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Progression through the spliceosome cycle requires Prp38p function for U4/U6 snRNA dissociation

Jian Xie, Kristopher Beickman, Elizabeth Otte and Brian C.Rymond[1]

T.H.Morgan School of Biological Sciences and the Markey Cancer Center, University of Kentucky, Lexington, KY 40506-0225, USA

[1]Corresponding author
e-mail: rymond@pop.uky.edu

The elaborate and energy-intensive spliceosome assembly pathway belies the seemingly simple chemistry of pre-mRNA splicing. Prp38p was previously identified as a protein required *in vivo* and *in vitro* for the first pre-mRNA cleavage reaction catalyzed by the spliceosome. Here we show that Prp38p is a unique component of the U4/U6.U5 tri-small nuclear ribonucleoprotein (snRNP) particle and is necessary for an essential step late in spliceosome maturation. Without Prp38p activity spliceosomes form, but arrest in a catalytically impaired state. Functional spliceosomes shed U4 snRNA before 5′ splice-site cleavage. In contrast, Prp38p-defective spliceosomes retain U4 snRNA bound to its U6 snRNA base-pairing partner. Prp38p is the first tri-snRNP-specific protein shown to be dispensable for assembly, but required for conformational changes which lead to catalytic activation of the spliceosome.

Keywords: pre-mRNA/snRNP/spliceosome/splicing/yeast

Introduction

The current model of spliceosome assembly was developed principally from the *in vitro* pattern of small nuclear ribonucleoprotein (snRNP) particle association with synthetic splicing substrates (reviewed in Moore *et al.*, 1993; Madhani and Guthrie, 1994; Krämer, 1996). In mammals and yeast, spliceosome assembly progresses by the sequential addition of the U1 snRNP→U2 snRNP→U4/U6.U5 tri-snRNP particles to the pre-mRNA. Before 5′ splice-site cleavage (chemical step I in splicing), the affinities of the U1 and U4 snRNAs for the splicing complex are greatly reduced and, under many (Pikielny *et al.*, 1986; Cheng and Abelson, 1987; Konarska and Sharp, 1987) although not all (Blencowe *et al.*, 1989) isolation conditions, the U4 snRNA is lost from the spliceosome. This model of spliceosome assembly is supported by the abridged spliceosome assembly profiles observed when splicing is inhibited by specific mutations in the pre-mRNA or when one of the many *trans*-acting components of splicing is removed. For example, when the essential co-factor adenosine triphosphate (ATP) is depleted before splicing-substrate addition, U1 snRNP binds the pre-mRNA, but the subsequent ATP-dependent additions of the U2 snRNP and the U4/U6.U5 tri-snRNP particles are blocked (reviewed in Rosbash and Seraphin, 1991). In a comparable fashion, U1 and U2 snRNP addition occurs but U4/U6.U5 tri-snRNP addition is prevented when Prp4p, Prp6p, or Prp8p is inactivated or when the U5 snRNA is metabolically depleted (Banroques and Abelson, 1989; Seraphin *et al.*, 1991; Brown and Beggs, 1992; Galisson and Legrain, 1993). This progression of subunit addition, rearrangement and dissociation defines a process termed the spliceosome cycle.

Fundamental to spliceosome assembly is the ordered formation and resolution of multiple snRNA–pre-mRNA and snRNA–snRNA base pair associations (Moore *et al.*, 1993; Madhani and Guthrie, 1994; Nilsen, 1998). It is generally accepted that the geometric restrictions of nucleic acid association impart considerable specificity to the spliceosome-assembly process. Proteins, on the other hand, are required for snRNP recruitment, for the stabilization and resolution of the spliceosome's RNA interactions and, in the most general sense, for the formation of an enzyme superstructure compatible with catalysis. Yet, comparatively little is known about these protein factors which advance spliceosome assembly and activation.

Two groups of RNA-binding proteins, the serine–arginine enriched phosphoproteins of metazoa (the SR and SR-related proteins) and the DExD/H-box proteins of the spliceosome have received the most attention as effectors of snRNP recruitment and snRNP reorganization, respectively. The SR protein family consists of at least eight related members. Each member is characterized by the presence of one or more RNA recognition motif (RRM) and multiple clustered serine–arginine dipeptides (reviewed in Fu, 1995; Manley and Tacke, 1996). The SR proteins and a structurally distinct group of SR-related proteins function at each snRNP addition step in mammalian spliceosome assembly and participate in both constitutive and regulated pre-mRNA splicing. Protein–protein interactions between the phosphorylated SR motif and other spliceosomal components promote, at least in part, the spliceosome-assembly process. Curiously, the canonical SR proteins, so prevalent in metazoa, are absent in yeast.

The spliceosomal DExD/H-box proteins are a group of at least seven structurally related proteins [Prp2p, Prp5p, Prp16p, Prp22p, Prp28p, Prp43p, Snu246p (also called Brr2p, Rss1p, Slt22p)] which roughly resemble a subtype of RNA helicase (Lauber *et al.*, 1996; Lin and Rossi, 1996; Noble and Guthrie, 1996; Xu *et al.*, 1996; Arenas and Abelson, 1997; see references in Krämer, 1996). These DExD/H-box proteins are implicated in multiple stages of spliceosome assembly, in both chemical steps of splicing, and in spliceosome disassembly. RNA-dependent ATP hydrolysis has been observed for Prp2p, Prp5p, Prp16p and Snu246p and, in each case, this activity is believed to be important for conformation changes within

the spliceosome (Schwer and Guthrie, 1992; Kim and Lin, 1993, 1996; Plumpton *et al.*, 1994; Umen and Guthrie, 1995; O'Day *et al.*, 1996; Xu *et al.*, 1996). To date, however, the yeast DExD/H-box proteins have not shown strict RNA substrate specificity or demonstrated RNA helicase activity *in vitro*. This has led to speculation that like the prototypical DEAD-box helicase, eif4A (Rozen *et al.*, 1990), the spliceosomal DExD/H-box proteins may require auxiliary factors for complete catalytic activity (Strauss and Guthrie, 1994; Kim and Lin, 1996).

The challenge ahead is to integrate the intricate choreography of the spliceosome cycle with the mechanism of substrate recognition and splice-site activation. Toward this end we have investigated the function of the essential splicing factor, Prp38p, in the spliceosome cycle (Blanton *et al.*, 1992). Prp38p is a small (28 kDa) protein with an extremely acidic, serine-rich C-terminus. *In vivo* and *in vitro* studies with the temperature sensitive (ts) *prp38-1* mutant suggested that Prp38p might function within U6-snRNA-bearing complex at a late stage of spliceosome assembly. Here, we establish that Prp38p is an authentic component of the yeast U4/U6.U5 tri-snRNP particle. Unlike other tri-snRNP-specific proteins characterized to date, Prp38p functions as a maturation factor; spliceosomes assemble without Prp38p, but arrest in a pre-catalytic state prior to U4 snRNA release. The data support a model in which Prp38p recruits (or activates) an RNA unwinding activity necessary for U4/U6 snRNA dissociation and subsequent U6 snRNA integration into the active site of the spliceosome.

Results

Spliceosome assembly progresses without Prp38p

In vitro heat inactivation of the ts Prp38-1p protein blocks pre-mRNA splicing without an obvious reduction in the amount of splicing complex formed (Blanton *et al.*, 1992). Either Prp38p is dispensable for much of spliceosome assembly or the Prp38-1p derivative retains partial activity (i.e. assembly competence) even after it has lost the ability to support splicing. To resolve this question, two new yeast strains were prepared. One strain expressed a novel ts allele, *prp38-2*, that exhibits a more thorough block to splicing than observed for *prp38-1*. The Prp38-2 protein contains a cysteine to tyrosine substitution at position 87 (C87Y). This change is distinct from the glycine to aspartic acid substitution at amino acid 66 (G66D) introduced by the *prp38-1* lesion and is consistent with the *N*-ethylmaleimide-sensitivity of the native Prp38p protein (Blanton *et al.*, 1992). The second yeast strain expressed a nutritionally-regulated, hemagglutinin (HA)-tagged allele, *GAL1::PRP38HA*, as the sole source of Prp38p. Transcription of *GAL1::PRP38HA* is induced in galactose-based media and repressed in glucose-based media.

Under permissive conditions, the *prp38-1*, *prp38-2* and *GAL1::PRP38HA* alleles produced functional proteins that supported pre-mRNA splicing *in vivo* (Figure 1). A 2 h shift to the restrictive temperature of 37°C inhibited *RP51A* pre-mRNA splicing in the *prp38-1* culture and blocked splicing in the *prp38-2* culture (Figure 1, lanes 3 and 4, 5 and 6). In both cases, pre-mRNA levels increased and mRNA levels decreased after the temperature shift.

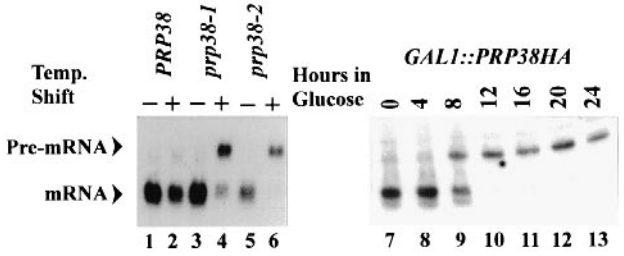

Fig. 1. Cellular pre-mRNA splicing requires functional Prp38p. *In vivo* pre-mRNA splicing was monitored by Northern blot analysis with an *RP51A* intron-plus-exon probe. RNA was extracted from cultures bearing a wild-type *PRP38* allele (lanes 1 and 2), the ts mutant alleles *prp38-1* (lanes 3 and 4), or *prp38-2* (lanes 5 and 6), and from cultures that expressed the nutritionally regulated *GAL1::PRP38HA* allele (lanes 7–13). Yeast cultures were grown continuously at the permissive temperature of 23°C (–), or on the permissive growth medium (galactose, lane 7) and after a 2 h shift to the restrictive temperature of 37°C (+), or the indicated number of hours in repressing medium (lanes 8–13). Primer extension with an *RP51A* exon two primer (Teem and Rosbash, 1983) was used to confirm that the RNA that accumulated under the restrictive conditions was pre-mRNA and not the similarly sized lariat intermediate.

Splicing in the wild-type control culture, in contrast, was largely unaffected by this treatment (Figure 1, lanes 1 and 2). Metabolic depletion of Prp38HAp through transcriptional repression of *GAL1::PRP38HA* also arrested pre-mRNA splicing (Figure 1, lanes 7–13). Like other *GAL* promoter fusions (for example Brown and Beggs, 1992; Lockhart and Rymond, 1994), a 6–8 h delay was observed between the time of media change and the onset of splicing inhibition. Presumably, this lag reflects the interval required to make Prp38HAp limiting for pre-mRNA splicing through the combined affects of transcriptional repression, cell division and protein turnover. Culture growth stopped ~18 h after the shift to glucose medium.

Consistent with the *in vivo* observations, extracts prepared from the *prp38-2* mutant were splicing-competent, but selectively temperature sensitive (Figure 2A). A 30 min pre-incubation at 35°C blocked splicing in the *prp38-2* extract (Figure 2A, lanes 10–12), but had little effect on a wild-type control extract (Figure 2A, lanes 4–6). Native gel electrophoresis (Figure 2B) showed that spliceosome assembly in the splicing-competent extracts advanced through the three complexes resolved by this system (Pikielny *et al.*, 1986), the U1 and U2 snRNP-dependent prespliceosome (complex III), the snRNP-complete complex I intermediate, and the U4-depleted mature spliceosome (complex II). Similar levels of splicing complex were formed in the Prp38-2p inactivated extract, although, here no prominent band was observed for the mature spliceosome (Figure 2B, compare lanes 1–9 and 13–15 with lanes 10–12). The intensity of the complex I band in the 15 min time point would mask the presence of moderate amounts of mature spliceosome in the mutant extract. However, as shown below, Prp38p-deficient extracts display a unique arrest in spliceosome maturation. This defect probably accounts for the absence of an obvious complex II band, rather than the gel resolution.

Extracts prepared from glucose-repressed *GAL1::PRP38HA* cultures efficiently supported spliceosome assembly (Figure 2B, lanes 16–18), but not splicing (Figure 2A, lanes 16–18). Here again, spliceosome assembly appeared arrested at the complex I stage. In some

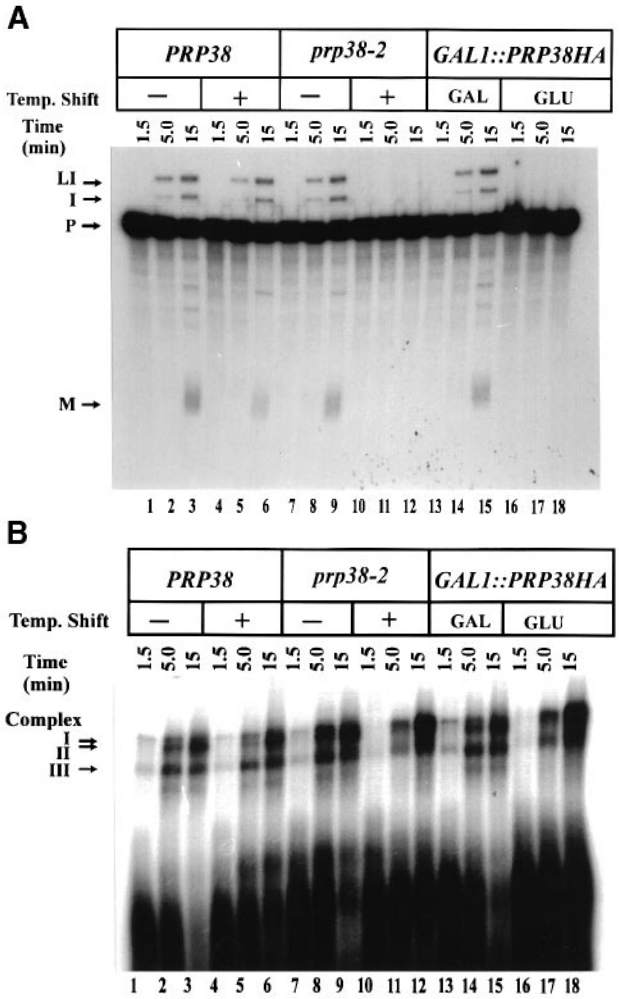

Fig. 2. A time course of *in vitro* splicing and spliceosome assembly of ^{32}P-labeled *RP51A* pre-mRNA, T7Δ2 (Rymond and Rosbash, 1988). (**A**) Pre-mRNA was added to a wild-type (*PRP38*) extract or mutant (*prp38-2*) extract that was pre-incubated at 35°C for 30 min (+) or maintained at 23°C (–). In parallel, Prp38HA complete (GAL) and depleted (GLU) extracts were monitored for pre-mRNA splicing. Samples were withdrawn at the indicated times and assayed for splicing on a denaturing 12% polyacrylamide gel. The positions of the pre-mRNA (P), lariat intermediate (LI), excised intron (I) and mRNA (M) bands are noted. (**B**) Spliceosome assembly was monitored in parallel on a 3% polyacrylamide/0.5% agarose gel. The positions of the previously characterized complex I, II and III bands (Pikielny *et al.*, 1986) are noted. These complexes are equivalent to complexes B, A2-2 and A2-1, respectively, in an alternate nomenclature (Cheng and Abelson, 1987).

experiments, trace levels of spliced products were detected with the Prp38HAp-depleted extract (see below and Figure 3). While this weak splicing probably results from residual Prp38HAp, the possibility that inefficient splicing occurs without Prp38p has not been rigorously ruled out. As expected for a Prp38p-dependent arrest, splicing in the Prp38HAp-depleted extract was complemented by an extract heat-inactivated for the unrelated splicing factor, Prp2-1p, but not by an extract heat-inactivated for Prp38-2p (Figure 3). A similar level of complementation was observed when a micrococcal nuclease-treated (i.e. snRNP-free) extract was mixed with a heat-inactivated *prp38-1* extract (Blanton *et al.*, 1992). Together, the concordance of the results obtained with the *prp38-1*, *prp38-2* and *GAL1::PRP38HA* alleles establish that

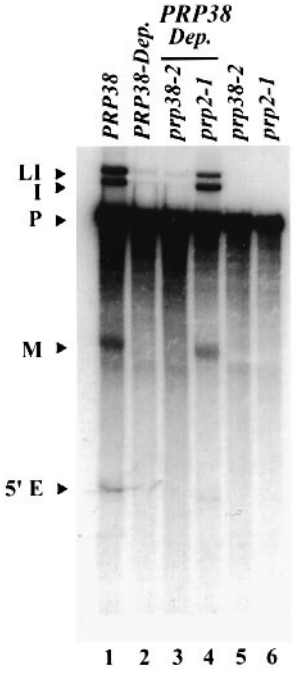

Fig. 3. Complementation of Prp38p depleted extracts. *In vitro* pre-mRNA splicing was monitored in a wild-type extract (*PRP38*), an extract depleted of Prp38HAp (*PRP38*-Dep.), and in extracts temperature-inactivated for Prp2p (*prp2-1*) or Prp38p (*prp38-2*). In lanes 3 and 4, the indicated extracts were premixed before substrate addition (see Materials and methods) to assay for the restoration of splicing through complementation. The positions of the pre-mRNA (P), lariat intermediate (LI), excised intron (I), mRNA (M) and 5′ exon bands (5′E) are noted on the left.

Prp38p is essential for cellular pre-mRNA splicing, but unnecessary for the recruitment of most spliceosomal components.

U4 snRNA release is inhibited in Prp38p-defective spliceosomes

An affinity-selection procedure was used to determine whether the removal of Prp38HAp interfered with snRNP addition to the splicing substrate (Figure 4). Biotin-substituted pre-mRNA was incubated under standard splicing conditions in Prp38HAp-complete and Prp38HAp-depleted extracts. At various time points, the assembled splicing complexes were recovered by streptavidin chromatography and assayed for snRNA content. As reported with the development of this technique (Grabowski and Sharp, 1986), non-specific snRNA association with unmodified pre-mRNA or with the streptavidin matrix was negligible (Figure 4, lanes 6 and 12).

The assembly results obtained with the Prp38HAp complete extract support the conclusions of the native gel study (Figure 2B) and are consistent with many other published studies on yeast spliceosome assembly (for example Pikielny *et al.*, 1986; Cheng and Abelson, 1987). The earliest complexes (1.5 min) contained the U1 and U2 snRNAs and some of the U4, U5 and U6 snRNAs. This snRNA distribution reflects the presence of the prespliceosome (U1, U2) and complex I (U1, U2, U4, U5, U6) in the affinity-purified pool. As assembly progresses, prespliceosomes are converted into spliceosomes and the U1 snRNA becomes less stably bound. Reflecting these changes, the spliceosomal yields of U5 and U6 snRNA

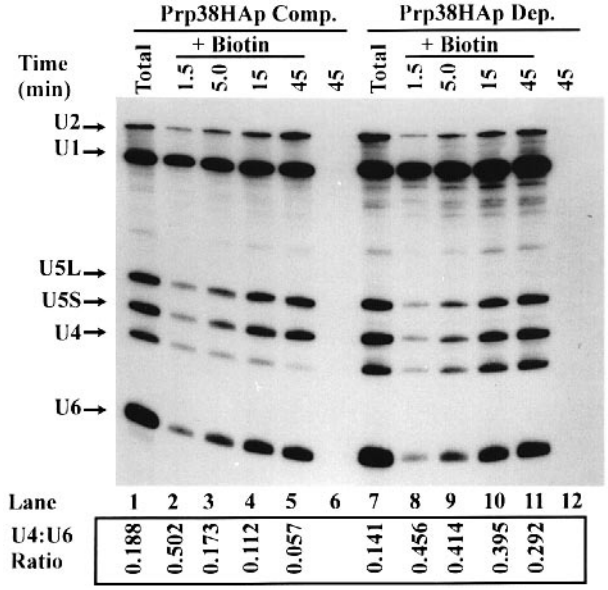

Fig. 4. U4 snRNA release from the spliceosome is inhibited without Prp38HAp. Biotin-substituted *SpRP51a* pre-mRNA (lanes 1–5, 7–11; Rymond *et al.*, 1987) or unmodified RNA (lanes 6 and 12) was incubated in Prp38HAp-complete (Comp.) and depleted (Dep.) extracts for the indicated times. The associating snRNAs were recovered by streptavidin–agarose chromatography and assayed by Northern blot. The positions of the spliceosomal snRNAs are shown on the left of this figure. The U4 snRNA to U6 snRNA signal ratios (uncorrected for sp. act.) are listed below the lane numbers. Total, unselected snRNAs from the indicated yeast extract.

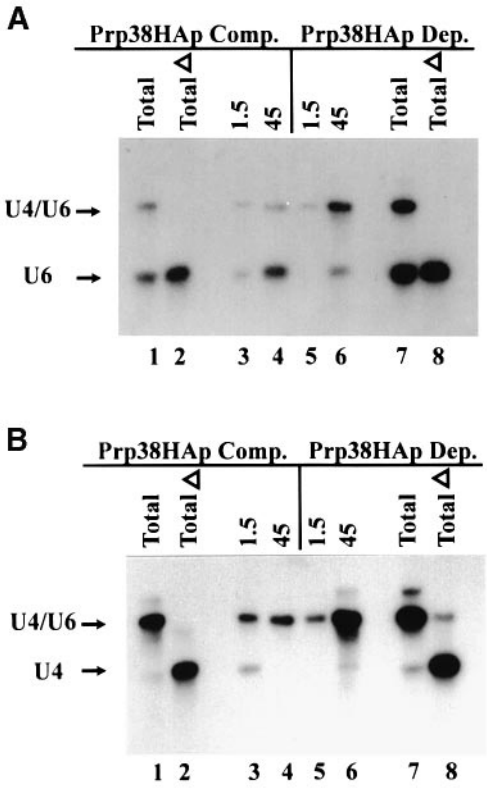

Fig. 5. U4/U6 snRNA dissociation is blocked by removal of Prp38HAp. (**A**) RNA from unfractionated Prp38HAp-complete (Comp.) and depleted (Dep.) extracts was fractionated on a non-denaturing 4% polyacrylamide/0.5% agarose gel either directly (lanes 1 and 7), or after dissociation of the U4/U6 helices by a 100°C step (lanes 2 and 8). Hybridization with a U6 snRNA-specific probe revealed the positions of the U4/U6 and the free U6 snRNA. Spliceosomal complexes were on biotin-substituted *RP51A* RNA at 1.5 min (lanes 3 and 5) and 45 min (lanes 4 and 6) of incubation in the indicated extracts. (**B**) The Western blot membrane described in Figure 4A is shown after hybridization with a U4-specific probe.

increased with time far more than that of U1 snRNA (Figure 2B, compare lanes 2 and 5). Inconsistencies in the stability and electrophoretic transfer of the exceptionally large U2 snRNA made it a less reliable marker of spliceosome assembly by this method. U4 snRNA is normally released from the yeast splicing complex before the first pre-mRNA cleavage–ligation step (Pikielny *et al.*, 1986; Cheng and Abelson, 1987). Here, this was observed as a time-dependent decrease in yield of spliceosomal U4 snRNA when compared with the U5 and U6 snRNAs. Quantifying the U4 snRNA and U6 snRNA bands by phosphoimage analysis showed that the U4/U6 signal ratio decreased approximately an order of magnitude over the 45 min incubation (Figure 2B, lanes 2–5).

The pattern of snRNP addition in the Prp38HAp-depleted extract (Figure 2B, lanes 8–11) was similar to that seen in the splicing-competent extract (Figure 2B, lanes 2–5) with one obvious distinction. Without Prp38HAp, the U4 snRNA was no longer efficiently released from the spliceosome. After 45 min of splicing, the U4/U6 ratio remained ~65% of that observed at 1.5 min (Figure 2B, lanes 8 and 12). In contrast, when Prp38HAp was present, the U4/U6 snRNA signal ratio dropped much more rapidly and, at 5 min, was <35% of that observed at 1.5 min (Figure 2B, lanes 2 and 3). Persistence of U4 snRNA in the Prp38p deficient spliceosome was consistent with the accumulation of an authentic, snRNP-complete complex I band in the gel mobility analysis (Figure 2B). The minor release of U4 snRNA observed correlated well with the weak splicing observed with the Prp38HAp-depleted extract. Inhibited U4 snRNA release was also observed with the *prp38-2* extract after temperature inactivation, but not with an extract inactivated for the unrelated splicing factor, Prp2p (Yean and Lin, 1991; J.Xie and B.C.Rymond, unpublished data). Thus, the removal of Prp38p activity results in a clear and specific defect in the spliceosomal reorganization events that lead to U4 snRNA release.

U4 snRNA remains bound to U6 snRNA in Prp38-depleted spliceosomes

The absence of Prp38p might prevent dissociation of the U4/U6 snRNAs or, alternatively, cause a hyperstabilization of the 'free' U4 snRNA within the spliceosome. To address this, RNAs isolated from Prp38HA-complete and Prp38HAp-depleted spliceosomes were fractionated on a native polyacrylamide gel under conditions that resolve the U4/U6 snRNA hybrid from the free U4 and U6 snRNAs. For comparison, total extract RNA (i.e. non-spliceosomal) was run in parallel. The resulting transfer was then sequentially hybridized with probes specific for the U4 (Figure 5B) and U6 snRNAs (Figure 5A). Since U6 snRNA is naturally present in a 2- to 5-fold molar excess over the U4 snRNA in yeast (Li and Brow, 1993; B.C.Rymond, unpublished observations), much of the total U6 snRNA signal was found in the free U6 band (Figure 5A, lanes 1 and 7). A U4/U6 hybrid band was also observed. As expected, this band could be resolved

into constituent U4 and U6 snRNA forms by preincubation at 100°C prior to electrophoresis (Figure 5A, lanes 1 and 2, 7 and 8).

The splicing complex snRNAs were assayed for U4/U6 association after 1.5 and 45 min of assembly. When compared with the total snRNA pool, a greater fraction of the early (1.5 min) spliceosomal U6 snRNA was found in the U4/U6 state (Figure 5A, compare lanes 1 and 3, 7 and 5). Presumably, this bias reflects the initial recruitment of the U4/U6 base-paired snRNAs to the spliceosome. In concordance with the continued spliceosome assembly noted above, spliceosomal U6 snRNA levels increased greatly between the 1.5 and 45 min time points. For the Prp38HAp-complete extract, spliceosome maturation over the 45 min incubation period shifted most (87%) of the U6 signal to the free U6 form (Figure 5A, lanes 3 and 4). Free U4 snRNA and U4/U6 tethered snRNAs were detected in the Prp38HAp-complete 1.5 min spliceosome (Figure 5B, lane 3) while virtually no free U4 snRNA was observed in the total RNA pool (Figure 5B, lane 1). This greater abundance of free U4 snRNA in the early spliceosome presumably reflects the rapid dissociation of the U4/U6 helices after tri-snRNP addition. Furthermore, this result shows that U4 snRNA release from the spliceosome is not concurrent with U4/U6 snRNA dissociation. This differs from the observation that for U6, the absolute amount of the U4 snRNA signal in the Prp38HAp-complete extract changed little over the 45 min incubation. As less free U4 snRNA was observed at 45 min (Figure 5B, lane 4) than at 1.5 min (Figure 5B, lane 3), the U4/U6 complexes that persist late in assembly may be defective in maturation through U4 release.

For complexes assembled in the Prp38HAp-depleted extract, nearly all of the spliceosomal U4 (>95%) and U6 (76%) snRNAs remained in the U4/U6 state (Figure 5A and B, lanes 5 and 6). This result provides clear evidence that Prp38p is important for the resolution of the U4/U6 snRNA helices and not simply for the displacement of freed U4 snRNA from the spliceosome.

Prp38p is a U4/U6.U5 tri-snRNP-specific protein

Two observations suggested that Prp38p was snRNP-associated: (i) the decreased intracellular U6 snRNA abundance observed with extended periods of *prp38-1* temperature inactivation (Blanton *et al.*, 1992); and (ii) the inhibited spliceosomal U4 snRNA release seen with Prp38p-depleted (or inactivated) extracts. To test directly for snRNP association, Prp38HAp extracts were incubated with the anti-HA antibody, HA.11, and the immune pellets assayed for snRNA content (Figure 6). At 100 mM NaCl, the U4, U5 and U6 snRNAs, and a trace amount of U1 and U2 snRNAs were found in the immune precipitates. In contrast, a background level of the U1 and U2 snRNAs, but no U4, U5 or U6 snRNAs were observed if the irrelevant monoclonal antibody, mAb63, was used with the Prp38HAp extract (Figure 6, lane 8) or if the HA.11 antibody was used with an untagged extract (Figure 6, lane 9). The stability of the Prp38HAp–snRNP association was similar to that reported for other moderately salt-sensitive snRNP proteins (for example Snu246p, Lauber *et al.*, 1996; U1-C, Tang *et al.*, 1997). The U4, U5, U6 RNAs were efficiently recovered at 200 mM NaCl; significantly reduced snRNA recovery was observed at

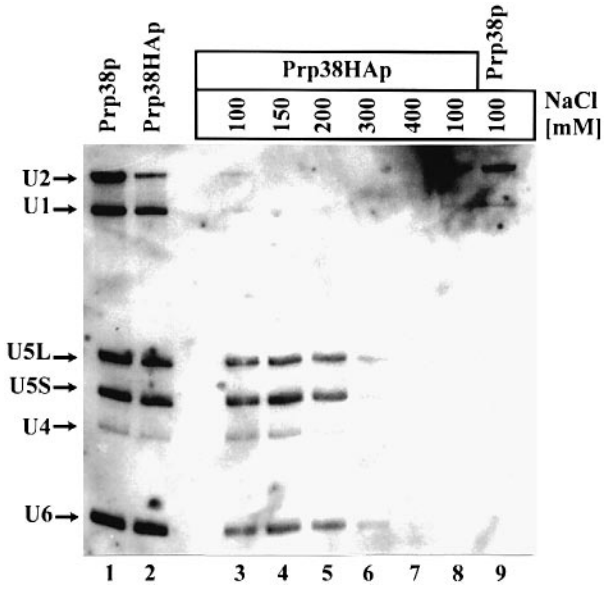

Fig. 6. Prp38HAp interacts with U4, U5 and U6 snRNA bearing complex(es). A Northern blot of RNA from unfractionated extract (lanes 1 and 2) and immune precipitate pellets with the anti-HA antibody, HA.11 (lanes 3–7 and 9) and the irrelevant antibody, mAb63 is shown. Extracts were prepared from yeast that expressed the epitope-tagged Prp38HAp protein (lanes 2–8) or from an untagged strain (Prp38p; lanes 1 and 9). The numbers in the box refer to the salt concentration used in the immune precipitation experiments. The positions of the spliceosomal snRNAs are shown on the left.

300 mM NaCl and no snRNAs were observed at 400 mM NaCl.

Yeast snRNP complexes were fractionated on a 10–30% linear glycerol gradient in 50 mM NaCl (Figure 7A). As previously described (Bordonne *et al.*, 1990), this gradient resolves the free U6 snRNP (Figure 7A, fractions 5–9), the U4/U6 snRNP (Figure 7A, fractions 11–15), the U5 snRNP (Figure 7A, fractions 17–23) and the U4/U6.U5 tri-snRNP (Figure 7A, fractions 25–29). While most of the U5 and U6 snRNA signals reside above fraction 25, only in the U4/U6.U5 tri-snRNP fraction did the U4, U5, and U6 snRNAs efficiently co-precipitate with the anti-HA antibody (Figure 7B). In contrast with the selective tri-snRNP precipitation, U1 and U2 snRNAs were recovered in each immune pellet in amounts that did not directly parallel the abundances of these snRNAs in the gradient fractions. Non-specific precipitation of these large snRNAs occurs with anti-HA antibodies under low salt conditions even in the absence of an HA-tagged protein (e.g. Tang *et al.*, 1997; McLean and Rymond, 1998). Consistent with interpretation, the U1 and U2 snRNAs were lost from the immune pellets at 100 mM NaCl while the U4, U5 and U6 snRNAs were observed up to at least 200 mM NaCl (Figure 6; data not shown).

When expressed at natural levels from its native promoter, the levels of Prp38p were too low to be detected by Western blot from individual gradient fractions. This occurred whether or not the samples were first precipitated with antibodies against Prp38HAp. To circumvent this problem, the tri-snRNP fractions were pooled and then compared with the remainder of the gradient samples for the presence of Prp38p (Figure 7C). All the detectable Prp38HAp co-fractionated with the U4/U6.U5 tri-snRNP particle, although it is possible that Prp38p exists elsewhere

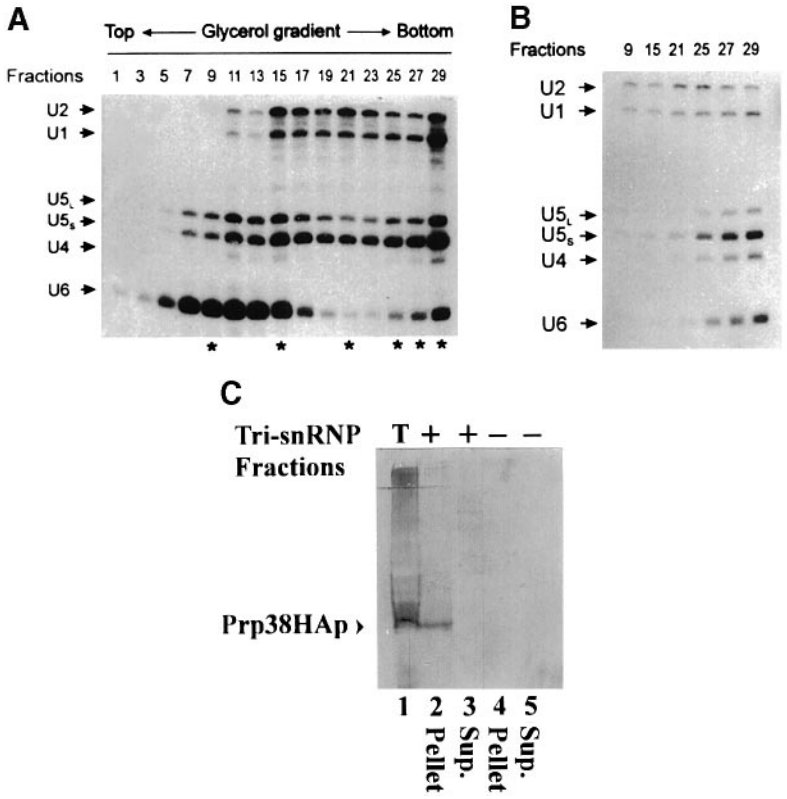

Fig. 7. Prp38HAp co-fractionates with the U4/U6.U5 tri-snRNP particle. (**A**, **B**) An extract prepared from the *GAL1::PRP38HA* yeast strain was fractionated on a 10–30% linear glycerol gradient and the indicated odd-numbered fractions were assayed in parallel for the snRNA in (A) and for the co-precipitation of snRNA with Prp38HAp in (B). The asterisks show the samples used for immune precipitation. The chosen samples contain the free U6 snRNP (9), the U4/U6 snRNP (15), the U5 snRNP (21) and the U4/U6.U5 tri-snRNP (25–29) particles. The labels to the left of each panel identify the spliceosomal snRNAs. (**C**) Prp38HAp was recovered by immune precipitation from a parallel gradient of a *PRP38HA* extract. Here, two pools were used for immune precipitation, one from gradient fractions 1–23 (–, lacks tri-snRNP) and one from 25–29 (+, tri-snRNP enriched). In each case, 50% of the recovered immune pellet (pellet) and 10% of the supernatant (Sup.) were assayed by Western blot with the HA.11 antibody to identify Prp38HAp. T, unfractionated *GAL1::PRP38HA* extract.

in the gradient in low abundance or in a form unrecognized by the HA.11 antibody. These results support a Prp38p–U4/U6.U5 tri-snRNP association and argue against the stable interaction of Prp38p with the U6, U4/U6 or U5 snRNP precursors.

Discussion

Prp38p is one of a small group of proteins uniquely associated with the U4/U6.U5 tri-snRNP particle. Previous studies with yeast and mammalian tri-snRNP specific proteins showed that members of this group are essential for stable tri-snRNP assembly and for U4, U5 and U6 snRNA addition to the spliceosome (Abovich *et al.*, 1990; Behrens and Lührmann, 1991; Galisson and Legrain, 1993). This study establishes the first link between a tri-snRNP specific protein and spliceosome maturation events that result in the dissociation of the U4/U6 intermolecular helices.

Formation of the U4/U6.U5 tri-snRNP particle requires both the release of certain U4/U6-associated proteins and the acquisition of novel tri-snRNP-specific proteins (reviewed in Seraphin and Mattaj, 1991). The co-fractionation and immune precipitation experiments presented here show that much, or all, of the Prp38p is tri-snRNP bound. Curiously, a previous biochemical characterization did not report a Prp38p-size protein (i.e. 28 kDa) in the yeast tri-snRNP particle (Fabrizio *et al.*, 1994). Perhaps the most probable explanation for this apparent discrepancy is that Prp38p was lost (e.g. salted off, proteolyzed) though the sequential anti-m_3G chromatography and glycerol gradient fractionation steps used in this earlier study. We note, however, that a band of ~30 kDa was present in fractions next to the tri-snRNP peak and that these same fractions had significant levels of other tri-snRNP-associated proteins (for example, Prp4p and Prp6p). The presence of Prp38p in a subset of the tri-snRNP would be consistent with the fact that we routinely recover <50% of the tri-snRNP snRNAs by immune precipitation. Additional work is needed to resolve whether the tri-snRNP fraction without stably bound (or antibody-accessible) Prp38p is an artifact of the isolation conditions or a legitimate intermediate of tri-snRNP assembly.

No clear human counterpart of Prp38p has been reported, although recently an SR-related protein similar in size to Prp38p was identified as a unique component of the human U4/U6.U5 tri-snRNP particle (Fetzer *et al.*, 1997). Like Prp38p, the human 27 kDa protein lacks an RRM element. Both Prp38p and the human 27 kDa tri-snRNP protein can be phosphorylated *in vitro* and, at least for the human protein, a tri-snRNP associated protein kinase is responsible (Fetzer *et al.*, 1997; B.C.Rymond, unpublished data). Beyond size and serine richness, the structural similarity between Prp38p and the human 27 kDa

protein is limited. Nevertheless, given the similar complexity of the yeast and mammalian U4/U6.U5 tri-snRNP particles (Fabrizio *et al.*, 1994), it is possible that this 27 kDa phosphoprotein functions like Prp38p in spliceosomal reorganization.

A number of proteins have been implicated by genetic or biochemical evidence in U4/U6 snRNA dynamics. These include the DExD/H-box proteins, Snu246p (Lauber *et al.*, 1996; Lin and Rossi, 1996; Noble and Guthrie, 1996; Xu *et al.*, 1996), Prp28p (Jamieson *et al.*, 1991; Strauss and Guthrie, 1991, 1994), the likely U4/U6 annealing protein, Prp24p (Shannon and Guthrie, 1991; Ghetti *et al.*, 1995; Jandrositz and Guthrie, 1995), the U4/U6 snRNP-protein, Prp4p (Ayadi *et al.*, 1997, and references within) and, as shown here, Prp38p. Spliceosomal U4 snRNA release does not occur when ATP levels are reduced (Tarn *et al.*, 1993) or in the absence of Prp38p. While Prp38p might act directly in this process, the lack of obvious RNA binding domains or ATPase elements within Prp38p argue for an indirect role in U4 snRNA release. Snu246p is perhaps the most attractive DExD/H-box candidate to mediate U4/U6 snRNA dissociation as it enters the spliceosome with the U4/U6.U5 tri-snRNP particle and interacts genetically with the U2 snRNA, a target for U6 snRNA interaction after U4 snRNA release.

The function of Prp38p is still speculative but could involve the recruitment or activation of the putative unwinding activity. Precedents for DExD/H-box protein auxiliary factors include the splicing factor, Spp2p (Roy *et al.*, 1995), and the translation initiation factor, eif4B (Rozen *et al.*, 1990). Alternatively, Prp38p might act simply to promote a conformational change within the spliceosome needed to expose the U4/U6 helices or otherwise provide opportunity for U4 snRNA displacement. The U4/U6-associated protein, Prp4p, is a credible target of Prp38p interaction as certain mutations within Prp4p cause spliceosomal arrest with bound (though not necessarily U6 associated) U4 snRNA (Ayadi *et al.*, 1997). Subsequent to U4/U6 dissociation, the U6 snRNA is integrated into the presumed active site of the spliceosome through base pair contacts with the U2 snRNA and the pre-mRNA 5′ splice site (reviewed in Nilsen, 1998). At this point, Prp38p might remain in the complex (perhaps to function again in splicing) or be released to join a newly reconstituted U4/U6.U5 tri-snRNP particle in spliceosome assembly.

Materials and methods

Yeast strains, plasmid constructs

MGD353-46D (*a, trp1-289, ura3-52, leu2-3,112, ade2, cyh^R*)
KBY2 [α*, prp38::LEU2, trp1–289, ura3-52, leu2-3,112, pYCplac22 (TRP1, PRP38HA)*]
JXY1-6d [α*, prp38::LEU2, trp1-289, ura3-52, leu2-3,112, ade2, pBM150(GAL1::PRP38HA, URA3)*]
KBY20 [α*, prp38-1, prp16-2, ade2, ura3-52, leu2-3,112, trp1-289, his3, pBM150(GAL1::PRP38HA, URA3)*]
ts368 (α*, prp2-1, ade1, ade2, ura1, tyr1, his7, lys2, gal1*)

To prepare the galactose-dependent, epitope-tagged allele, *GAL1::PRP38HA*, a YCp50-based genomic clone of *PRP38* (Blanton *et al.*, 1992) was amplified by the polymerase chain reaction with oligonucleotides (5′CTT*AGATCT*GGACTACAATGGCTGTCAATG3′ and 5′CCAAGAAGAGGTCTATGA**AGCGTAGTCTGGAACGTCGTATGGGTA**TTCGCTGGTGTCGTT; the *Bgl*II site is in italics, the HA codons are in bold) and the DNA fragment blunt-end ligated into the *Sma*I site of vector pTZ19R (USB/Amersham). This subclone was sequenced to confirm the insertion of the HA epitope after the penultimate *PRP38* codon. This DNA was subcloned as a *Bgl*II–*Bam*HI fragment into the *Bam*HI site of the yeast expression vector pBM150 (Johnston and Davis, 1984) to produce *GAL1::PRP38HA*. A diploid strain heterozygous for a *LEU2* disruption of *PRP38* (Rymond, 1993) was transformed with the *GAL1::PRP38HA* plasmid. After meiotic induction, haploid segregants were then scored for the galactose-dependent growth and for the presence of the *GAL1::PRP38HA* plasmid and the *prp38::LEU2* disrupted chromosomal allele. To place *PRP38HA* under its own promoter, a 600 bp *Kpn*I fragment from *GAL1::PRP38HA* that contained the 3′ end of this gene, including the *HA*-encoding epitope, was subcloned into a *Kpn*I-digested plasmid *YCplac22-PRP38*. This plasmid contained all *PRP38* sequences from the *Pst*I site upstream of the promoter to the *Kpn*I site within the coding sequence (Blanton *et al.*, 1992). The genetic techniques of yeast transformation, yeast mating, meiotic induction and tetrad dissection were carried out according to standard protocols (Kaiser *et al.*, 1994).

Chemical mutagenesis of *PRP38* was performed with a 2.2 kbp *Hin*dIII–*Pvu*II subclone of this gene (Blanton *et al.*, 1992) in vector YCplac22 (Gietz and Sugino, 1988). Hydroxylamine at 200 mg/ml was incubated with the plasmid DNA at 42°C under conditions otherwise as described (Rose and Fink, 1987). The mutated DNA was amplified in *Escherichia coli* (strain TG1) and the recovered plasmid pool then transformed into the *GAL1::PRP38HA* yeast strain. The yeast transformants were selected on galactose-based media lacking uracil and tryptophan and screened for ts *PRP38* mutations by replicating to 37°C on glucose-based media. DNA sequence analysis of the entire *PRP38* gene was used to identify the location of the *prp38-2* lesion. Subsequently, 5-fluoro-orotic acid selection was used to isolate yeast that had lost the *GAL1::PRP38HA* plasmid and retained the ts *prp38-2* allele.

Splicing and spliceosome assembly assays

Yeast extracts were prepared by spheroplast formation and Dounce homogenization as described by Lin *et al.* (1985) or by the ground cell pellet method published by Umen and Guthrie (1995). Temperature inactivation of the *prp38-2* (or *prp2-1*) extract was performed by mixing 1 µl of 0.6 M $KHPO_4$ (pH 7.0) with 4 µl yeast splicing extract and rapidly transferring this to 35°C for 30 min. Spliceosome assembly was initiated by the addition of 1 µl of 25 mM $MgCl_2$, 1 µl of 30% PEG–8000, 1 µl of 20 mM ATP and 2 µl (~100 000 c.p.m.) of *SpRP51a* pre-mRNA. For unknown reasons, extracts prepared from low density cultures using the Dounce homogenization method were more sensitive to *in vitro* temperature inactivation and were used exclusively for this purpose. To prepare extracts metabolically depleted for Prp38HAp, 10 ml of a *GAL1::PRP38HA* starter culture grown to an OD_{600} of ~2.0 in 1% yeast-extract, 2% bactopeptone (YP) galactose was collected by centrifugation and resuspended in 2 l of YP glucose medium. Cultures were grown 10–17 h in YP glucose medium at 30°C and the extracts were prepared by the method of Umen and Guthrie (1995). Complementation studies were carried out by first mixing equal volumes of the Prp38p-depleted extract with the appropriate heat inactivated extract at room temperature for 10 min, followed by a 30 min incubation on ice. Splicing was then performed for 20 min after the addition of pre-mRNA and required co-factors as described above.

Native gel electrophoresis of splicing complexes were carried out in 0.5× TBE buffer as described (Pikielny *et al.*, 1986) in a matrix composed of 3% polyacrylamide (59:1, acrylamide:bis-acrylamide)/0.5% agarose. For the affinity-recovery of splicing complexes, 300 µl splicing reaction volumes were used with 50 ng of biotin-substituted *SpRP51a* pre-mRNA prepared as previously published (Rymond *et al.*, 1987), except that biotin-16-UTP (Boehringer Mannheim) was used as the modified nucleotide. At each time point, 40 µl of the reaction was removed and mixed in a siliconized microfuge tube with an equal volume of Q buffer [400 mM KCl, 2 mM $Mg(OAC)_2$, 100 mM HEPES pH 7.5] and 20 µl of immobilized streptavidin (Boehringer Mannheim). Afterwards, for 45 min at 4°C, the beads were washed four times with 800 µl of NET-2 buffer (50 mM Tris–HCl pH 8.0, 50 mM KCl, 1 mM DTT and 0.5% NP-40). To help reduce background, the streptavidin beads were transferred to a fresh siliconized microfuge tube for the final wash. The snRNAs were then released by the addition of an equal volume of 2× PK buffer (200 mM Tris–HCl pH 7.5, 25 mM EDTA, 300 mM NaCl, 2% SDS, 2 mg/ml proteinase K) for 10 min at 37°C. The samples were phenol extracted and the snRNAs concentrated by ethanol precipitation. Subsequent blotting and snRNA detection steps were as previously described in detail (Blanton *et al.*, 1992). The relative intensities of the snRNA bands were determined directly with a Storm

860 phosphoimager (Molecular Dynamics) or by quantifying X-ray film images with an LKB model 2222–010 Ultroscan XL laser densitometer.

Extracts were fractionated on 10–30% glycerol gradients by the method of Bordonne *et al.* (1990). The gradients were separated into 0.4 ml fractions and processed by immune precipitation or direct phenol extraction. Unbound proteins were concentrated by precipitation with acetone for Western blot analysis with anti-HA antibodies 12CA5 (Boehringer Mannheim) or HA.11 (Babco). Immune precipitations (with 20 µl of total yeast extract or 150 µl of gradient fractions) were carried out essentially as described previously (Lockhart and Rymond, 1994). The yeast extract was mixed with 10–20 µl of antibody-bound protein A/protein G beads (Oncogene) in a total volume of 100 µl (total extract) or 300 µl (gradient fractions) HNT buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 0.05% Triton X-100) at room temperature for 30 min. The NaCl concentration of the HNT buffer was adjusted between 50–300 mM as needed. The unbound extract was separated from the antibody-bound material by centrifugation at 4000 *g* for 1 min. The beads were washed 6× with 300 µl of HNT. The bound material was then released by the addition of 100 µl of 1× PK buffer for 10 min at 37°C. The sample was phenol extracted and the snRNAs concentrated by ethanol precipitation for Northern blot analysis (Blanton *et al.*, 1992).

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