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Roles of PRP8 protein in the assembly of splicing complexes

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Three different approaches have been used to investigate the roles of the yeast U5 snRNP protein PRP8 in spliceosome assembly: genetic depletion of PRP8 protein *in vivo*, heat inactivation of temperature-sensitive prp8 protein in protoplasts and inhibition of PRP8 function with antibodies *in vitro*. In each case, U5 and U4/U6 snRNPs failed to assemble into the forming spliceosomes. In addition, extract prepared from PRP8-depleted cells and extract containing inactivated PRP8 protein had substantially reduced amounts of U4/U6.U5 triple snRNP complexes. Thus, functional PRP8 protein is required for the stable formation of U4/U6.U5 complexes without which spliceosomes fail to form. As spliceosome formation was also blocked by anti-PRP8 antibodies that apparently do not disrupt triple snRNPs, PRP8 protein may play a separate role in the assembly of triple snRNPs into spliceosomes. As a consequence of PRP8 depletion the levels of the U4, U5 and U6 snRNAs declined dramatically. We discuss this in the context of the known genetic interactions between *PRP8* and putative RNA helicase (DEAD box protein) genes and propose that PRP8 protein plays a role in regulating dynamic RNA–RNA interactions in spliceosome assembly, possibly ensuring the correct directionality of these events.

Key words: pre-mRNA splicing/protein/snRNPs/spliceosome/yeast

Introduction

The process of nuclear pre-mRNA splicing involves many *trans*-acting factors that assemble in an ATP-requiring fashion to form a large complex, the spliceosome, in which the two transesterification reactions take place (reviewed in Green, 1991; Ruby and Abelson, 1991). *In vitro*, the assembly of the spliceosome is highly organized and a number of distinct spliceosome assembly complexes can be resolved by techniques such as native gel electrophoresis and gradient sedimentation (e.g. Pikielny *et al.*, 1986; Bindereif and Green, 1987; Cheng and Abelson, 1987; Konarska and Sharp, 1987; Seraphin and Rosbash, 1989; Michaud and Reed, 1991).

The most studied of the *trans*-acting factors that make up the spliceosome are the four small nuclear ribonucleoprotein particles (snRNPs) containing the U1, U2, U4+U6 and U5 RNAs (for reviews see Guthrie and Patterson, 1988; Lührmann *et al.*, 1990). U1 snRNP is first to interact with the substrate, followed by U2, to form a pre-spliceosome complex with which the U4/U6 and U5 snRNPs then associate. The U5 and U4/U6 snRNPs appear to associate simultaneously with the pre-spliceosome (Pikielny *et al.*, 1986; Bindereif and Green, 1987; Cheng and Abelson, 1987; Konarska and Sharp, 1987) and in both yeast and mammals are found in a single U4/U6.U5 triple snRNP particle (Cheng and Abelson, 1987; Lossky *et al.*, 1987; Konarska and Sharp, 1987; Black and Pinto, 1989) that is thought to be a functional intermediate in spliceosome assembly. U4/U6 and U5 snRNPs do not assemble independently into spliceosomes when one or the other has been specifically depleted (Barabino *et al.*, 1990; Lamm *et al.*, 1991; Seraphin *et al.*, 1991).

A U4/U6.U5 triple snRNP complex has recently been purified from HeLa nuclear extracts and shown to contain several triple snRNP specific polypeptides (Behrens and Lührmann, 1991). Proteins from the triple snRNPs were found to promote the association *in vitro* of purified U5 and U4/U6 snRNPs (Behrens and Lührmann, 1991) indicating a role for at least one of these proteins in triple snRNP assembly.

Splicing is inhibited in HeLa cells following heat shock and this correlates with a deficiency in the U4/U6.U5 triple snRNP (Bond, 1988; Shukla *et al.*, 1990). Recently, Utans *et al.* (1992) showed that the U4/U6.U5-specific proteins identified by Behrens and Lührmann (1991) promote the recovery of triple snRNP formation, spliceosome assembly and splicing activity in extracts from heat shocked cells. This, in combination with results obtained in snRNP depletion studies, constitutes good evidence for the U4/U6.U5 triple snRNP being a functional intermediate in mammalian spliceosome assembly.

Both snRNP and non-snRNP proteins are extremely important both in spliceosome assembly and during the splicing reaction (see Green, 1991; Ruby and Abelson, 1991). Among the non-snRNP factors identified in yeast are several proteins (PRP2, PRP5, PRP16, PRP22 and PRP28) with amino acid sequence motifs similar to those characteristic of ATP-dependent RNA helicases (DEAD box proteins; reviewed in Koonin, 1991; Wassarmann and Steitz, 1991; Schmid and Linder, 1992). Of these, PRP2 (Kim *et al.*, 1992) and PRP16 (Schwer and Guthrie, 1991) are known to have RNA-stimulated ATPase activities. A number of base-pairing interactions occur and are disrupted in the course of splicing (e.g. unfolding the intron, U1:5′ splice site, U2:branch point, U4:U6 and U5:substrate RNA; Newman and Norman, 1992) and the action of RNA helicases could account, at least in part, for the ATP requirement of the splicing reaction. In particular, a conformational change occurs in the spliceosome that destabilizes the U4:U6 base-pairing interaction (Pikielny *et al.*, 1986; Cheng and Abelson, 1987; Lamond *et al.*, 1988). This destabilization of U4 from U6 has been proposed to result in the exposure of residues in U6 RNA that are important in the splicing reaction (Brow and Guthrie, 1989;

Guthrie, 1989) and is presumed to be an ATP-dependent unwinding process (Brow and Guthrie, 1988), possibly involving PRP28 (Strauss and Guthrie, 1991).

PRP8 is a 280 kDa protein essential for splicing in yeast and is specifically associated with the U5 snRNP and with U4/U6.U5 triple snRNPs (Lossky *et al.*, 1987). Several observations indicate that PRP8 is a pivotal component of the spliceosome. First, PRP8 is highly conserved over a diverse range of eukaryotic organisms both immunologically and in its very large size as well as in its association with U5 snRNPs (Anderson *et al.*, 1989; Pinto and Steitz, 1989; Paterson *et al.*, 1991). Secondly, PRP8 is present in yeast spliceosomes throughout the splicing reaction and in a post-splicing complex containing excised intron (Whittaker *et al.*, 1990). Thirdly, PRP8 and its human homologue can be cross-linked to pre-mRNA in a splicing specific fashion, indicating a close contact with the substrate RNA (Garcia-Blanco *et al.*, 1990; Whittaker and Beggs, 1991). Fourthly, genetic interactions have been found between PRP8 and putative RNA helicases (Jamieson *et al.*, 1991; Strauss and Guthrie, 1991), including PRP28.

Despite these observations, the steps at which PRP8 function is required have not been defined, nor have the consequences of lack of PRP8 function been assessed. Using *in vivo* depletion, heat inactivation and antibody inhibition to eliminate PRP8 function, we demonstrate here that PRP8 is required for the stable formation of U4/U6.U5 triple snRNPs and for the assembly of triple snRNPs into spliceosomes *in vitro*. In addition, the absence of PRP8 protein *in vivo* causes the levels of the U4, U5 and U6 snRNAs to decline dramatically. We discuss this in the context of the known genetic interactions between *PRP8* and putative RNA helicase genes.

Results

Experimental systems

To study the first PRP8-dependent step in splicing we assessed the phenotype of PRP8 depletion. As PRP8 is an essential splicing factor, we constructed a yeast strain, JDY8.05, which contains a disruption at the chromosomal *PRP8* locus, *prp8::LEU2* and plasmid pJDY13 from which *PRP8* expression is regulated by the *GAL1* promoter and control sequences: inducible by galactose, repressible by glucose (see Materials and methods). This strain grew with a doubling time of ~2.5 h in galactose medium. Following transfer to glucose medium the growth rate declined after 4–5 h and eventually stopped (Figure 1A). Strain JDY8.06, a derivative of JDY8.05, in which pJDY13 is replaced by pY8500 (Jackson *et al.*, 1988) which carries the *PRP8* gene with its own promoter, grew equally as well as JDY8.05 in galactose medium, but also grew in glucose medium as expected (data not shown).

Figure 1B is a Northern blot showing the effect on the splicing of actin and *RP28* transcripts of repressing *PRP8* transcription (lanes 3–5) or of incubating the temperature-sensitive strain SPJ8.31 (*prp8-1*) at the restrictive temperature (lanes 1 and 2). In each case spliced mRNA became depleted and the ratio of unspliced to spliced RNA increased. The levels of mRNA of non-spliced transcripts did not become depleted in strain JDY8.05 grown on glucose (e.g. *URA3* in Figure 6A). Compared with SPJ8.31 grown at 36°C, the defect was not as severe in strain JDY8.05 on glucose, particularly at early time points, presumably because

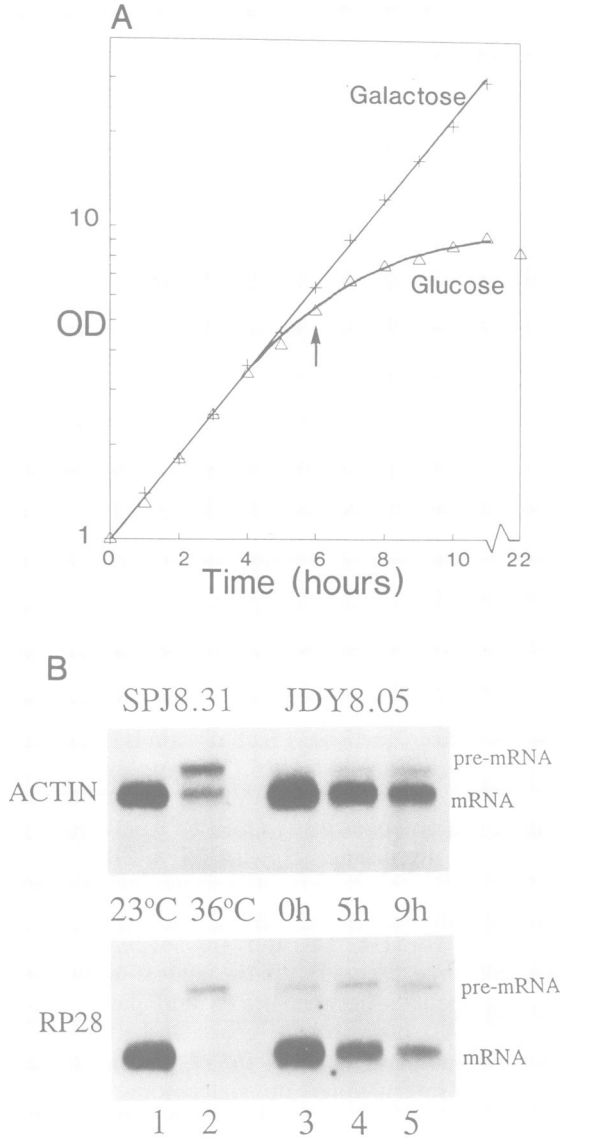

Fig. 1. Repression of *PRP8* transcription results in a rapid decline in cell growth and accumulation of unspliced pre-mRNA. **(A)** Growth curve. A mid-log culture of strain JDY8.05 grown in YEP-GR [2%(w/v) each of galactose and raffinose] was spun down (time 0), and half the cells (ΔPRP8; △) were shifted into YEPD while the other half (+PRP8; +) were returned to YEP-GR medium. The cultures were diluted to maintain conditions suitable for logarithmic growth (monitored as relative OD_{600nm}). The arrow indicates the time at which splicing extracts were prepared. **(B)** Northern Blot. Total RNA was extracted from strain SPJ8.31 (*prp8-1*) grown at the permissive temperature (23°C; lane 1) or after growth at 36°C for 4 h (lane 2) and from strain JDY8.05 grown in YEP-GR (lane 3) or after growth in YEPD for 5 or 9 h (lanes 4 and 5). RNA (25 μg) was denatured and electrophoresed through 1.5% agarose, blotted to Hybond-N and hybridized sequentially to ^{32}P-labelled DNA fragments encoding actin and rp28. The positions of unspliced precursor RNA (pre-mRNA) and spliced messenger RNA (mRNA) are marked.

depletion of PRP8 was gradual whereas the temperature-sensitivity of SPJ8.31 was more rapidly manifested (growth almost stopped after 2 h; data not shown).

Splicing extract made from strain JDY8.05 after 6 h growth in glucose (ΔPRP8) had hardly any detectable splicing activity, i.e. lack of intermediates (IVS-E2, E1) and products (E1-E2, IVS) (Figure 2A, lane 4), whereas extract from cells grown in galactose (+PRP8; lane 3) spliced normally. Thus, the *in vivo* splicing defect was reproducible *in vitro*.

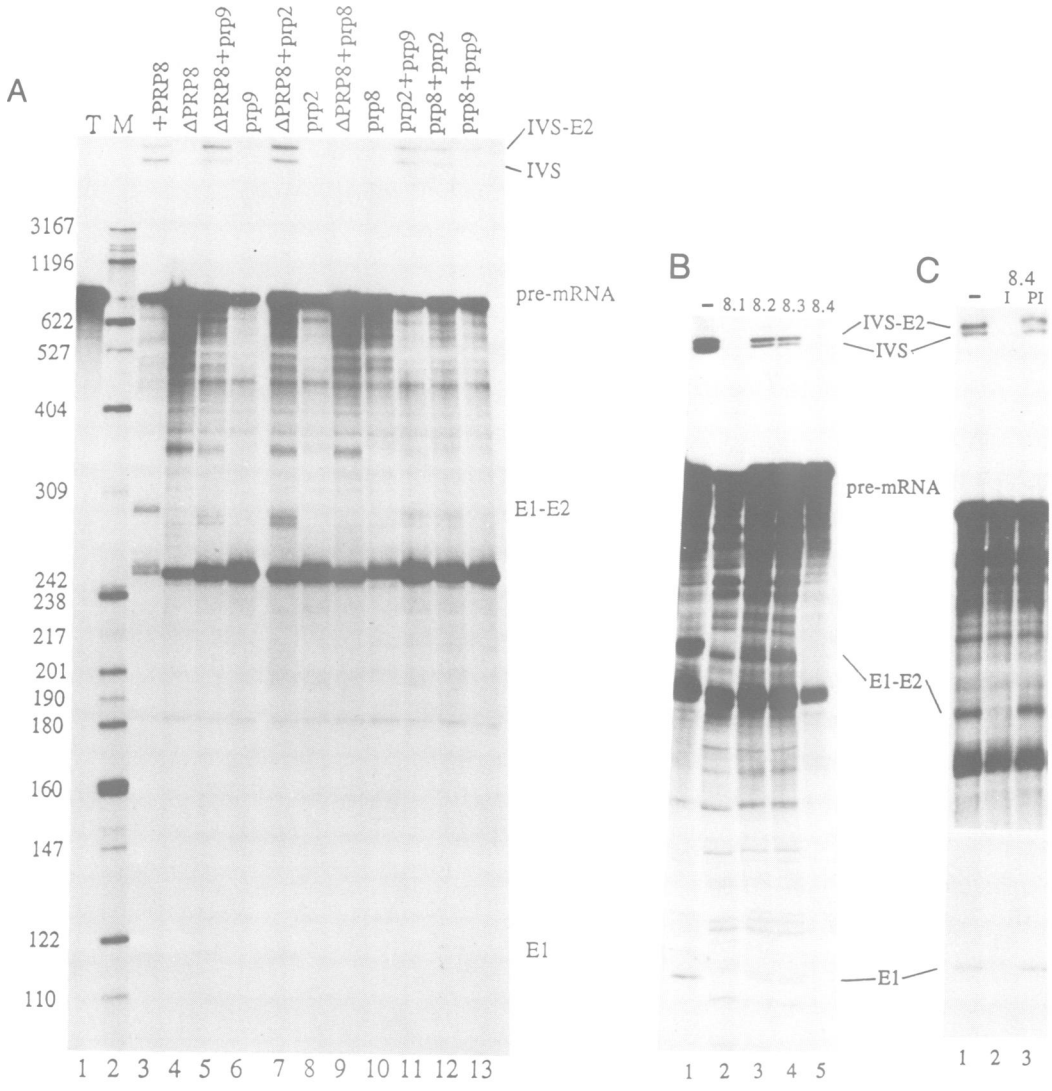

Fig. 2. *In vitro* splicing assays. **(A)** Splicing reactions (10 μl) were carried out with rp28 RNA and either 5 μl of a single extract or 2.5 μl each of two extracts, to test complementation. Lane T, rp28 transcript; lane M, end-labelled fragments of pBR322 DNA digested with *Msp*I and *Ava*I + *Nhe*1; +PRP8, extract from JDY8.05 grown in YEP-GR; ΔPRP8, extract from JDY8.05 grown for 6 h in YEPD; prp2, prp9 and prp8 are inactive extracts from heat treated strains temperature-sensitive for that prp protein. In this experiment the complementation between prp8 and prp9 extracts was poor. IVS-E2; lariat intron−exon2 intermediate species. IVS; lariat excised intron. pre-mRNA; rp28 substrate RNA. E1−E2; spliced mRNA product. E1; exon1 intermediate species. **(B)** Effect of anti-PRP8 antibodies on splicing. Splicing reactions (10 μl) containing +PRP8 extract, which had been treated (see Materials and methods) with anti-PRP8 IgG from the serum indicated (lanes 2−5) or with phosphate buffered saline (−; lane 1), were incubated for 15 min and half of each was analysed as in (A), while the remainder was fractionated by native gel electrophoresis (see Figure 3C). **(C)** Reactions with extract treated with phosphate buffered saline (lane 1), IgG from serum 8.4 (lane 2) or from the corresponding pre-immune serum (lane 3). The centre of panel C was trimmed to fit the other panels and still show exon 1.

A second method of examining PRP8 function utilized inactive extracts prepared from yeast strains carrying temperature-sensitive *prp* alleles. For this we developed a protoplast heat inactivation protocol (see Materials and methods). In our hands this procedure was more reproducible for heat inactivation of *prp8* strains than a previously published method (Lustig *et al.*, 1986) and gave extracts in which events late in spliceosome formation could be studied, unlike a published *in vivo* heat inactivation protocol (Abovitch *et al.*, 1990). Figure 2A shows splicing assays of extracts from *prp2-1*, *prp9-1* and *prp8-1* strains treated in this way (lanes 6, 8 and 10), and the ability of each inactive extract to complement the defect in the others (lanes 11, 12 and 13), which indicates the specificity of the inactivation process for the mutant gene product. Complementation of the splicing defect of the ΔPRP8 extract by prp2 or prp9 extract (lanes 5 and 7) but not by prp8 extract (lane 9) provides evidence that PRP8 activity is limiting in both the heat inactivated prp8 extract and in the ΔPRP8 extract.

As a third method of investigating PRP8 function, we used antibodies against different non-overlapping regions of PRP8 protein (Lossky *et al.*, 1987; Jackson *et al.*, 1988) to inhibit *in vitro* splicing reactions. Figure 2B shows complete inhibition of splicing by 8.1 and 8.4 antibodies (lanes 2 and 5), whereas 8.2 and 8.3 antibodies had only a modest effect (lanes 3 and 4). Immunoglobulins (IgG) prepared from pre-immune serum had no effect on splicing at a concentration equivalent to that of anti-PRP8 IgG which inhibited splicing completely (Figure 2C, lanes 2 and 3). These inhibition results are consistent with the observation that 8.1 and 8.4 antibodies interact with native PRP8 protein associated with U5 snRNPs (Lossky *et al.*, 1987) whereas 8.2 and 8.3

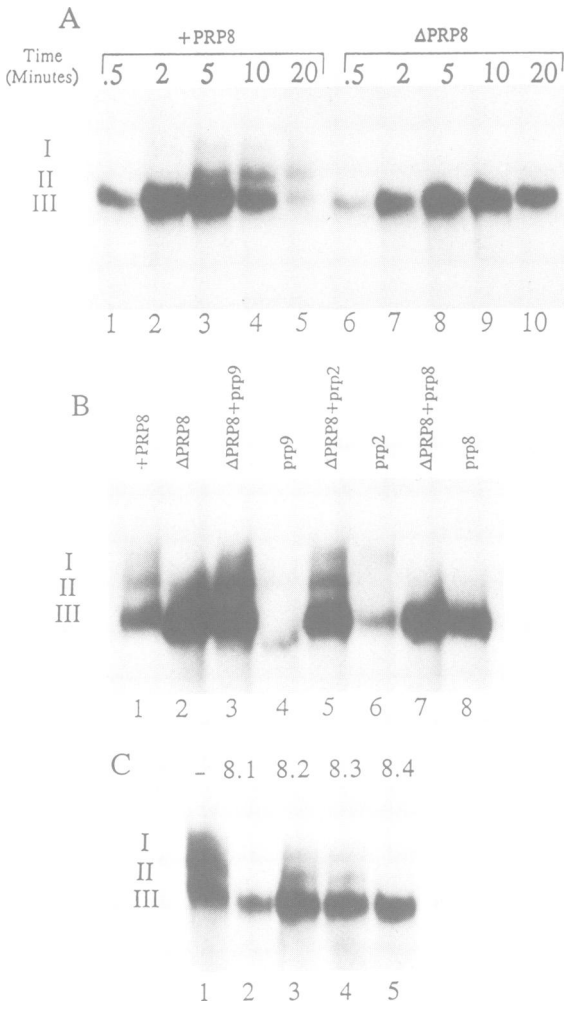

Fig. 3. Analysis of splicing complexes by native gel electrophoresis. **(A)** Time course. Splicing reactions were carried out with rp28 RNA and extract from JDY8.05 (+PRP8; lanes 1–5, ΔPRP8; lanes 6–10) were incubated for the times indicated and the splicing complexes were analysed by non-denaturing gel electrophoresis. Complexes were designated I, II and III, according to Pikielny *et al.* (1986), II being the active spliceosome, as confirmed by RNA analysis. **(B)** Complementation assays. Complexes were analysed from 8 min, 10 μl reactions set up as in Figure 2A. Lanes as marked. **(C)** Effect of antibodies. Aliquots (5 μl) of the splicing reactions used for the RNA analysis in Figure 2B were fractionated by non-denaturing gel electrophoresis.

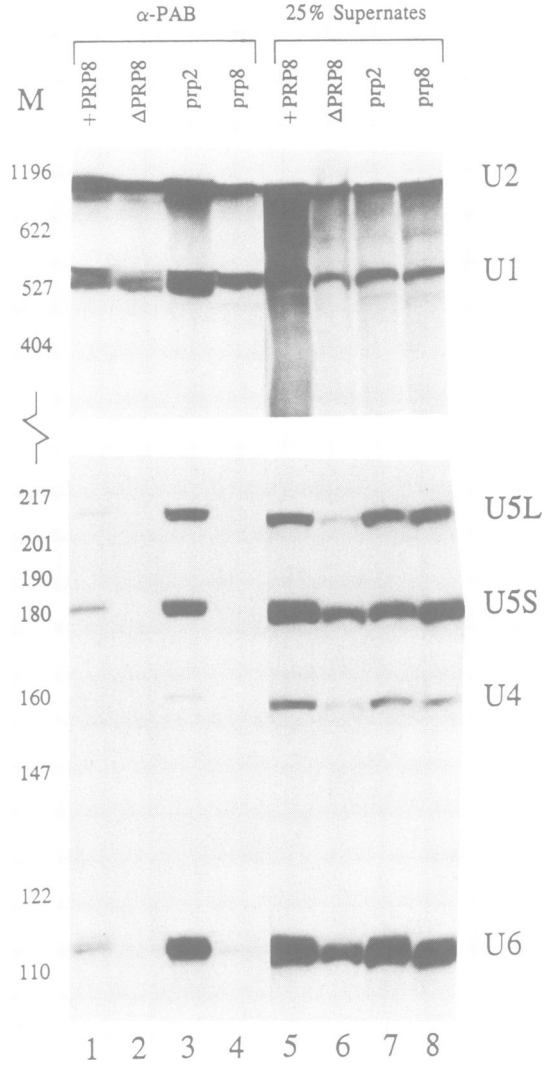

Fig. 4. Analysis of snRNA content of splicing complexes. Splicing reactions (50 μl) containing non-radioactive rp51A transcript were incubated for 8 min and the complexes formed on the RNA were affinity-selected with antibodies against poly(A)-binding protein. RNA was extracted from the immunoprecipitates (lanes 1–4; ΔPAB) and supernates (lanes 5–8) and the snRNA content analysed by Northern blotting. U5L and U5S are two forms of the U5 snRNA that differ by only 35 nucleotides at the 3′ end. Probes were uniformly labelled gel-purified fragments of *snR* genes. Extracts used were as marked above the lanes, M; markers as in Figure 2A.

antibodies recognize only denatured forms of PRP8 protein (Lossky *et al.*, 1987; S.Teigelkamp and J.D.Beggs, unpublished results), presumably because the epitopes are not available in the native protein.

Thus these three independent ways of impairing PRP8 function caused inhibition of splicing prior to the first cleavage–ligation reaction.

Spliceosome assembly is inhibited by loss of PRP8 function

The effects of impairing PRP8 function on spliceosome assembly were investigated using native gel electrophoresis, essentially as described by Pikielny *et al.* (1986). In this system, three complexes designated I, II and III were detected, with complex II, the last to form, being the active spliceosome. Figure 3A shows a time course of complex formation in +PRP8 and ΔPRP8 extracts. While +PRP8 extract produced the normal pattern and kinetics of complex formation (lanes 1–5), ΔPRP8 extract (lanes 6–10) produced only complex III.

Complex formation with inactive extracts from temperature-sensitive mutants and complementation between them and the ΔPRP8 extract is shown in Figure 3B. Inactive prp2 extract (Figure 2B, lane 6) formed complexes III and I consistent with the known prp2 defect, which is after formation of the complete spliceosome (Cheng and Abelson, 1987; Lin *et al.*, 1987), while inactive prp9 extract (lane 4) formed few if any complexes, consistent with it being required for early steps in spliceosome assembly (Abovitch *et al.*, 1990; the small amount of complex III reflects incomplete inactivation of prp9 protein in this extract). Whereas inactive prp2 and prp9 extracts complemented

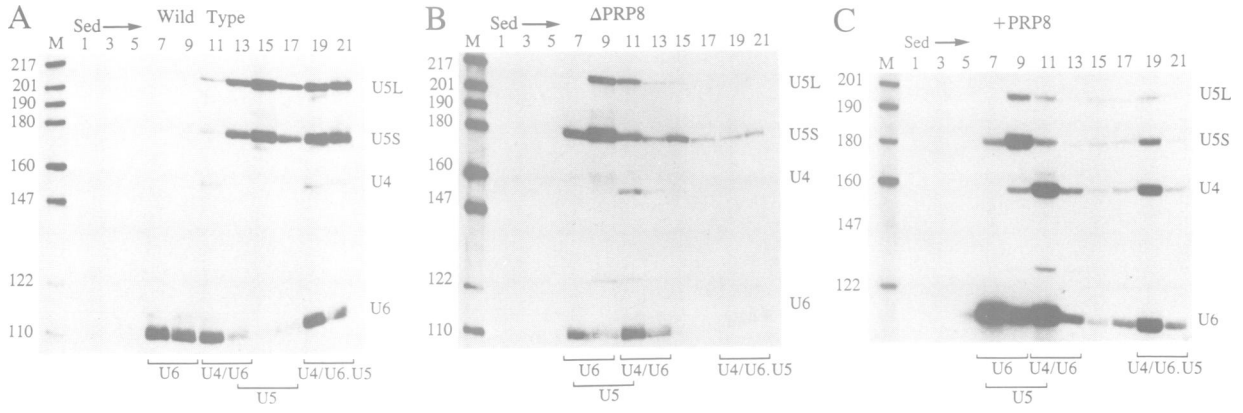

Fig. 5. Effect of PRP8 depletion on the sedimentation properties of snRNPs. Samples were prepared and glycerol gradients run as described (see Materials and methods). The snRNA content of alternate fractions was analysed by Northern blotting. **(A)** Wild type, BJ2412 extract; **(B)** ΔPRP8, PRP8 depleted extract; **(C)** +PRP8, extract from strain JDY8.05 grown in YEP-GR. Positions of free U6, U4/U6, free U5 snRNPs and U4/U6.U5 triple snRNPs are indicated. M, markers as in Figure 2A). Note that the U4 probe used in (C) was at a higher specific activity than that used in (A) and (B).

ΔPRP8 extract (lanes 3 and 5), heat inactivated prp8 extract, which itself produced only complex III (lane 8), did not (lane 7). Thus *in vivo* depletion and heat inactivation of PRP8 protein blocked spliceosome assembly at the same point.

Figure 3C shows complexes formed in reactions pre-incubated with 8.1, 8.2, 8.3 or 8.4 antibodies. Consistent with their effects on splicing reactions, 8.1 and 8.4 antibodies prevented spliceosome formation and resulted in accumulation of complex III, whereas 8.2 and 8.3 antibodies allowed formation of a reduced amount of complexes II and I. Thus, all three methods of eliminating PRP8 activity resulted in spliceosome assembly being blocked at the same stage. Complex III is the first splicing-specific complex seen in this gel system, which is consistent with spliceosome assembly being blocked at a point after the stable binding of U2 snRNPs, but before the addition of U4/U6 and U5 snRNPs.

Fractionation of splicing complexes by native gel electrophoresis provides an informative profile of the intermediates of the spliceosome assembly pathway. However, the stringent conditions employed tend to result in loss of the U1 snRNP and of the destabilized U4 snRNP (Konarska and Sharp, 1986, 1987; Pikielny *et al.*, 1986; Cheng and Abelson, 1987; Lamond *et al.*, 1988). In order to verify the snRNA content of the complex that accumulates in the absence of functional PRP8 protein, we affinity-selected splicing complexes under conditions that permit the isolation of intact spliceosomes, i.e. containing all the relevant snRNAs. Extracts were incubated with a polyadenylated RP51A transcript and complexes were immunoaffinity-selected with antibodies against poly(A)-binding protein (Whittaker *et al.*, 1990). Complexes that formed with +PRP8 extract contained all the snRNAs required for splicing (Figure 4, lane 1), as did inactivated prp2 extract (lane 3). Complexes immunoprecipitated from ΔPRP8 extract or heat inactivated prp8 extract (lanes 2 and 4) carried only U1 and U2 snRNAs. Examination of supernates from the immunoprecipitations (lanes 5–8) confirmed that all the snRNAs were present. Thus, in the absence of PRP8 protein or in the presence of inactive PRP8 protein (and by inference when PRP8 function is blocked by antibodies) neither U5, nor U4 or U6 snRNAs assembles into splicing complexes.

Loss of PRP8 function leads to loss of U4/U6.U5 triple snRNPs

Since the assembly of U5 and U4/U6 snRNPs into spliceosomes was blocked by loss of PRP8 function, it was important to assess whether there was any effect on the stability of these snRNPs or on the formation or stability of the U4/U6.U5 triple snRNP complex. The various U4, U5 and U6 snRNP complexes present in wild type or PRP8-depleted extracts were fractionated by glycerol gradient sedimentation and their distribution was analysed by Northern blotting of the snRNAs. Figure 5A shows the result from a typical wild type gradient. U4/U6 and U4/U6.U5 particles, and free U5 and U6 snRNPs were resolved as in other similar analyses (Bordonne *et al.*, 1990; Shannon and Guthrie, 1991). Gradients of ΔPRP8 extract (e.g. Figure 5B) showed two marked differences to those of wild type extract. First, free U5 snRNPs had a reduced sedimentation velocity, the majority being in approximately the same position as free U6 particles. This implies a change in composition, in keeping with loss of a U5-associated protein. Secondly, U4/U6.U5 particles were hardly detectable. The depletion of U4/U6.U5 particles is consistent with the failure of incomplete U5 snRNPs to associate stably with U4/U6 snRNPs. +PRP8 extract (Figure 5C) contained a lower level of triple snRNP particles than wild type and most of the free U5 snRNPs sedimented as in the ΔPRP8 extract. This sedimentation pattern, intermediate between those of wild type and ΔPRP8 extract, was consistent with an observed low level of galactose-induced *PRP8* expression (Western blotting showed the level of PRP8 protein in JDY8.05 to be at least 20-fold lower than in wild type cells; data not shown) and also with the limiting amount of intact U5 snRNPs being preferentially assembled into stable U4/U6.U5 particles.

Heat inactivated prp2 extract contained a distribution of snRNPs identical to wild type (data not shown). Thus, despite the heat labile nature of the triple snRNP complex in mammalian cells (Bond, 1988; Shukla *et al.*, 1990), the yeast U4/U6.U5 triple snRNP was not affected by the heat treatment used here. Heat inactivated prp8 extract contained a similar distribution of snRNPs to that seen on PRP8 depletion (data not shown), implying that the heat inactivated protein was not stably associated with the U5 particle. This

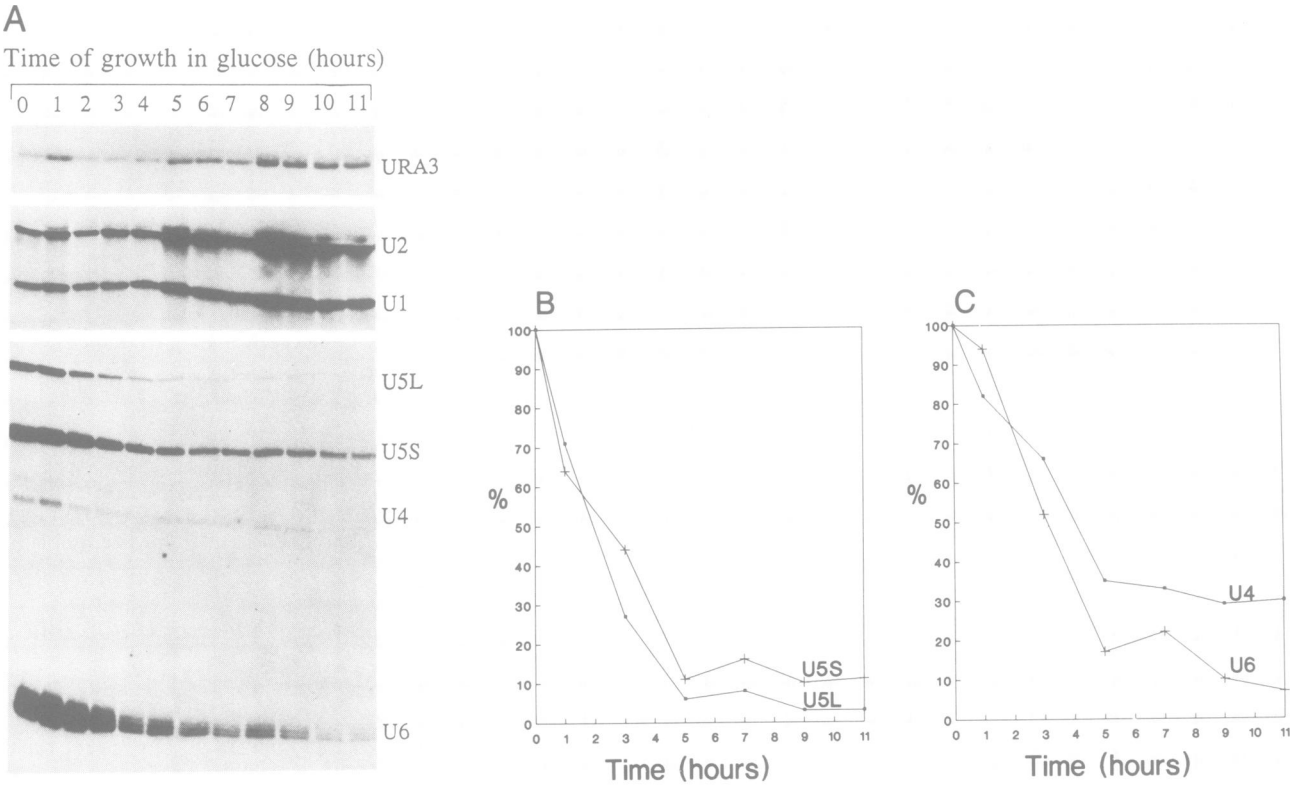

Fig. 6. Effect of PRP8 depletion on the snRNA content of JDY8.05. **(A)** Total RNA was extracted from JDY8.05 at 1 h intervals following the switch from YEP-GR to YEPD as in Figure 1A and analysed for the levels of various transcripts by Northern blotting. The *URA3* transcript is a non-spliced control. **(B)** and **(C)** Levels of snRNAs were estimated by densitometric scanning of autoradiographs of Northern blots of two independent time courses. Values were normalized against U1, averaged between the two experiments and plotted as a percentage of the starting amount as 100%. (B) U5L and U5S; (C) U4 and U6.

was verified by the failure of anti-PRP8 antibodies to immunoprecipitate U5 snRNA from heat treated prp8 extract (data not shown). Thus depletion and inactivation of PRP8 were apparently functionally equivalent in terms of snRNP stability and snRNP–snRNP interactions.

Depletion of PRP8 results in destabilization of a subset of snRNAs

In a number of experiments the amount of certain snRNAs appeared lower in ΔPRP8 extracts than in wild type and other extracts. We therefore analysed the levels of splicing-associated snRNAs in strain JDY8.05 following transfer to glucose medium. Figure 6A shows a Northern blot of a typical experiment. Whereas the levels of U1 and U2 snRNAs remained essentially constant compared to a control transcript (*URA3*), U5L, U5S, U4 and U6 snRNAs dropped following the switch to the repression medium. This decrease occurred in the initial 4–5 h when the level of PRP8 was decreasing due to repression of transcription but during which logarithmic growth was maintained.

To quantify this effect accurately, autoradiographs of blots from several time courses were analysed by densitometry. Levels of U4, U5L, U5S and U6 were normalized against U1 and plotted as a percentage of the initial amount. Figure 6B and C show the averaged results of two independent experiments. U5L decreased by ~20-fold, U5S by ~10-fold, U6 by 7- to 10-fold and U4 by 3-fold. In yeast, as in other organisms, U6 snRNA is in excess over U4 snRNA (Cheng and Abelson, 1987; Siliciano *et al.* 1987; Xu *et al.*, 1990) and therefore the rate of loss of U6 was much faster than that of U4. This non-stoichiometric loss of U4 and U6 is discussed below.

To control for any effect of change in carbon source on the levels of the snRNAs, this experiment was performed with strain JDY8.06; no change was observed in the relative amounts of snRNAs during 9 h following the transfer from galactose to glucose (data not shown).

Discussion

In this work three approaches have been used to study the role of the PRP8 protein in snRNP interactions and in spliceosome assembly; genetic depletion *in vivo*, heat inactivation of temperature-sensitive prp8 protein in protoplasts, and inhibition with PRP8 antibodies *in vitro*. The *in vivo* depletion of an intrinsic snRNP protein has not previously been reported. This approach has allowed a more definitive investigation of the function of PRP8.

Previous analyses of heat inactivated splicing factors did not resolve the complexes that assembled when prp8 was inactivated (Lin *et al.* 1987; Abovitch *et al.*, 1990). The results presented here show that the block in spliceosome assembly caused by lack of PRP8 activity is at a point after the binding of U1 and U2 snRNPs to the pre-mRNA but before the association of the U4/U6.U5 triple snRNP. PRP4 and PRP6 proteins may also be required at this stage of spliceosome assembly. Banroques and Abelson (1989) showed that PRP4 antibodies, which do not prevent triple snRNP assembly, inhibit splicing reactions, causing the accumulation of a complex co-migrating with U1/U2 pre-

spliceosomes and Abovitch *et al.* (1990) demonstrated that heat inactivated prp4 and prp6 extracts fail to add U6 snRNA to pre-spliceosomes.

The U4/U6.U5 triple snRNP has been confirmed as a true intermediate in the assembly of U4/U6 and U5 snRNPs into spliceosomes in HeLa *in vitro* systems (see Introduction) and a study of yeast U5 snRNA depletion (Seraphin *et al.*, 1991) indicated that this was also true for yeast. In this work the failure of U5 and U4/U6 snRNPs to assemble into spliceosomes upon depletion or heat inactivation of PRP8 correlated with loss of U4/U6.U5 triple snRNPs. This demonstrates an important role for PRP8 in the formation of triple snRNPs and provides further evidence for this complex being a functional intermediate in spliceosome assembly in yeast. PRP8 is not the only yeast splicing factor that is required for stable U4/U6.U5 snRNP complexes. A mutant U4 RNA that has a deletion of the 5′stem−loop does not bind the U4/U6 snRNP protein PRP4 and fails to form U4/U6.U5 complexes, suggesting the need for PRP4 and/or this region of the U4 RNA (Bordonne *et al.*, 1990).

Here we show that 8.1 and 8.4 antibodies inhibit spliceosome formation. Previously, 8.4 antibodies were shown to co-precipitate the U4/U6 snRNP with the U5 snRNP, apparently as a triple snRNP, whereas 8.1 antibodies immunoprecipitated only the U5 snRNP and apparently disrupted the U4/U6.U5 association under the same conditions (Lossky *et al.*, 1987). At that time the possibility could not be ruled out that 8.4 antibodies precipitated U4/U6 snRNPs that were associated with PRP8 independently of U5 snRNPs, rather than in a triple snRNP complex. However, depletion of PRP8 did not change the sedimentation of either free U6 or U4/U6 snRNPs, and thus the independent association of PRP8 with either of these particles seems extremely unlikely. Taken together, all these data indicate that the region of PRP8 recognized by 8.1 antibodies is required for the association of U5 snRNPs with U4/U6 snRNPs to form triple snRNP complexes, whereas the region recognized by 8.4 may be important for the incorporation of U4/U6.U5 complexes into forming spliceosomes. However, it cannot be ruled out that the inhibition observed with the antibodies was due to steric effects caused by the antibodies preventing nearby interactions taking place.

Depletion of PRP8 resulted in a substantial reduction in the levels of the U4, U5 and U6 snRNAs while the U1 and U2 snRNAs were unaffected. Similar effects were observed following incubation of the *prp8-1* strain at the restrictive temperature (data not shown). The loss of U5 snRNA could be explained by destabilization of the U5 snRNP in the absence of PRP8, which is one of its component proteins. The loss of U4 and U6 snRNAs was more surprising. That this was due to decreased transcription of U4, U5 and U6 snRNAs on depletion of PRP8 seems unlikely and even if snRNA synthesis ceased immediately upon transferring cells to repressing medium, the levels of the snRNAs should not have declined so rapidly simply due to cell division. The possibility that the U4/U6 snRNP is a transient intermediate in the assembly of the U4/U6.U5 particle and that it is intrinsically unstable *in vivo* also seems unlikely as genetic depletion of U5 snRNA had little effect on the U4 and U6 snRNAs (Seraphin *et al.*, 1991; J.D.Brown and J.D.Beggs, unpublished observations). Our favoured interpretation is that U5 snRNPs lacking PRP8 interact with U4/U6 snRNPs to produce an aberrant, unstable complex, as a consequence of which the RNAs become exposed to nucleases. Several observations, both biochemical and genetic, discussed below, make this model attractive.

In a genetic selection for suppressors of the *prp8-1* temperature-sensitivity, several independent cold-sensitive mutations were isolated in a gene, *DED1*, which encodes a DEAD box protein and hence a putative RNA helicase (Jamieson *et al.*, 1991). As the *prp8-1* defect has the same spectrum of phenotypes at the non-permissive temperature as PRP8 depletion, this implies that the modification in DED1 stabilizes the triple snRNP complex, possibly by reducing an RNA unwinding activity and thus redressing the balance between opposing effects of DED1 and PRP8.

A possible interaction between PRP8 and another putative helicase, PRP28, was suggested by the isolation of a *PRP8* allele that suppresses a mutation in *PRP28* (Strauss and Guthrie, 1991). A genetic interaction has also been found between *PRP28* and *PRP24*, which encodes a U6-associated protein (Shannon and Guthrie, 1991). On the basis of these observations, Strauss and Guthrie (1991) proposed that PRP28 may be responsible for the unwinding of U4 from U6 and that the U4/U6 association is stabilized in the triple snRNP by the action of PRP8 protein.

Thus, there are at least two putative RNA helicases with which PRP8 might interact or whose activity might be counteracted or regulated by PRP8. In the situation where PRP8 is not present the activity of these helicases might be unregulated, leading to disruption of RNA base-pairings usually only unwound in the spliceosome. This would expose the RNAs to the action of nucleases and hence lead to their degradation. The greater loss of U5 and U6 snRNAs over U4 snRNA, especially the non-stoichiometric loss of U4 and U6, may indicate that U5 and U6 become more exposed to the action of nucleases following the inappropriate action of helicases. The yeast U4 snRNA has been shown to be less sensitive than U6 to micrococcal nuclease digestion (Xu *et al.*, 1990).

Whittaker *et al.* (1990) proposed that PRP8 might function as a scaffold in the spliceosome and results presented here and elsewhere (see Introduction) are consistent with this. We propose that PRP8 plays a role in regulating or monitoring dynamic RNA−RNA interactions in spliceosome assembly and in the spliceosome, perhaps ensuring the correct directionality of events.

Splicing is generally believed to involve a cycle of assembly and disassembly events *in vivo* such that spliceosome components dissociate from one another after the reaction is complete and reassemble on a new transcript. As PRP8 is known to be present with excised intron in a post-splicing complex (Lossky *et al.*, 1987), it will be interesting to see whether PRP8 also plays a role in the disassembly process that presumably reverses many of the interactions involved in spliceosome assembly.

Materials and methods

Yeast strains

Saccharomyces cerevisiae strains: BJ2412 (wild type) and SPJ8.31 (*prp8-1*) were described previously (Lossky *et al.*, 1987; Jamieson *et al.*, 1991); DJY85 (MATa/α, *prp2-1*, *ura3*, *ade1*/+, *ade2*, *trp1*/+, *his3*/+, *tyr1*/+, *lys2-801*/+, *can1*/+) was obtained from D.Jamieson, Edinburgh; JDY9.11 (MATa/α, *prp9-1*, *ura*, *ade*, *lys2*/+, *leu2*/+, *trp1*/+, *arg*/+, *tyr1*/+, *gal1*/+) is a diploid formed by mating J17 with J93 (J.Warner, New York).

Yeast growth media and genetic manipulations were as described in Rose *et al.* (1990).

Construction of the GAL–PRP8 strain

To aid construction of the transcriptionally regulated *GAL–PRP8* gene, a unique *Nhe*I site was introduced by site-directed mutagenesis close to the 5′ end of the *PRP8* coding sequence. This changes the fifth amino acid from proline to alanine, but has no apparent effect on PRP8 function. The *GAL1* promoter (copied by PCR from pBM125; Johnson and Davis, 1984) was joined to this modified *PRP8* gene via a linker fragment encoding the first nine base pairs of *PRP8* coding sequence and an *Nhe*I site, and the *GAL–PRP8* fusion was cloned into the polylinker of pRS313 (CEN, *HIS3*; Sikorski and Hieter, 1989) to produce pJDY13. Sequencing of the *GAL1* promoter fragment revealed a single base change presumably caused by PCR error; whether this affected expression of *PRP8* from the promoter was not established. To make a strain conditionally expressing *PRP8*, the diploid strain JDY0.2 (MATa/α, *ura3-52*, *leu2-3,-112*, *ade2*/+, *his3*-Δ1/+, *trp1-289*/+) was transformed with a DNA fragment encoding *LEU2* flanked by sequences from upstream (540 bp) and downstream (~2500 bp) of *PRP8* (including only a few base pairs of *PRP8* coding sequence). A Leu$^+$ transformant carrying the *prp8::LEU2* gene replacement at one *PRP8* locus was identified by Southern blotting and tetrad analysis. Transformation with pY8000 (2 μm, *URA3*, wild type *PRP8*; Jackson *et al.*, 1988) followed by sporulation, selection of a *prp8::LEU2* haploid segregant and plasmid shuffling with pJDY13 generated JDY8.05 (*ura3-52*, *leu2-3,-112*, *ade2*, *his3*-Δ1, *trp1-289*, *prp8::LEU2*, pJDY13). Strain JDY8.06 was obtained by transformation of JDY8.05 with pY8500 (CEN, *URA3*, wild type *PRP8*; Jackson *et al.*, 1988) and selecting clones that had lost pJDY13.

Nucleic acid methods

To produce RNAs as substrates for *in vitro* splicing reactions, pT7rp28 (Whittaker and Beggs, 1991) and pSPrp51A (Pikielny and Rosbash, 1986) DNAs were linearized with *Eco*RI and *Bam*HI respectively and transcribed *in vitro*. Full-length transcripts were purified by electroelution from a polyacrylamide gel using a UEA Unidirectional Electroelutor (IBI).

Yeast total RNA was prepared by the method of Hopper *et al.* (1978). Denaturing agarose gel electrophoresis of RNA was as described in Jackson *et al.* (1988). RNA was transferred to Hybond-N membrane as recommended by the supplier (Amersham).

DNA fragments of cloned genes were radiolabelled by the random priming method (Feinberg and Vogelstein, 1984) to produce probes for experiments shown in Figures 1B and 4. Pre-hybridization and hybridization was at 42°C in 1×P buffer [0.2% (w/v) each of BSA, polyvinyl pyrolidone and Ficoll-400, 50 mM Tris–HCl pH 7.5, 0.1% (w/v) sodium pyrophosphate and 1% (w/v) SDS], 50% (v/v) formamide, 1 M NaCl, 10 μg/ml denatured sonicated salmon sperm DNA (Stratagene), washing was 2×10 min at 42°C in 2×SSC, 2×30 min at 65°C in 2×SSC plus 0.5% SDS, 2×30 min at room temperature in 0.1×SSC. For experiments shown in Figures 5 and 6, probing was with kinased oligonucleotides as described in Lossky *et al.* (1987), using the oligonucleotides described in Whittaker *et al.* (1990) plus the *URA3*-specific oligonucleotide 5′-CATCAAAAGGCCTCTAGGTT-3′ (OSWEL, Edinburgh). RNAs detected by Northern blotting were quantified by densitometric scanning using a Shimadzu Dual-Wavelength Chromato Scanner model CS-930 (Howe, London).

Splicing extract preparation and in vitro splicing reactions

Yeast whole cell extracts were prepared as described by Lin *et al.* (1985). To prepare heat inactivated extracts, sphaeroplasts were incubated at 36°C in osmotically stabilized medium for between 30 min and 2.5 h depending on the strain, chilled on ice and spun down at 4°C. Washing, lysis and subsequent steps were as Lin *et al.* (1985). This treatment had no effect on extracts from wild type cells. *In vitro* splicing reactions were performed as described by Lin *et al.* (1985). For complementation experiments equal amounts of extracts were mixed and pre-incubated at 25°C for ~10 min before assembling the splicing reaction. For inhibition studies 5 μl of splicing extract were incubated with about 10 μg IgG [purified according to the method of Ey *et al.* (1978) as in Harlow and Lane (1988)] at 25°C for 20–30 min prior to addition of other components of the splicing reaction. The anti-PRP8 antibodies have been described (Lossky *et al.*, 1987; Jackson *et al.*, 1988). Reaction products were fractionated on 6% polyacrylamide–8M urea gels and visualized by autoradiography. Native gel electrophoresis was carried out according to Pikielny *et al.* (1986) except the EDTA concentration in the gel and running buffer was 10 mM. Affinity-purification of splicing complexes using antibodies against poly(A)-binding protein was as described by Whittaker *et al.* (1990).

Glycerol gradient sedimentation

Prior to centrifugation, aliquots of splicing extract (1 mg protein) were incubated for 15 min under splicing conditions without pre-mRNA. Samples were then diluted 3-fold in buffer A and layered onto 11 ml 10–30% glycerol gradients as described by Bordonne *et al.* (1990). Centrifugation was for 14 h at 37 000 r.p.m. in a TST41.14 rotor (Sorvall). 0.5 ml fractions were collected and numbered from the tops of the gradients. RNA was extracted and snRNAs were analysed by Northern blotting.

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