that the stage at which the prp2Δ spliceosomes were blocked was the transition between complexes A1 and A2-2. It was suggested that PRP2 protein is a factor extrinsic to the spliceosome.

In this study we have used anti-PRP2 antibodies to investigate the function of PRP2 protein and its interaction with spliceosomes. We demonstrate co-precipitation with PRP2 protein of pre-mRNA and splicing intermediates, but not of the reaction products. Splicing complexes do not co-precipitate with heat-inactivated prp2 protein from a heat sensitive *prp2* strain. By elution of complexes from non-denaturing gels we have identified the particular spliceosomal complexes with which PRP2 protein interacts.

* To whom correspondence should be addressed

MATERIALS AND METHODS

Production of antisera and antibodies

The lacZ-PRP2 fusion plasmid pFP2.5 contains a 1.4kb internal fragment (from *Hind*III to *Taq*I) of the *PRP2* gene fused to the 3 end of *lacZ* in pUR289 (26). Fusion protein (FP2.5) expressed in BMH71-18 cells (27) was partially purified as previously described (28). Rabbits were injected at monthly intervals with 50−100μg of the partially purified fusion protein, and serum was collected 12−14 days after each injection. Although PRP2 protein was the major species precipitated by unfractionated antiserum raised against fusion protein FP2.5, one or more cross-reacting species were sometimes weakly precipitated. Antibodies used in this work were affinity purified to optimise PRP2 specificity.

Antibodies against the trimethylguanosine snRNA cap structure were a generous gift from R. Lührmann (Philipps-Universitat Marburg).

Affinity purification of antibodies

PRP2-specific antibodies were immunoaffinity purified from sera by a method based on that of Robinson *et al* (29) using FP2.6, a fusion containing the whole of PRP2 protein fused to *E.coli* trpE protein. The plasmid encoding FP2.6, consists of a 3kb *Bam*HI fragment encoding the whole of PRP2 (see pBM-PRP2 below) in the *Bam*HI site of pATH1 (30). Fusion protein expression was induced (31) and cells were resuspended in SDS sample buffer—(62.5 mM Tris-HC1 pH6.8, 2% SDS, 100 mM DTT, 20% glycerol, 0.01% bromophenol blue) and boiled for 5 minutes. The lysates were fractionated on preparative 8.5% acrylamide, SDS gels and electroblotted to a nitro-cellulose membrane (Schleicher and Schuell) in 20 mM Tris pH8.3, 150 mM glycine. The proteins were visualised by staining with Ponceau-S (Sigma) and the region of the membrane containing FP2.6 was excised. The membrane strips were incubated with 5% (w/v) Bovine serum albumin (BSA; Sigma) in TBS (50 mM Tris-HC1 pH8.0, 150 mM NaCl) for one hour at 37°C and then in a 1:300 dilution of antiserum in TBS plus 1% (w/v) BSA for two hours at room temperature, or overnight at 4°C, and washed three times in TBS. Antibodies were eluted with 1.5 ml of 0.1 M glycine, pH2.2, 50% (v/v) glycerol for 4 minutes and neutralised with 75 μl 1M Tris base. Purified antibodies were stored at −70°C.

Synthesis of RNA substrates and *in vitro* splicing

The RP51A (32) and actin (33) plasmid DNAs used as templates were linearised by digestion with *Dde*I and *Bam*HI, respectively. Transcription reactions (10 μl) contained 10 units of SP6 (for RP51A) or T7 RNA polymerase (for actin) in 40 mM Tris-HCl pH7.5, (plus 10 mM NaCl, for T7 transcription), 6 mM $MgCl_2$, 10 mM DTT, 500 μM ATP, CTP and GTP, 25 μM UTP, 40 μCi [α-^{32}P]UTP, 1U RNasin (Promega) plus 200 ng linearised template DNA and were incubated for 15 minutes at 37°C. For oligonucleotide-directed RNase H cleavage the precursor RNA was incubated with 0.2 nmol. of oligo A or oligo C at 65°C for 30 minutes in 10 μl digestion buffer (Bethesda Research Labs.). *E. coli* RNase H (1U; Bethesda Research Labs.) was added and the reaction was incubated at 37°C for 30 to 60 minutes.

Splicing extracts were prepared (34) from strain BJ2412 (*a/α*, gal2, leu2, pep4-3, prb1-1122, prc1-407, trp1, ura3-52; E. Jones, Carnegie Mellon) or DJY85 (*a/α* ura3/ura3 ade1/ADE1 ade2/ade2 HIS3/his3 tyr1/TYR1 LYS2/lys2 TRP1/trp1 can1/CAN1 prp2-1/prp2-1; D. Jamieson, Edinburgh). *In vitro* splicing reactions (34) contained one part whole cell extract to one part 35% saturated ammonium sulphate precipitate (35P1) of whole cell extract (35). A 10 μl reaction contained 5 μl splicing extract, 1 μl H_2O, 1 μl precursor RNA in 2 mM ATP, and 3 μl reaction buffer containing 10% (w/v) polyethylene glycol, 200 mM potassium phosphate, pH 7.5, 8.3 mM $MgCl_2$, 25.6% (v/v) IgG buffer (250 mM potassium phosphate, 67 mM sodium citrate, 17% (v/v) glycerol, pH 7.0). To heat-inactivate prp2 extracts the mixture was incubated for one hour at 32°C prior to the addition of 1 μl of H_2O (or PRP2 protein preparation to be assayed for PRP2 activity), pre-mRNA and ATP (2 mM final conc.). All splicing reactions were incubated at 25°C.

Extracts from cells overproducing PRP2 protein

Yeast cell extracts containing high levels of PRP2 protein were produced from KY117 cells (a, *his3-Δ200, lys2-801, ade2-101, trp1-Δ1, ura3-52*; ref. 36) containing plasmid pBM-PRP2 which carries the PRP2 gene under the control of the yeast GAL1 promoter (see below). To construct pBM-PRP2, pY2016 (37) was digested with *Afl*III (a gift from N Brown), single stranded ends were filled in with Klenow enzyme and *Bam*HI linkers added. The resulting 3kb *Bam*HI fragment was purified and ligated to pBM125 (38) digested with *Bam*HI.

Cultures (10ml) of KY117 cells carrying pBM125 or pBM-PRP2 were grown to stationary phase on yeast minimal medium supplemented with histidine, lysine, adenine, tryptophan (40 μg/ml each) and 2% (w/v) glucose. Cells were washed with yeast minimal medium containing 2% raffinose plus 2% galactose instead of glucose, and resuspended in 10 ml of this medium. The cell suspension was diluted 1:100 in the same medium, containing 0.5 mg/ml casamino acids (Difco), plus 40 μg/ml adenine and tryptophan and grown overnight at 30°C, until the OD_{600nm} was 0.4. The extract was prepared as for splicing extract, except that following digestion of cell walls, the cells were incubated in the above growth medium, containing 1.2 M sorbitol.

Immunoprecipitations

Antibodies were bound to protein A-Sepharose (PAS;Sigma) and washed three times with NTN buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.1% (v/v) NP-40). Splicing reactions (10 μl) were stopped by placing on ice and adding an equal volume of Q buffer (400 mM KCl, 2 mM MgOAc, 20 mM EDTA, 64 mM Tris-HCl pH7.5) plus 6 μg yeast RNA. The reactions were incubated with the PAS-bound antibodies (PAS-IgG) for one hour at 4°C and then washed three times with NTN buffer. The immunoprecipitates were collected and treated with proteinase K followed by extraction with phenol and chloroform. The RNA was recovered by precipitation with ethanol and analysed by electrophoresis through 6% polyacrylamide−8 M urea gels, followed by autoradiography.

Non-denaturing gels and electroelution

For non-denaturing gel electrophoresis (modified from ref.17) samples were quenched with Q buffer and yeast total RNA (6μg) plus 0.2 sample volume of loading buffer (2.5×TBE, 50% (v/v) glycerol, 0.5% (w/v) each xylene cyanol and bromophenol blue) were added. The 1.5 mm thick vertical gels contained 0.5% (w/v) agarose, 3% (w/v) polyacrylamide (60:1 acrylamide:bis acrylamide), TBE (89 mM Tris, 89 mM boric acid pH 8.3, 2 mM EDTA). The gels were de-gassed and polymerized by

addition of ammonium persulphate to 0.05% (w/v) and 3-dimethylamino-propionitril (Fluka Chemie A.G.) to 0.4% (v/v) final concentrations. Electrophoresis was in TBE at 120V for eight hours at 4°C with recirculation of buffer. Following electrophoresis and autoradiography, bands were excised and placed in dialysis tubing, with 0.5 ml TBE. The complexes were electroeluted in TBE at 150V for two hours at 4°C.

Oligodeoxynucleotides

Oligodeoxynucleotides A and C (39) were synthesised by the OSWEL DNA Service, Edinburgh.

RESULTS

Immunoprecipitation of spliceosomes with antibodies to PRP2 protein

To determine whether PRP2 protein associates with spliceosomes during splicing reactions *in vitro*, immunoprecipitations were performed using affinity purified PRP2- specific antibodies. Lanes 1 to 3 of Figure 1A show that, as expected, splicing intermediates and products were formed only when ATP was present and when the RP51A pre-mRNA contained a functional UACUAAC sequence. When PRP2 protein was immunoprecipitated from these reactions with anti-PRP2 antibodies, co-precipitation of significant levels of ^{32}P-labelled RNA species occurred only from a standard splicing reaction (lane 7) and not when the pre-mRNA lacked a branchpoint sequence (lane 9), or when ATP was omitted from the reaction (lane 8). This co-precipitation did not occur when no antibodies (lane 5), or when antibodies from preimmune serum (lane 6) were bound to the protein A-Sepharose (PAS). Anti-PRP2 antibodies co-precipitated the precursor and both intermediate RNA species,but did not precipitate the spliced mRNA or the excised intron. Anti-trimethylguanosine (anti-m_3G) antibodies, in contrast, precipitated precursor, intermediates and the lariat intron product of the reaction (lane 4). The co-precipitation of pre-mRNA with PRP2 protein under splicing conditions, and the co-precipitation of intermediates, suggest that the anti-PRP2 antibodies immunoprecipitated spliceosomes. It appears that PRP2 protein associated with spliceosomes prior to step 1 of the splicing reaction and was present throughout step 1. Although no co-precipitation of either of the reaction products was detected, it is not clear whether PRP2 protein dissociated from the spliceosomes before step 2, or immediately after completion of this step.

An interesting feature of the pattern of RNA precipitation was the presence of the species X, at levels significantly above those expected from its proportion in the splicing reaction. The RNA X is a prematurely terminated transcript produced during SP6-transcription of the substrate RNA; its 3′ end lies between the UACUAAC sequence and the 3′ splice site. (In these experiments, the SP6-transcribed RNA was not fractionated, in order to include this species in splicing reactions). When X was gel-purified and used as the sole substrate for a splicing reaction, it underwent step 1 of splicing, but not step 2 (data not presented) as would be expected for a transcript terminating in this region (39).

Figure 1B shows a similar experiment, but using a yeast actin transcript as the substrate RNA. Anti-PRP2 antibodies co-precipitated precursor and intermediates from this reaction (lane 3; exon 1 was visible after a longer exposure). As with the RP51A transcript, a species X′ terminating between the UACUAAC sequence and the 3′ splice site was co-precipitated more efficiently than the full length precursor RNA suggesting that the selective immunoprecipitation of such truncated transcripts is a general phenomenon, and not specific to RP51A RNA.

To investigate this phenomenon in more detail, two oligodeoxynucleotides (A and C, corresponding exactly to oligonucleotides A and C used by Rymond *et al* (39) that

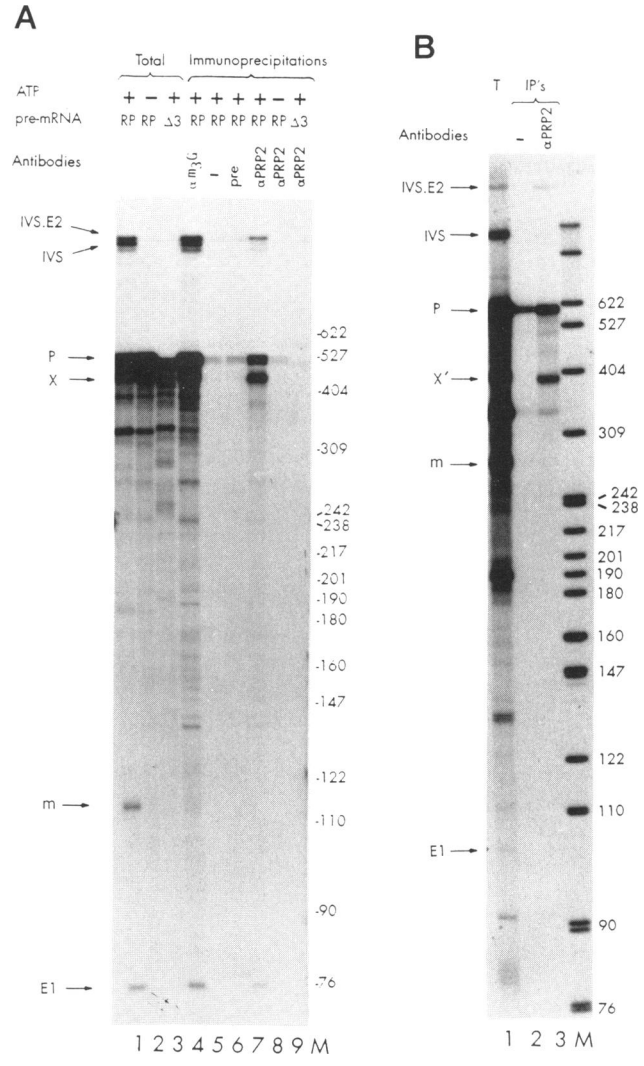

Figure 1. Co-precipitation of RNA with PRP2-specific antibodies. A. Immunoprecipitation of RP51A RNA. RP51A (RP) and RP51A TACTAAC deletion mutant (Δ3) RNAs were incubated in splicing reactions for 20 minutes prior to immunoprecipitation. Lanes 1 to 3: RNA extracted from 1 μl of unfractionated reactions using RP51A pre-mRNA with (lane1) or without (lane 2) 2 mM ATP. Lane 3: Δ3 pre-mRNA with 2 mM ATP. Lanes 4 to 9: RNA from immunoprecipitates from 9 μl splicing reactions; lanes 4 to 7, from reaction 1; lane 8, from reaction 2; lane 9, from reaction 3. Immunoprecipitations were with anti-trimethylguanosine (anti-m_3G) antibodies (lane 4), protein A-Sepharose control (without IgG; lane 5), preimmune IgG (lane 6) or anti-PRP2 antibodies (lanes 7 to 9). M: markers with sizes indicated in nucleotides. IVS-E2−lariat intermediate, IVS−lariat intron product, P−pre-mRNA, X-truncated RNA (see Results), m−mRNA, E1−exon 1. The identities of the intermediates and products of the reactions were based on size, ATP dependence and kinetics of appearance. The upper, stronger pair of bands in the lariat region of the gel are the full size IVS-E2 and IVS species: the lower, fainter band is a degradation product of the IVS. B. Co-precipitation of actin RNA. Actin pre-mRNA was incubated in a 20 μl splicing reaction for 20 minutes prior to immunoprecipitation. Lane 1: RNA from 1 μl of the reaction. Lanes 2 and 3: RNA extracted from immunoprecipitates from 9 μl of the splicing reaction. Immunoprecipitations were with protein A-Sepharose alone (lane 2), or protein A-Sepharose-bound PRP2 antibodies (lane 3). X′ — truncated pre-mRNA (see Results).

hybridize to the region of the RP51A transcript between the UACUAAC sequence and the 3′ splice site (Figure 2A), were incubated with the RP51A transcript, followed by digestion with *E.coli* RNase H. The cleaved transcripts were used as substrates for splicing *in vitro*. In agreement with the results of Rymond *et al* (39), oligo C-cleaved substrates efficiently underwent step 1 of the reaction, while oligo A-cleaved substrates did not (Figure 2B, lanes 2 and 3). However, oligo A-cleaved transcripts were preferentially co-precipitated by anti-PRP2 antibodies as was species X (the 3′ terminus of species X maps between the cleavage sites of oligonucleotides A and C), whereas oligo C-cleaved transcripts are co-precipitated with the same efficiency as full length transcripts (Figure 2B, lanes 4 to 9). Thus, preferential co-precipitation of precursors only occurs when there are less than approximately 27–33 nucleotides downstream from the UACUAAC sequence.

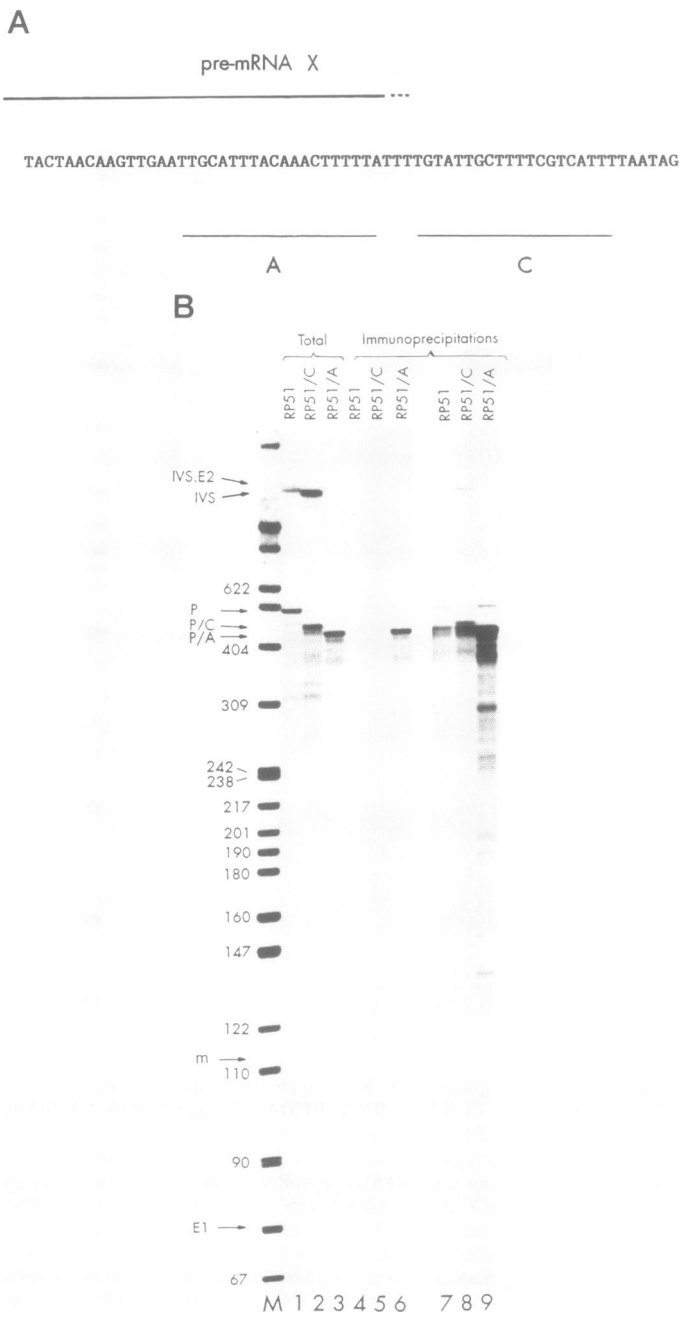

Figure 2. Preferential co-precipitation of transcripts terminating between the UACUAAC sequence and the 3 splice site of the RP51A transcript. A. Sequence from TACTAAC to 3 splice site of the RP51A intron. Regions of hybridization of oligodeoxynucleotides A and C and approximate position of the 3 ends of species X are indicated. B. Immunoprecipitation of full length and RNase H-cleaved RP51A transcripts. Lanes 1 to 3: RNA from 1 μl of unfractionated reactions using full length pre-mRNA (lane 1), oligo C-cleaved pre-mRNA (P/C, lane 2), or oligo A-cleaved pre-mRNA (P/A, lane 3). Lanes 4 to 6: RNA from anti-PRP2 immunoprecipitations from 9 μl of reactions in lanes 1 to 3, respectively. Lanes 7 to 9: long exposure of lanes 4 to 6. Species X migrates at a position between the oligo A- and oligo C-cleaved RNAs, and can be seen most clearly in lanes 7 and 8.

Heat-inactivated prp2 protein is not associated with spliceosomes

The increased sensitivity of detection of co-precipitation available with oligo A-cleaved transcripts, was utilized to determine whether heat-inactivated prp2 protein interacts with spliceosomal complexes. Rymond *et al* (39) demonstrated that spliceosomal complexes I and III (17) form on oligo A-cleaved RP51A transcripts.

Splicing extract from a temperature sensitive *prp2-1* strain was

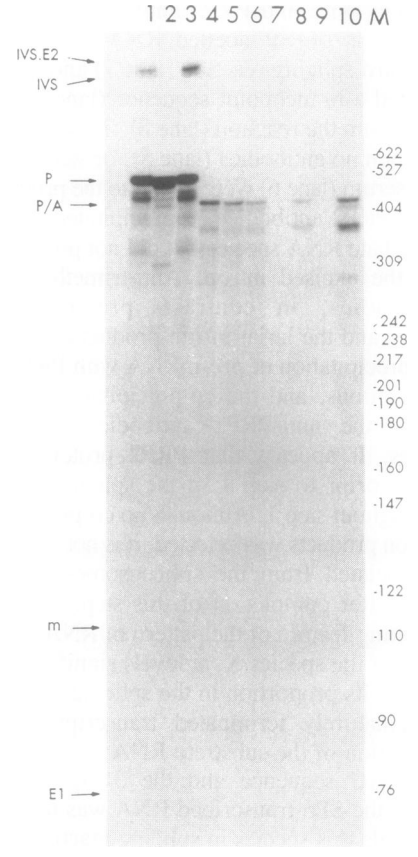

Figure 3. Immunoprecipitations from heat-inactivated prp2-1 splicing extracts. Lanes 1 to 6: RNA extracted from 1 μl of unfractionated reactions. Lanes 1 to 3: reactions with full length RP51A substrate, performed with untreated prp2-1 extract (lane 1), heat-inactivated prp2-1 extract (lane 2), or heat-inactivated prp2-1 extract complemented with wild type PRP2 protein (lane 3). Lanes 4 to 6 are as lanes 1 to 3, but using oligo A-truncated RP51A pre-mRNA. Lane 7: protein A-Sepharose control immunoprecipitation from 9 μl of reaction in lane 4. Lanes 8–10: anti-PRP2 immunoprecipitations from 9 μl of reactions in lanes 4–6 respectively. M: markers with sizes indicated in nucleotides. P/A indicates oligo A-cleaved pre-mRNA. The origin of the band at approximately 370 nucleotides in lanes 8 and 10 is uncertain; while generally present in such experiments, its intensity is variable.

inactivated by heating, and used for reactions with full length RP51A substrate (Figure 3 lanes 1 to 3) and oligo A-cleaved substrate (Figure 3, lanes 4 to 6). Splicing activity of the heat-inactivated prp2-1 splicing extract was restored (Figure 3, lane 3) by addition of a protein extract derived from a yeast strain which overproduces PRP2 protein. The heat-inactivation of the splicing activity was due to specific heat-inactivation of the prp2-1 protein, since the amount of complementing extract used in this experiment was such that control (non-overproducing) extract did not restore activity (not shown), and this amount of complementing extract had no splicing activity in the absence of prp2-1 extract (not shown).

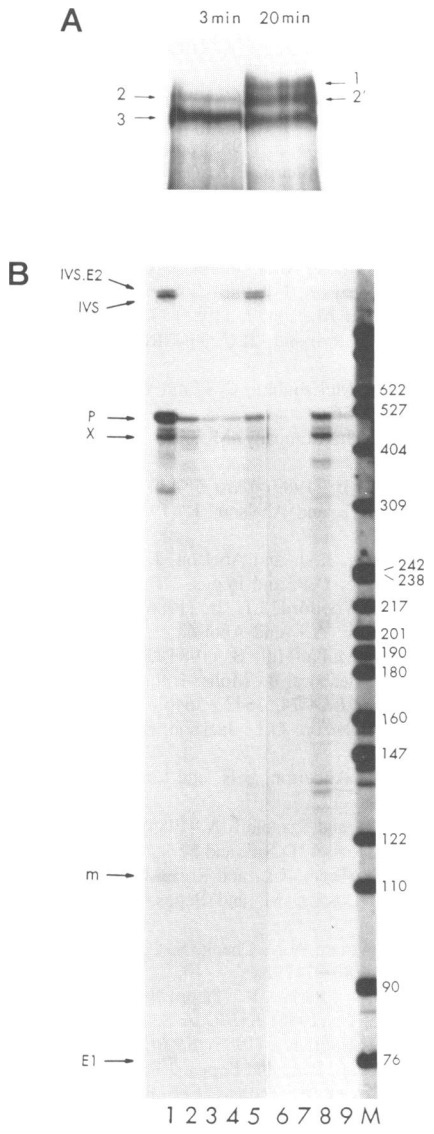

Figure 4. Kinetics of interaction of PRP2 protein with spliceosomes. A. Non-denaturing gel electrophoresis of spliceosomal complexes. Splicing reactions (20 μl) were incubated for the times indicated prior to non-denaturing gel electrophoresis. B. Immunoprecipitation of eluted spliceosomal complexes. Bands 3 and 2 from the 3 minute sample, and 1 and 2′ from the 20 minute sample were eluted from the gel shown in Figure 4A and 10% of the eluted material was extracted with phenol-chloroform (lanes 2 to 5 respectively). The remaining 90% was immunoprecipitated with anti-PRP2 antibodies (lanes 6 to 9 respectively). Lane 1: RNA extracted from 1 μl of the unfractionated 20 minute splicing reaction. Lanes 1 to 5 are a 24 hour exposure; lanes 6 to 10 are a 1 week exposure. M: markers, with sizes indicated in nucleotides.

Immunoprecipitations from the reactions using the oligo A-cleaved substrate RNA were carried out with the results shown in Figure 3, lanes 7 to 10. When the prp2-1 protein was heat-inactivated (lanes 5 and 9), no co-precipitation of substrate RNA was observed, although heat-inactivated PRP2 protein was efficiently precipitated by anti-PRP2 antibodies (not shown). When the prp2-1 deficiency was complemented with wild type PRP2 protein (lanes 6 and 10), however, co-precipitation of substrate RNA was restored. These results demonstrate that heat-inactivation of prp2-1 protein prevents its interaction with spliceosomal complexes. Similar results have been obtained with extracts made from a *prp2-4* yeast strain, which contains an independently isolated *prp2* allele (data not shown). These observations also provide further evidence that the observed co-precipitation of substrate RNA is dependent upon PRP2 protein.

The kinetics of interaction of PRP2 protein with spliceosomes

To investigate with which specific complexes PRP2 protein interacts, different spliceosome complexes, formed on RP51A RNA, were fractionated by non-denaturing gel electrophoresis. A typical time course of appearance of the different complexes is shown in Figure 4A. After three minutes of incubation, two complexes, labelled 2 and 3 were visible, while after twenty minutes a further complex, 1 was visible. These complexes were electroeluted and the ^{32}P-labelled RNA was analysed by denaturing gel electrophoresis. After 3 minutes, complexes 3 and 2 contained only pre-mRNA (Figure 4B, lanes 2, 3). After 20 minutes, complex 1 contained mainly pre-mRNA plus a small amount of intermediates and lariat product (lane 4), while complex '2' contained the majority of intermediates plus some pre-mRNA and is termed complex 2′ as it clearly differs from the complex 2 present after only 3 minutes (compare lanes 3 and 5). It is possible that, in addition to active spliceosomes, this band contained some residual complex 2.

Anti-PRP2 immunoprecipitations were performed on the material eluted from the non-denaturing gels (Figure 4B, lanes 6−9). While there was no detectable co-precipitation of RNA from complexes 2 and 3, strong co-precipitation of pre-mRNA was observed from complex 1 and weaker co-precipitation from complex 2′. It is therefore likely that the majority of pre-mRNA and species X co-precipitated from whole splicing reactions came from complex 1, while the majority of intermediates co-precipitated came from complex 2′. The reason for the relatively inefficient co- precipitation of the reaction intermediates in elution experiments is not known. This experiment defines the point of initial interaction of PRP2 protein with spliceosomal complexes as the transition from complex 2 to complex 1. Based on (a) their relative electrophoretic mobilities, (b) the kinetics of their appearance and (c) their RNA composition, complexes 2 and 1 are probably equivalent to complexes A2-1 and A1 respectively of Cheng & Abelson (16).

DISCUSSION

This paper describes experiments in which antibodies were used to investigate the interaction of the yeast PRP2 protein with spliceosomes. Functional PRP2 protein associates with complexes containing precursor RNA and the reaction intermediates, whereas heat-inactivated prp2 protein does not. These observations and direct co-precipitation of gel purified spliceosomes indicate that PRP2 protein interacts directly with spliceosomes, rather than playing a more indirect role by, for

example, modifying other splicing factors outwith the spliceosome. It was important to establish this since, unlike most of the factors which have been implicated in RNA splicing in yeast, PRP2 protein is not required for the assembly of early forms of the spliceosome (16, 25).

Despite numerous attempts, using the same immunoprecipitation conditions which detect spliceosome association, it has not been possible to co-precipitate any snRNAs with PRP2 protein except in the form of spliceosomes (data not shown). This indicates that PRP2 protein is not stably associated with free snRNPs, and is compatible with the fact that the five snRNAs known to be required for splicing are assembled into spliceosomes prior to the association of PRP2 protein with splicing complexes (16).

Preferential co-precipitation of transcripts (X and X) which have their 3 end between the UACUAAC sequence and the 3 spice site was observed. In both the actin and RP51A genes there is a very T rich sequence (13/19 nucleotides in actin, 12/15 nucleotides in RP51A) in this region. It seems that during transcription *in vitro* the bacteriophage polymerases pause or terminate at this sequence, due to the limiting amounts of UTP in the transcription reaction. Oligodeoxynucleotide-directed RNase H cleavage of this region in the RP51A pre-mRNA revealed that transcripts with less than approximately 27–33 nucleotides 3 to the UACUAAC sequence were preferentially co-precipitated. This figure is in agreement with the minimum of 29 nucleotides which was previously reported to be required for step 1 of the splicing reaction to occur (39). Although they observed a slightly different pattern of complex formation, Rymond *et al* (39) found that oligo A-cleaved pre-mRNA was trapped in the most slowly migrating complex. Thus, the relatively inefficient co-precipitation of full length transcripts with PRP2 may be explained by the fact that pre-mRNA is present in this complex for a very short time, the complex being rapidly converted to the active spliceosome. By contrast, RNAs which have approximately 30 nucleotides or less 3 to the UACUAAC sequence are trapped in this complex and therefore are efficiently co-precipitated by anti-PRP2 antibodies.

Our results indicate that PRP2 protein associates transiently with spliceosomal complexes prior to the initiation of the RNA splicing reaction and that it is present throughout step 1 of the reaction. It is difficult to determine at what point it dissociates from the spliceosome; the lack of co-precipitation of splicing products may indicate that it leaves before step 2 or immediately upon completion of step 2. Post-splicing complexes have been detected, that contain lariat intron product complexed with snRNPs (40, 41), whereas the spliced mRNA is released as a ribonucleoprotein complex which apparently does not contain snRNPs (42). Although PRP2 protein does not appear to associate with such post-splicing complexes, a possibility which cannot be excluded is that PRP2 protein epitopes are not available for antibody binding in post-splicing complexes or that a much weaker association between PRP2 and such complexes could be disrupted under these immunoprecipitation conditions.
Although similar qualifications apply to the conclusion that PRP2 protein does not associate with complexes 3 and 2 (Figure 4), this is compatible with the observation (16) that such early complexes can form in the absence of active PRP2 protein.

Future experiments will be directed towards determining the specific function of PRP2 protein and identifying the other splicing factors with which PRP2 protein interacts.

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