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Proteomic analysis identifies a new complex required for nuclear pre-mRNA retention and splicing

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Using the proteomic tandem affinity purification (TAP) method, we have purified the *Saccharomyces cerevisie* U2 snRNP-associated splicing factors SF3a and SF3b. While SF3a purification revealed only the expected subunits Prp9p, Prp11p and Prp21p, yeast SF3b was found to contain only six subunits, including previously known components (Rse1p, Hsh155p, Cus1p, Hsh49p), the recently identified Rds3p factor and a new small essential protein (Ysf3p) encoded by an unpredicted split ORF in the yeast genome. Surprisingly, Snu17p, the proposed yeast orthologue of the seventh human SF3b subunit, p14, was not found in the yeast complex. TAP purification revealed that Snu17p, together with Bud13p and a newly identified factor, Pml1p/Ylr016c, form a novel trimeric complex. Subunits of this complex were not essential for viability. However, they are required for efficient splicing *in vitro* and *in vivo*. Furthermore, inactivation of this complex causes pre-mRNA leakage from the nucleus. The corresponding complex was named pre-mRNA REtention and Splicing (RES). The presence of RES subunit homologues in numerous eukaryotes suggests that its function is evolutionarily conserved.

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Introduction

Pre-mRNA splicing occurs by two sequential transesterification reactions that are catalysed by a large and dynamic ribonucleoprotein complex: the spliceosome. Every splicing reaction necessitates the stepwise assembly of a full spliceosome onto the pre-mRNA. Interestingly, two different but evolutionarily related spliceosomes co-exist in most eukaryotic cells. Formation of major spliceosomes involves the U1, U2, U4, U5 and U6 snRNPs, while that of the minor spliceosomes requires U11, U12, $U4_{ATAC}$, U5 and $U6_{ATAC}$ snRNPs. For major spliceosomes, U1 snRNP binding to the intron 5′ splice site allows the formation of a commitment complex for yeast or complex E in mammals (Séraphin and Rosbash, 1989a; Michaud and Reed, 1991). This step is followed by pre-spliceosome formation through U2 snRNP addition. Finally, the U4/U6.U5 triple snRNP joining generates the complete spliceosome (Pikielny *et al*, 1986; Konarska and Sharp, 1987). After remodelling steps, during which the U1 and U4 snRNPs dissociate, the first splicing reaction occurs. A second remodelling step precedes the last transesterification reaction. Recently, an alternative scenario, suggesting that a pre-assembled complex of all snRNPs binds the pre-mRNA as a single unit, was proposed (Stevens *et al*, 2002). In this model, the spliceosome also must undergo remodelling before each catalytic step. In the cell, molecular mechanism must act in parallel to splicing to ensure that pre-mRNAs are not exported to the cytoplasm before their complete processing. This process is not well understood. Many factors involved in spliceosome assembly have associated pre-mRNA leakage and splicing inhibition phenotypes (Legrain and Rosbash, 1989). Pre-mRNA leakage is then likely to result indirectly from pre-mRNA accumulation, even though this is not always the case (Rutz and Séraphin, 2000). More recently, the new yeast factor, Mlp1, was directly implicated in nuclear pre-mRNA retention without affecting splicing itself (Galy *et al*, 2004).

U2 and U6 snRNPs have been implicated in catalysis of splicing in the active spliceosome (Valadkhan and Manley, 2001), while U5 snRNP is known to interact with exons (Newman and Norman, 1992; Wyatt *et al*, 1992). Spliceosome remodelling allows the U2 and U6 snRNAs to form a base pair (Brow, 2002). In addition, these snRNAs interact with the pre-mRNA: U2 snRNA is involved in branchpoint adenosine definition (Parker *et al*, 1987), while U6 snRNA selects the 5′ splice site (Kandels-Lewis and Séraphin, 1993; Lesser and Guthrie, 1993). The U2 snRNP is a large structure containing the U2 snRNA, seven Sm proteins and two U2-specific proteins: U2A′ and U2B″ (Lea1p and Msl1p in *Saccharomyces cerevisiae*) (Lührmann *et al*, 1990; Tang *et al*, 1996; Caspary and Séraphin, 1998). In addition, two multisubunit complexes associate with U2 snRNP: SF3a and SF3b (Brosi *et al*, 1993). SF3a is composed of three polypeptides (SF3a120, SF3a66, SAP130, in human; Prp9p, Prp11p and Prp21p in yeast; Krämer, 1996), while human SF3b was reported to contain seven proteins (SAP130, SAP155, SAP145, SF3b49, SF3b14b, p14 and SF3b10)(Gozani *et al*, 1996; Das *et al*, 1999; Will *et al*, 2002). Yeast homologues of five of these factors have been characterized (Wells

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et al, 1996; Igel *et al*, 1998; Caspary *et al*, 1999; Wang and Rymond, 2003). Interestingly, human SF3a acts exclusively during the splicing of major introns (Will *et al*, 1999), while SF3b is required for splicing of both types of introns by associating with U2 and U12 snRNPs, respectively (Das *et al*, 1999; Will *et al*, 1999). Thus, both factors are essential for splicing. Furthermore, many SF3b subunits can be crosslinked to the branchpoint region of the pre-mRNA (Staknis and Reed, 1994; Gozani *et al*, 1996; Query *et al*, 1996; McPheeters and Muhlenkamp, 2003). Interestingly, the human SF3b p14 subunit that crosslinks directly to the branchpoint lies in the centre of a shell made by the other SF3b subunits in a structural model of the complex obtained by cryo-electron microscopy (Golas *et al*, 2003).

We have used the TAP proteomic approach developed in our laboratory (Rigaut *et al*, 1999; Puig *et al*, 2001), to characterize the yeast U2 snRNP-associated complexes. Identification of the SF3a subunits by mass spectrometry revealed the presence of the three previously known factors: Prp9p, Prp11p and Prp21p, but no additional component. In-depth analysis of SF3b revealed six subunits, four of which had been identified previously, namely Rse1p, Hsh155p, Cus1p and Hsh49p (Wells *et al*, 1996; Igel *et al*, 1998; Caspary *et al*, 1999; Puig *et al*, 2001). We also found Rds3p that was independently identified while this work was in progress (Wang and Rymond, 2003). Interestingly, we identified a novel, unpredicted small yeast protein, as a new subunit of this complex. This subunit, encoded by a small split yeast ORF, is essential for yeast growth. Surprisingly, however, Snu17p, which was suggested to represent the yeast orthologue of the human p14 branchpoint-binding protein (Gottschalk *et al*, 2001; Wang and Rymond, 2003), was consistently absent from our purifications. TAP purification revealed that Snu17p is present in an independent trimeric complex. Subunits of this new complex, called pre-mRNA REtention and Splicing (RES), are dispensable for yeast cell viability. These proteins are, however, conserved in eukaryotes and required for efficient pre-mRNA splicing *in vivo* and *in vitro*. More importantly, inactivation of RES subunits induces nuclear pre-mRNA leakage. Overall, our proteomic analysis of the yeast SF3a and SF3b splicing factors established definitively their composition, revealing altogether the presence of unsuspected subunits. It also led to the identification and functional characterization of the new RES complex. These findings have implication for our understanding of the composition and organization of the mammalian splicing machinery.

Results

Yeast SF3a contains three subunits

The yeast SF3a subunits, Prp9p, Prp11p and Prp21p, were identified genetically and, based on their homology to human subunits and *in vitro* studies, suggested to be SF3a components (reviewed in Krämer, 1996). However, as yeast SF3a was not purified, it remained possible that more subunits were present and/or that these three proteins did not form the predicted complex. Thus, we purified SF3a using a Prp9p TAP fusion. Purified products were fractionated by SDS–PAGE. This consistently revealed three proteins present in apparent relative stoichiometric amount (Figure 1A). These factors were identified by mass spectrometry as Prp9p, Prp11p and Prp21p. We conclude that yeast SF3a contains only three subunits.

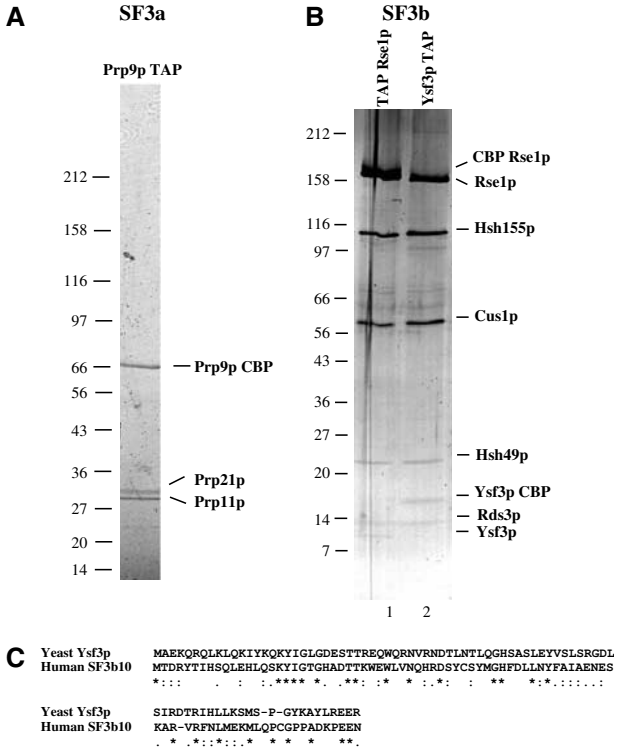

Figure 1 Characterization of yeast SF3a and SF3b complexes. (**A**) TAP purification of yeast SF3a using a PRP9 TAP fusion identifies three subunits. The TAP purified material was fractionated on a 5–20% gradient SDS–PAGE gel and stained with Coomassie Blue. Proteins were identified by MALDI. (**B**) TAP purification of yeast SF3b complex using TAP Rse1 or Ysf3 TAP fusions identifies six subunits. The purified material was fractionated on a 5–20% gradient SDS–PAGE gel, which was then stained with silver. Proteins were identified by MALDI. (**C**) Sequence alignment of human SF3b10 protein and its yeast homologue Ysf3p. Symbols indicate identity (*), high (:) or low (.) similarity.

Characterization of yeast SF3b reveals new subunits

TAP purification of the yeast U2 snRNP led us previously to identify and characterize Rse1p, a yeast homologue to human SF3b130 (Caspary *et al*, 1999). Hsh155p was also identified in the purified material (data not shown). We used a N-terminal TAP-tagged Rse1p to characterize yeast SF3b (Puig *et al*, 2001). Mass spectrometry analysis identified previously known components of yeast SF3b (Rse1p, Hsh155p, Cus1p and Hsh49p; Puig *et al*, 2001) as well as a new 14 kDa protein: Rds3p (data not shown). While this work was in progress, the presence of Rds3p in yeast SF3b was independently reported (Wang and Rymond, 2003). Additional smaller products were consistently seen in SF3b purifications (e.g., Figure 1B, lane 1), but we were unable to identify them in the yeast protein database through mass spectrometry (data not shown). The recent purification of human SF3b revealed the presence of two new small subunits of 14 and 10 kDa, namely SF3b14 and SF3b10 (Will *et al*, 2002). While the similarity between SF3b14 and Rds3p was noted, SF3b10 was reported to have no homologues in yeast. BLAST searches against the yeast genome database revealed, however, a sequence that, when translated, showed significant similarity to human SF3b10

(Figure 1C). The corresponding ORF had not been predicted because a putative intron disconnects the initiating ATG from the downstream coding sequence. To confirm that this new gene, yeast splicing factor 3b subunit (*YSF3*), was active, we performed a 5′ RACE analysis. Sequencing of the resulting product confirmed that this region was transcribed and that the putative intron was removed, thus generating the predicted coding sequence (data not shown). To confirm that the cognate polypeptide was made and that it was a subunit of yeast SF3b, we fused the TAP tag to the C-terminus of Ysf3p. Western blot analysis confirmed that Ysf3p was expressed and the profile of the six subunits recovered after TAP purification, gel fractionation and silver staining was identical to the one observed following purification of TAP Rse1p (Figure 1B, compare lanes 1 and 2; note the expected mobility change of Rse1p and Ysf3p due to the tag). Mass spectrometry identified unequivocally the six subunits consistently recovered in such purifications in apparent relative stoichiometric amounts as Rse1p, Hsh155p, Cus1p, Hsh49p, Rds3p and Ysf3p. To confirm this finding and to rule out the presence of an undetected co-migrating subunit, we TAP tagged the remaining subunits of the yeast SF3b complex, namely Hsh155p, Cus1p, Hsh49p and Rds3p. Parallel TAP purification with the six SF3b subunits revealed that the major proteins recovered co-migrated (except for the tagged subunit that was enlarged by the expected mass) and that no additional subunit could be detected (data not shown). Overall, these results demonstrated that yeast SF3b contains only six subunits, including the newly identified Rds3p and Ysf3p factors.

Snu17p/Ist3 belongs to a new evolutionally conserved trimeric complex: RES

While seven proteins have been reported to be present in human SF3b (Will *et al*, 2002), our analysis failed to detect the yeast homologue to the human p14 subunit (Table I). More importantly, we could not detect the Snu17p factor, which was suggested to represent the yeast p14 homologue (Gottschalk *et al*, 2001; Will *et al*, 2001; Wang and Rymond, 2003). To ascertain that Snu17p was not a yeast SF3b subunit, we TAP purified Snu17p and associated factors. Consistent with our findings, the protein profile was different from the pattern observed in SF3b purifications (compare Figures 1B and 2A). Three proteins were present in apparent relative stoichiometric amounts. This new complex was named RES (see below). Mass spectrometry identified its subunits as Snu17p, Bud13p and the product of the YLR016c ORF (pre-mRNA leakage 1 (Pml1p), see below), as well as low levels of contaminating ribosomal and heat shock proteins that were not reproducibly found (data not shown). None of the RES subunits were detected or identified in the six TAP purifications performed with tagged SF3b subunits (see above).

Biocomputing analyses revealed that RES subunits are conserved and that they contain various domains (Table I). Like human p14, Snu17p possesses an RNA-binding domain (RBD). Interestingly, BLAST searches revealed that Snu17p is more related to the human CGI-79 protein than to p14. Phylogenetic analysis of proteins similar to these factors, rooted using the RBD domain of Hsh49p, indicates that p14 and Snu17p belong to two separate lineages (Figure 2B). In each species, besides yeast, two factors are present, one from each subfamily. However, no clear p14 orthologue is found in the yeast genome. This result is consistent with our finding that human and yeast SF3b have different compositions and that Snu17p belongs to a new complex. Bud13p contains a unique, phylogenetically conserved, C-terminal region of unknown function, while the C-terminus of Pml1p contains a conserved FHA domain, implicated in phosphothreonine

Table I Comparison of yeast and human SF3a, SF3b and RES subunits

Yeast	Human	Domains
SF3a		
Prp9p	SF3a60	2 × Zn finger
Prp11p	SF3a66	Zn finger
Prp21p	SF3a120	Swap domain
SF3b		
Rse1p	SAP130	β-Propeller repeats
Hsh155p	SAP155	HEAT repeats
Cus1p	SAP145	
Hsh49p	SF3b49	2 × RRM
Rds3p	SF3b14b	Zn finger
—	p14	RRM
Ysf3p	SF3b10	
RES		
Snu17p	CGI-79	RRM
Bud13p	MGC13125	
Pml1p	Snip	FHA domain

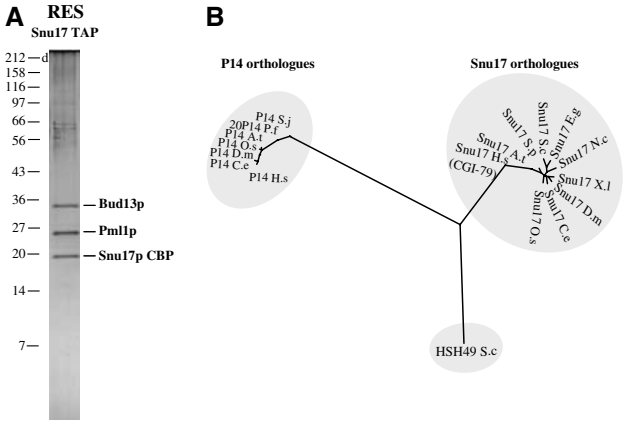

Figure 2 Identification of the new RES complex associated with Snu17p. (**A**) Proteins associated with Snu17p were purified by the TAP method. Purified material was fractioned on a 10% tricine PAGE gel and stained with Coomassie Blue. Proteins were identified by MALDI. In addition to Snu17p, Bud13p and Pml1p, we identified Ssa2p heat shock protein and some ribosomal proteins. These common contaminants were not reproducibly seen in other purifications (not shown). (**B**) Snu17 is not a P14 branchpoint-binding protein orthologue. A phylogenetic tree of Snu17 and human P14 homologue RNA-binding domains was built and rooted with the RNA-binding domain of Hsh49. Accession numbers of the sequences are as follows: P14 *Plasmodium falciparum* (Q8I5G8); P14 *Arabidopsis thaliana* (Q9FMP4); P14 *Oryza sativa* (Q7XZG6); P14 *Schistosoma japonicum* (Q86EF3); P14 *Caenorhabditis elegans* (Q8ITY4); P14 *Drosophila melanogaster* (Q9VRV7) P14 *Homo sapiens* (Q9Y3B4); Snu17 *C. elegans* (Q18318); Snu17 *D. melanogaster* (Q9VIS0); Snu17 *Xenopus laevis* (AAH56844) Snu17 *Schizosaccharomyces pombe* (O94290); Snu17 *O. sativa* (Q94GF0); Snu17 *Mus musculus* (Q8R0F5); Snu17 *Neurospora crassa* (CAE76416); Snu17/CGI-79 *H. sapiens* (Q9Y3I8); Snu17 *Eremothecium gossypii* (AAS51937); Snu17 *S. cerevisiae* (YIR005W); Hsh49 *S. cerevisiae* (YOR319W).

binding (Hammet *et al*, 2000). Interestingly, Pml1p was identified in complex containing all five snRNPs, supporting a role in splicing (Stevens *et al*, 2002).

SF3b subunits are essential for yeast growth, while inactivation of RES causes slow growth phenotypes

To understand the role of the newly identified factors, we disrupted the corresponding genes in yeast. Inactivation of *RDS3* and *YSF3* in a diploid strain followed by tetrad analysis revealed that they are, like all other SF3b subunits, essential for vegetative growth (data not shown). In contrast, all RES subunits were dispensable but their inactivation generated a slow growth phenotype that was exacerbated at 37°C (Figure 3). Interestingly, this phenotype was stronger for *BUD13* and *SNU17* inactivation than for *PML1*. Overall, the genetic analysis was consistent with the biochemical data indicating the presence of two functionally different complexes.

RES is not associated with snRNAs

SF3b subunits have been shown to co-precipitate the U2 snRNA in yeast and human cells, while Snu17p was also proposed to associate with this species (Gottschalk *et al*, 2001).We performed coimmunoprecipitation experiments to assess whether RES subunits were associated with the U2 snRNP. Extracts from strains expressing either Snu17p, Bud13p, Pml1p, or as positive controls the U2 snRNP protein Lea1p (U2A′), or SF3b subunits Rse1p and Ysf3p fused to the TAP tag were precipitated with IgG-coated beads and the presence of snRNAs in the pellets was analysed by primer extension. A wild-type extract served as a negative control. Surprisingly, for tagged Snu17p, Bud13p and Pml1p, like for the wild-type extract, we did not detect snRNA co-precipitation (Figure 4). In contrast, under these conditions, Lea1p, Rse1p and Ysf3p co-precipitated efficiently the U2 snRNA as well as small amounts of U5 and U6 snRNAs. Western blotting revealed that more than 50% of all tagged proteins were immunoprecipitated (data not shown). Thus, lack of snRNA co-precipitation by RES subunits does not result from inefficient pull-down. These data confirm that Snu17 is not an SF3b subunit, and indicates further that RES is not stably associated with an snRNP.

RES associates with pre-mRNA in vitro

In the absence of snRNA pull-down, we tested for a link between RES and splicing by performing *in vitro* splicing

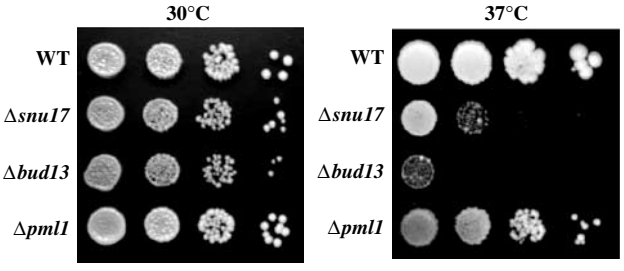

Figure 3 RES subunits are required for wild-type growth. Serial dilutions of strains harbouring deletion of the RES subunits (Δ*snu17*, Δ*bud13*, or Δ*pml1*) and an isogenic wild-type strain control were placed on YPDA plates and incubated at 30 and 37°C for 2 and 3 days, respectively.

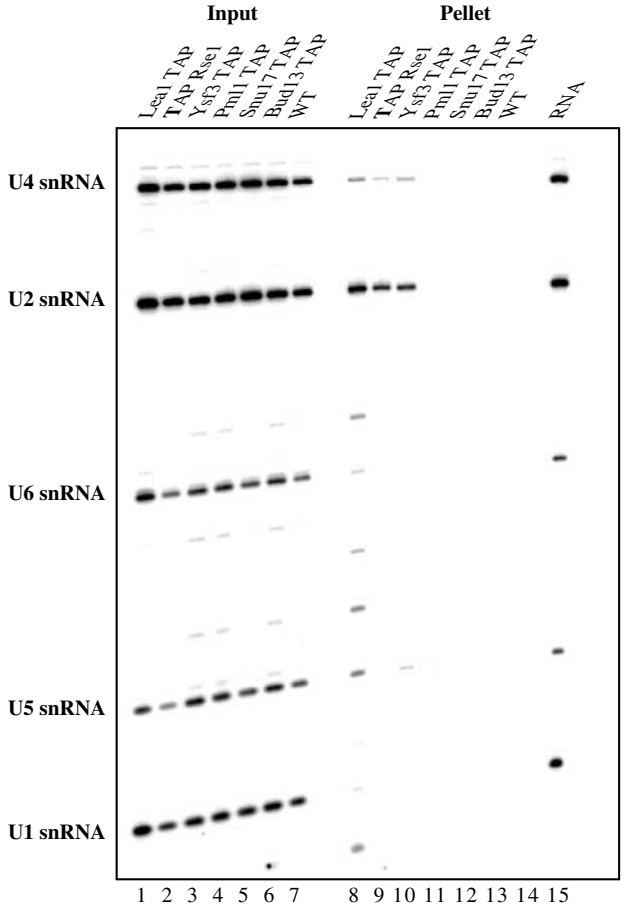

Figure 4 In contrast to SF3b, the RES complex is not stably associated with snRNAs. Extracts from a control wild-type yeast strain (WT) and strains harbouring TAP-tagged Lea1p, Rse1p, Ysf3p, Snu17p, Bud13p or Pml1p were immunoprecipitated with IgG-coated beads. RNA extracted from input and pellet was analysed by primer extension with primers specific for the U1, U2, U4, U5 and U6 snRNAs. The positions of the corresponding signals are shown on the left. RNA from a four times more extract was used for the pellets relative to the input fractions.

reactions and assaying its association with pre-mRNA, splicing intermediates and/or products. Extracts from a wild-type strain and strains expressing TAP-tagged Snu17p, Bud13p, Pml1p and Lea1p, Rse1p, Ysf3p were incubated either with radioactively labelled wild-type pre-mRNA or mutant pre-mRNA containing a branchpoint deletion as a negative control. After precipitation with IgG-coated beads, RNAs were extracted from pellets and detected by autoradiography following denaturing acrylamide gels electrophoresis. Supernatants and one input were analysed in parallel to control for precipitation efficiency and absence of RNA degradation. As expected (Figure 5), Lea1p, Rse1p and Ysf3 precipitated significant amount of pre-mRNA over the background observed with wild-type extract or with the mutant pre-mRNA lacking a branchpoint (Séraphin and Rosbash, 1991). In addition, Rse1p and Ysf3p co-precipitated low but significant amount of lariat intermediate, while Lea1p efficiently pulled down splicing intermediates and the lariat intron. The pre-mRNA was also co-precipitated with the tagged RES, even though to a lower extent than with the other tagged factors. The signal was significant and specific,

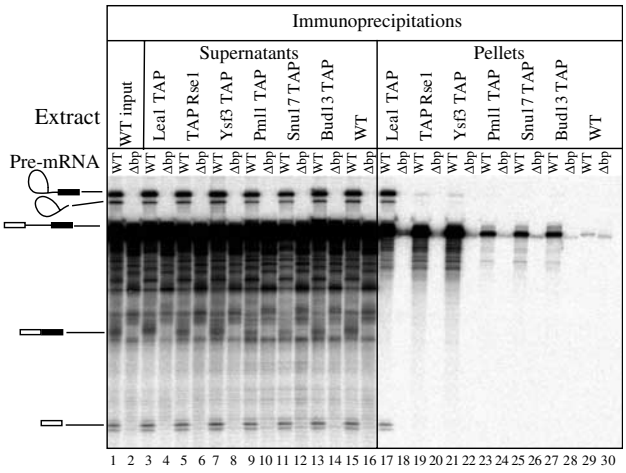

Figure 5 SF3b and RES associate with the pre-mRNA. Splicing reaction were assembled with a wild-type pre-mRNA and, as a negative control, a branchpoint deletion pre-mRNA mutant, using extracts prepared from an untagged wild-type strain or isogenic strains harbouring the TAP tag, fused either to Lea1p, Rse1p, Ysf3p, Snu17p, Bud13p or Pml1p. RNA immunoprecipitated with IgG sepharose (pellet) and RNA remaining in the supernant (supernatant) were extracted together with one input fraction, fractionated by denaturing acrylamide gel electrophoresis and detected by phosphorimaging. Structures of the various species are indicated on the left.

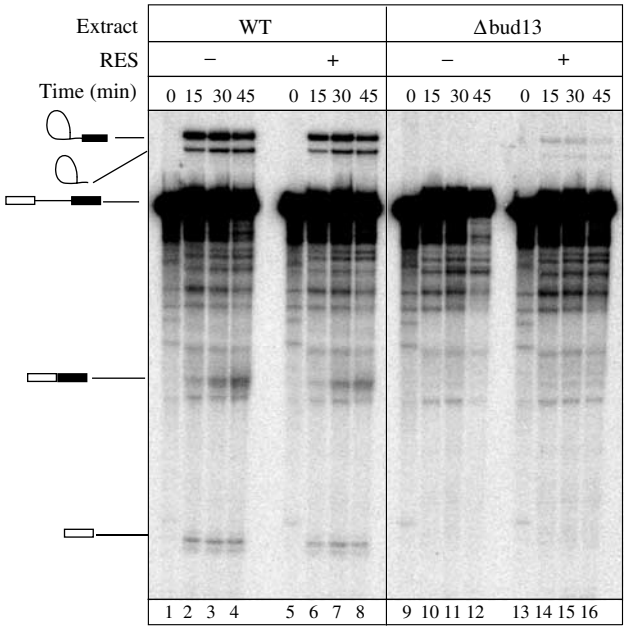

Figure 6 Extracts lacking Bud13p are defective in splicing *in vitro* and inhibition of splicing can be relieved by addition of purified RES complex. Splicing reactions were performed using extracts prepared from wild-type strain or isogenic Δ*bud13* strains. Products of 0, 15, 30 and 45 min reactions were fractionated by denaturing gels electrophoresis and detected by phosphorimaging. To reconstitute activity, TAP-purified RES complex was added to the reaction mixtures prior to addition of the pre-mRNA.

however, as it was consistently above background detected with the wild-type extract or the mutant pre-mRNA. Pre-mRNA co-precipitation indicated that RES associates with the spliceosome before step 1; the weaker signal relative to Lea1p, Rse1p and Ysf3p suggests that RES interaction is weaker, more transient and/or that the RES-tagged subunit is less accessible when incorporated into spliceosome.

Snu17p and Bud13p are required for efficient splicing in vitro

To gain further evidence for the function of the newly discovered proteins in splicing, we analysed *in vitro* splicing and spliceosome formation, by incubation of radiolabelled pre-mRNA in the extract lacking RES subunits. Interestingly, spliceosome formed in extracts lacking Bud13p or Snu17p migrated faster than complexes formed in wild-type extracts, while commitment complex formation and mobility were unaffected (data not shown). In contrast, removal of Pml1p did not alter spliceosome migration. Analysis of the reaction products revealed that *in vitro* splicing is significantly inhibited before the first catalytic step in the absence of Bud13p (Figure 6) or Snu17p (data not shown). Again, removal of Pml1p had no significant effect. To control for the specificity of this effect, we added TAP-purified RES complex to the reaction, which partially restored the *in vitro* splicing deficiency of Δ*bud13* extracts (Figure 6) and Δ*snu17* (data not shown). In contrast, purified RES addition to a wild-type extract did not affect splicing. These results demonstrate a direct role for RES in splicing *in vitro* and, together with the co-precipitation of pre-mRNA, they indicate that it acts before the first splicing step.

Analysis of the new SF3b subunit also provided evidence for their implication in splicing *in vitro* (data not shown). Strikingly, extracts containing an Rds3p TAP fusion formed commitment complexes, but were unable to assemble pre-spliceosomes (data not shown). This result is consistent with a role of SF3b in the commitment complex–pre-spliceosome transition.

RES is required for efficient splicing in vivo

Taking advantage of the viability and thermosensibility of the Δ*bud13,* Δ*snu17* and Δ*pml1* mutant strains, we tested the effect of RES subunit inactivation on pre-mRNA splicing *in vivo*. Reporter plasmids containing the RP51A intron or mutants thereof inserted within the *lacZ* reading frame were introduced in these strains and splicing of these construct assayed by β-galactosidase assays and by primer extension analysis (Jacquier *et al*, 1985).

Splicing of the wild-type reporter, assayed by the production of β-galactosidase, was essentially normal in the three mutant strains (at most two-fold reduction compared to an isogenic wild-type strain, Figure 7A). We obtained similar results for a branchpoint mutant (data not shown). In contrast, the reporter mRNA carrying a poor 5′ splice site (5′II: GUAU**a**U) was poorly spliced in strains lacking Snu17p or Bud13p grown at 25°C (100-fold reduction of β-galactosidase levels). This effect was strongly exacerbated at 37°C (500-fold reduction compared to wild type). Inactivation of Pml1p resulted in a milder phenotype as a 15-fold reduction of β-galactosidase could only be detected by combining the 5′ splice site mutation and the nonpermissive temperature.

To ascertain that splicing was affected by RES inactivation and to determine which step was inhibited, we assessed mRNA, pre-mRNA and lariat intermediate levels by a more direct and sensitive primer extension analysis. For the wild-type reporter, we observed an accumulation of pre-mRNA in

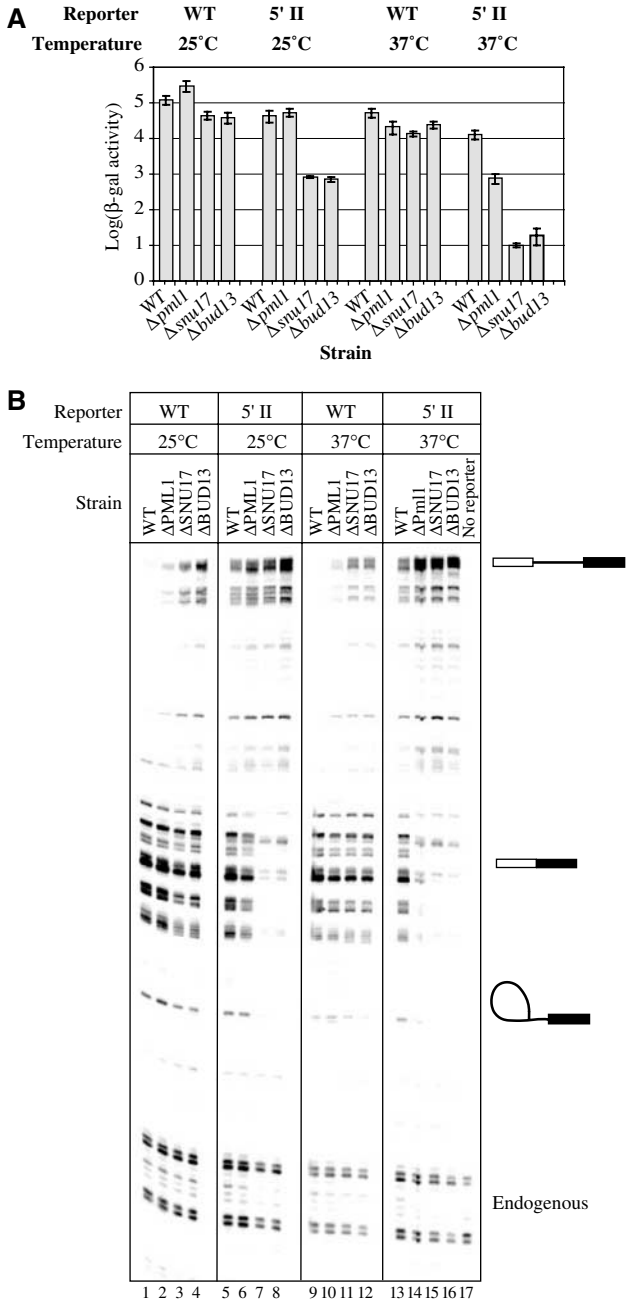

Figure 7 RES subunits are involved in splicing *in vivo*. The splicing efficiency of reporter constructs was analysed at two different temperatures (25 and 37°C) in a wild-type strain or mutant lacking *SNU17*, *BUD13* or *PML1*. Splicing reporters contained either the wild-type RP51A intron (WT) or a 5′ splice site mutant derivative (5′II), interrupting the reading frame of the *lacZ* gene. (**A**) β-Galactosidase activity of the wild-type and 5′II reporters. Given the wide variation, data are presented on logarithmic scale. In each strain, activities are normalized to the activity of a construct containing no intron in the *lacZ* gene. (**B**) Primer extension analysis of RNA present in the various strains. As a control, primer extension using RNA isolated from wild-type strain without reporters is depicted. Owing to multiple transcription initiation sites of the inducible GAL promoter, mRNAs appear as multiple bands. Positions of reporter-derived pre-mRNA, mRNA, lariat intermediate and endogenous RP51A mRNA are indicated.

the Δ*snu17* and Δ*bud13* strains, both at 25 and 37°C (Figure 7B), with no concomitant significant effect on the lariat intermediate or mRNA levels. For the 5′ splice site mutant reporter, mRNA levels were significantly reduced in the Δ*snu17* and Δ*bud13* backgrounds. In the Δ*pml1* strain, pre-mRNA accumulation was significantly lower, and a combination of high temperature and 5′ splice site mutation was required to detect some mRNA decrease. In all cases, the latter splicing block occurs concomitantly with a reduction of the lariat intermediate level indicative of a first step block. Overall, these data demonstrate that RES is required for efficient splicing. Furthermore, RES appears to act before the first splicing step and to be more critical for introns with weak 5′ splice sites.

RES is required for nuclear pre-mRNA retention in vivo

To check whether, in addition to splicing process itself, RES complex is also involved in pre-mRNA retention in the nucleus, we used a previously described reporter system composed of two related plasmids with either the pre-mRNA (pre-mRNA in-frame) or the mRNA (mRNA in-frame) encoding β-galactosidase (Rain and Legrain, 1997). Henceforth, β-galactosidase activity generated by the pre-mRNA in-frame construct allows for the estimation of pre-mRNA leakage from the nucleus, while enzymatic activity originating from the mRNA in-frame construct reports splicing. As a positive control, we have used isogenic Mlp1 knockout strain, which is known to have pre-mRNA leakage phenotype without associated splicing defect (Galy *et al*, 2004). For all the three RES knockouts, we observed a significant pre-mRNA leakage at 25°C, evidenced by increased β-galactosidase activity with the pre-mRNA in-frame construct (Figure 8A). This effect was not exacerbated at 37°C (data not shown). Splicing (mRNA in-frame construct) was not more affected in the Δ*pml1* background than in the Δ*mlp1* strain in contrast to the significant reduction observed for Δ*snu17* and Δ*bud13*. Thus, while mRNA leakage observed in the absence of Snu17 or Bud13 may result from poor splicing, the effect observed in Δ*pml1* is, like for Δ*mlp1* (Galy *et al*, 2004), likely to be direct. This conclusion is further supported by the observation that splicing defects are weak and thermosensitive with this mutant (see above), while pre-mRNA leakage is not. Primer extension analyses confirmed that, for Δ*pml1* as for Δ*mlp1*, there is no significant increase in pre-mRNA level in comparison to wild type (Figure 8B and C), while splicing of this reporter RNAs was defective for Δ*bud13* and Δ*snu17*, with a three-fold increase in pre-mRNA to mRNA ratio. These results indicate that the major function of Pml1 is nuclear pre-mRNA retention. Furthermore, the RES complex, at the edge between splicing and pre-mRNA retention, may connect these processes particularly for introns with weak 5′ splice sites.

Discussion

Using a proteomic strategy, we have characterized the yeast SF3a and SF3b splicing factors and identified a new complex, RES, required for efficient intron removal and nuclear pre-mRNA retention. These data reveal interesting similarities and unexpected differences between the yeast and mammalian splicing systems.

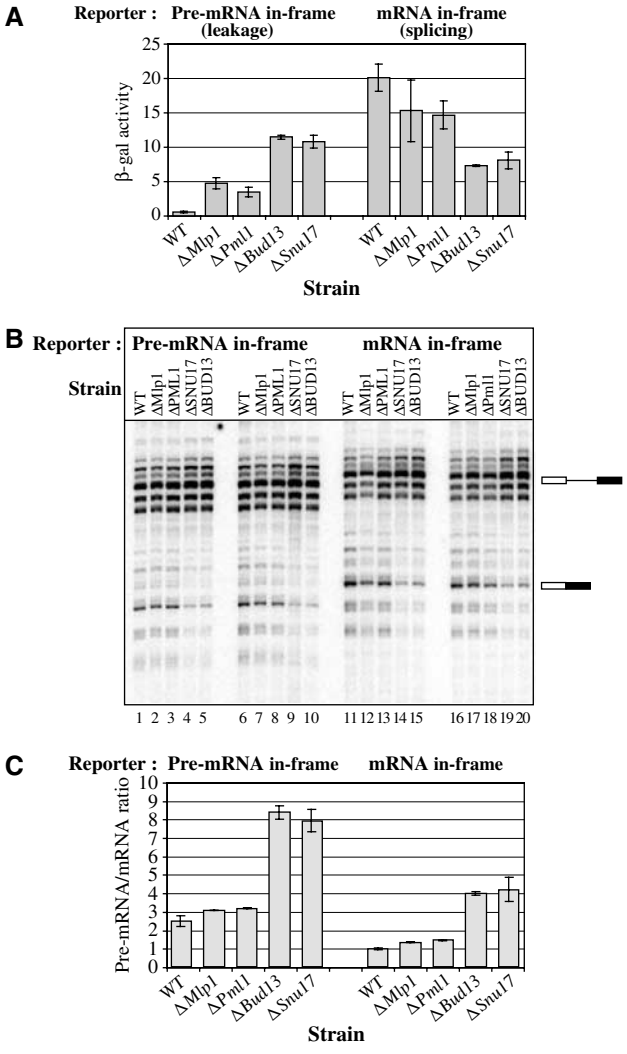

Figure 8 RES subunits are involved in nuclear pre-mRNA retention *in vivo*. Phenotypes of Δ*snu17*, Δ*bud13* or Δ*pml1* were analysed using reporters containing a synthetic intron reporting pre-mRNA leakage or splicing ('pre-mRNA in-frame' and 'mRNA in-frame', respectively). Isogenic wild-type and Δ*Mlp1* strains were used as controls. (**A**) β-Galactosidase activity of pre-mRNA retention reporters. Activity is expressed as a % of the activity of a construct containing no intron. (**B**) Primer extension analysis of the RNA produced by the reporters containing the pre-mRNA and mRNA in-frame. The experiment was made in duplicate using two independent transformants. Owing to multiple transcription initiation sites of the inducible GAL promoter, mRNAs appear as multiple bands. Positions of pre-mRNA and mRNA are indicated. (**C**) Quantification of the pre-mRNA/mRNA ratio from the primer extension. Error bars are calculated from the analysis of independent transformants.

Composition, organization and function of SF3 complexes

TAP purification and mass spectrometry analyses were used to determine the composition of the SF3a and SF3b complexes. Our data indicate that yeast SF3a contains only three subunits: Prp9p, Prp11p and Prp21p, with no trace of other partners. Thus, this complex appears to be homologous to its human counterparts (Table I).

Our results revealed the presence of two new subunits in yeast SF3b; it is thus composed of six proteins: Rse1p, Hsh155p, Cus1p, Hsh49p, Rds3p and Ysf3p. All of them are homologous to human SF3b subunits (Table I). However, human SF3b contains an additional protein, p14, which was shown to crosslink to the branchpoint adenosine (Query *et al*, 1997; Will *et al*, 2001). It was previously suggested that Snu17p is the yeast orthologue of p14 and a subunit of SF3b (Gottschalk *et al*, 2001; Wang and Rymond, 2003). Our data strongly argue against this conclusion, for several reasons:

(1) TAP purification of SF3b, using any of the six tagged subunits, failed to reveal a protein migrating at the position expected for Snu17. Furthermore, mass spectrometry analyses failed to identify this factor.
(2) TAP purification of Snu17p reveals that it belongs to a new splicing complex: RES and none of the characteristic SF3b subunits copurified with it. The presence of an unaccessible fraction of Snu17 in SF3b is unlikely because more than 80% of Snu17 TAP can be retained on an IgG column (data not shown). Furthermore, quantitative Western blotting indicates that SF3b and RES subunits are present in roughly similar quantities (Ghaemmaghami *et al*, 2003).
(3) Tagged Snu17p was unable to precipitate U2 snRNA, a characteristic feature of SF3b subunits.
(4) *SNU17* deletion indicates that, like other RES subunits but contrasting with SF3b subunits, it is not an essential gene.
(5) *SNU17* inactivation did not lead to commitment complex accumulation, as for SF3b mutants, but to a later-stage defect in the splicing process.
(6) Snu17p is more similar to the human CGI-79 protein than to p14. Furthermore, a true p14 orthologue is not found in *S. cerevisiae*.

Given this compelling evidence, why was Snu17 suggested to be a yeast SF3b subunit? First, Northern blot analysis of RNAs co-precipitating with Snu17 suggested a weak specific association with U2 snRNA (Gottschalk *et al*, 2001). However, the large size of U2 snRNA and differences in probe specific activity were not taken into account to correct the strong U2 signal before concluding that U2 snRNA was more specifically precipitated than other snRNAs (see Figure 2 in Gottschalk *et al*, 2001). It is conceivable that spliceosomes, rather than free snRNPs, were detected. Second, based on this result, Wang and Rymond (2003) detected a protein band corresponding in size to Snu17p in a purified SF3b fraction. However, this protein was not identified by mass spectrometry. It may thus simply correspond to a contaminant or a degradation product.

Taken together, the available data are consistent with the fact that yeast SF3b contains only six subunits. All available evidence, including our results on Rds3p and Ysf3p, indicate that SF3b is required for the commitment complex–pre-spliceosome transition as part of the U2 snRNP. Thus, biochemical characterization of Rds3p revealed that it was required for pre-spliceosome formation as part of the yeast SF3b complex (data not shown). It is unclear whether SF3b remains associated with U2 snRNA in the spliceosome throughout the splicing process. For each of the SF3b subunits that we tested, precipitation of the pre-mRNA was efficient but splicing intermediates were only recovered in small amounts and intron lariat was not detectable. This contrasted with core U2 snRNP protein such as Lea1 (Figure 5). This could suggest that SF3b subunits dissociate

from the spliceosome shortly after the first splicing step, during rearrangements leading to the positioning of the 3′ splice site in the catalytic site. However, we cannot formerly rule out that all SF3b subunits are not accessible after the first splicing reaction, even though this seems unlikely. We note that while SF3b subunits were detected in partially purified human spliceosomes blocked after the first catalytic step. However, as this preparation was not homogenous, SF3b may have been associated with the unspliced pre-mRNA still present (Jurica *et al*, 2002). Crosslinking analyses have shown that, in the spliceosome, SF3b subunits bind to the region surrounding the branchpoint, while p14 has been shown to bind to this sequence directly (Staknis and Reed, 1994; Gozani *et al*, 1996; Query *et al*, 1996; McPheeters and Muhlenkamp, 2003). The structural model of human SF3b obtained by cryo-electron microscopy indicates the presence of p14 in the centre of a globular cage-like structure made by the other SF3b subunits (Golas *et al*, 2003). It is thus tempting to speculate that pre-mRNA threads through SF3b, with the branchpoint contacting its central component. Rearrangement in the association of p14/SF3b with the pre-mRNA, allowing the attack of the 5′ splice site by the branchpoint adenosine, is thus also likely to occur before the first transesterification reaction. The absence of a p14 homologue in yeast SF3b can explain some differences between *in vitro* spliceosome formation in the yeast and human systems. Thus, yeast pre-mRNA lacking a functional 5′ splice site is unable to assemble pre-spliceosomes (Séraphin and Rosbash, 1989b) although this occurs in human splicing extracts (Query *et al*, 1997). Thus, due to the presence of p14, mammalian intron recognition may occur through an initial recognition of the branchpoint or the 5′ splice site; however, only the latter pathway would be available to yeast.

A new trimeric complex, RES, is required for efficient splicing and nuclear pre-mRNA retention

Purification of Snu17p-associated proteins identified a new protein complex, RES, containing, in addition to Snu17p, Bud13p and Pml1p proteins. Interaction between Snu17p and Bud13p proteins is also supported by global two-hybrid studies (Uetz *et al*, 2000; Ito *et al*, 2001). Furthermore, disruption of the RES subunit genes generates highly related phenotypes including slow growth and thermosensitivity. This supports their association in a complex. Pre-mRNA co-precipitation, together with *in vitro* and *in vivo* splicing assays, demonstrates that RES is required for efficient splicing. However, inactivation of *PML1* had a weaker effect than *SNU17* or *BUD13* in all the assays that we performed. In this vein, it is noteworthy that inactivation of either *SNU17* or *BUD13* also alters the yeast budding pattern (Ni and Snyder, 2001), possibly by reducing the splicing of transcript(s) encoding key factor(s) implicated in this process. All RES subunits have orthologues in higher eukaryotes (Table I). In addition, the Snu17p and Bud13p human orthologues, CGI-79 and MGC13125, respectively, were identified in a purified spliceosome fraction (Rappsilber *et al*, 2002), supporting their involvement in splicing.

What is the exact function of RES in splicing? Snu17p and RES are clearly not stably associated with the U2 snRNA as proposed previously (Gottschalk *et al*, 2001). *In vivo* and *in vitro* results, as well as the co-precipitation of pre-mRNA, indicate that it acts before the first catalytic step. Interestingly, pre-mRNA precipitation is weak. Furthermore, intermediates and splicing products are not precipitated, suggesting that RES interaction with the spliceosome is transient. As RES is not essential, this interaction is not obligatory either. Thus, RES can be seen as a factor enhancing splicing. This function becomes particularly important for weak introns carrying a poor 5′ splice site, but not for those carrying a weakened branchpoint. Given the similarity between Snu17p and p14, it will be of interest to find out which pre-mRNA and/or snRNA sequence(s) are recognized by the Snu17p RNA-binding domain.

A very interesting characteristic of the RES complex is the pre-mRNA leakage to cytoplasm that occurs in its absence. Moreover, for the Pml1p pre-mRNA leakage, phenotype occurs in the absence of significant splicing defect (at 25°C). By all criteria, Pml1p does not differ from Mlp1p, which has been demonstrated to specifically implicated in nuclear pre-mRNA retention (Galy *et al*, 2004). We conclude that the main function of Pml1p is also nuclear pre-mRNA retention. Interestingly, disruption of *MLP1* similarly to *PML1* generates very mild growth defect. Pml1p contains an FHA domain implicated in binding to phosphothreonine residues (Hammet *et al*, 2000). This raises the interesting possibility that Pml1p could be involved in phophorylation-dependent retention of pre-mRNA in the nucleus. It could thereby play a regulatory role in alternative splicing, particularly for intron carrying a weak 5′ splice site. Further analyses should reveal whether this is indeed the case and whether such feature extends to its mammalian orthologue.

Materials and methods

Yeast strain construction

Gene disruption and tagging on the chromosome were performed using PCR fragments following a published strategy (Puig *et al*, 1998) *SNU17*, *BUD13* and *PML1* genes were disrupted with the *S. pombe* HIS3 marker from pFA-HIS3MX6 (Wach *et al*, 1997) in the haploid strain BMA64-1a (MATa, ura3-1, trp1- 2, leu2-3,112, his3-11, ade2-1, can1-101). *RDS3* and *YSF3* genes were disrupted by integrating *K. tactis* TRP1 marker from pBS1479 in the diploid strain BSY320 (*ade2*, *arg4*, *leu2-3 112*, *trp1-289*, *ura3-52*).

Ysf3 TAP, Snu17 TAP, Bud13 TAP and Pml1 TAP strains were constructed as described previously (Rigaut *et al*, 1999). The TAP Rse1 fusion strain was described previously (Puig *et al*, 2001).The Δ*mlp1* strain was described (Galy *et al*, 2004).

TAP purification

All TAP purifications were performed as described previously from 2 l of culture (Rigaut *et al*, 1999). Purified proteins were concentrated by lyophilization, separated by SDS–PAGE and stained with Coomassie Blue or silver.

Mass spectrometry analysis

Proteins were identified following 'in gel' digestion (Pandey and Mann, 2000; Godovac-Zimmermann and Brown, 2001; Rappsilber and Mann, 2002). Coomassie-stained gels were treated directly, while silver-stained bands were rapidly destained using the Silver Quest decoloration kit (Sigma Aldrich). Digestion was carried out overnight with the addition of 50 ng of trypsin. A MALDI-Tof mass spectrometer (Voyager DE STR, Applied Biosystem) fitted with a pulsed nitrogen laser (337 nm) was used. Mass spectra were acquired in the reflectron mode. The total acceleration voltage was 20 kV, with a grid voltage of 68% and a delay extraction of 240 ns. Close external calibration was realized using a standard peptide mix solution ranging from 573 to 3496 Da (LaserBio Labs, SophiaAntipolis). Samples were prepared in the CHCA matrix at a final concentration of 10 mg/ml in acetonitrile/trifluoroacetic acid (70/0.1%) solution. In all, 1 µl of this sample solution corresponding

to a dilution of 1:2 was then deposited on the MALDI target and dried.

Database scans were performed by using MS Fit and Profound search engines. Protein identifications were obtained with a sequence coverage of 55–86% in average, and mass accuracies of about 15–60 ppm.

In vitro splicing analysis

Splicing reactions were as described before, except that the incubation was performed for 30 min at 25°C (Séraphin *et al*, 1988). Pre-mRNA was generated by *in vitro* transcription of plasmid pBS195 (wild type) or pBS199 (ΔUACUAAC) digested with *Dde*I. Reactions were stopped by addition of 200 µl of PK buffer (0.1 M Tris–HCl (pH 7.5), 12.5 mM EDTA (pH 8.0), 150 mM NaCl, 1% SDS) containing 80 µg of proteinase K (Sigma) and 10 µg of *Escherichia coli* tRNA. After incubation for 20 min at 37°C, RNA was extracted and analysed in a 15% polyacrylamide–7 M urea gel.

For complementation of *in vitro* splicing reaction with purified RES complex, it was TAP purified and dialysed against buffer D (20 mM HEPES-KOH (pH 7.9), 150 mM KCl, 8% glycerol, 0.5 mM phenylmethylsulphonyl fluoride and 0.5 mM dithiothreitol. A volume of 1 µl of purified material (or as control buffer D) was added to 10 µl splicing reactions.

Immunoprecipitation and primer extension

Immunoprecipitation and primer extension were as described previously (Séraphin, 1995). Immunoprecipitation of pre-mRNA was performed similarly: Briefly, 50 µl splicing reactions were preformed. In all, 10 µl of the reaction was extracted and kept as an input, while the remaining 40 µl was diluted in 500 µl of IPP150 buffer (10 mM Tris (pH 8), 150 mM NaCl, 0.1% NP40) and used for immunoprecipitation.

In vivo splicing assays

The Δ*snu17*, Δ*bud13*, Δ*pml1* strains and an isogenic control were transformed with reporters: RP51A wild-type intron (HZ18), 5′II (HZ12) (Jacquier *et al*, 1985), pre-mRNA in-frame (pLG-Nde°Acc°), mRNA in-frame or no intron (pLG-SD5) (Jacquier *et al*, 1985; Rain and Legrain, 1997). Reporters were assayed for β-galactosidase activity at two temperatures, 25 and 37°C. Strains were grown overnight at 25°C in a synthetic medium without uracil containing 2% lactate (pH 5.5), 2% glycerol and 0.05% glucose to an OD_{600} of 0.5–0.8. Cultures were maintained at 25°C or shifted to 37°C for 1 h before a 2 h induction of β-galactosidase. β-Galactosidase activity was tested as described previously (Rutz and Séraphin, 2000). All the experiments were performed in duplicate using two independent transformants. Error bars present standard deviation. Primer extensions to analyse splicing and retention reporters were performed as described previously (Jacquier *et al*, 1985).

Biocomputing

BLAST was used for database searches. Multiple sequence alignments were done with ClustalW. The neighbour-joining tree was computed, excluding positions with gaps and correcting for multiple substitutions using Clustal W (Thompson *et al*, 1994).

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