The yeast U2A'/U2B'' complex is required for pre-spliceosome formation

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Human U2 snRNP contains two specific proteins, U2A' and U2B'', that interact with U2 snRNA stem-loop IV. In *Saccharomyces cerevisiae*, only the counterpart of human U2B'', Yib9p, has been identified. Database searches revealed a gene potentially coding for a protein with striking similarities to human U2A', henceforth called *LEA1* (looks exceptionally like U2A'). We demonstrate that *Lea1p* is a specific component of the yeast U2 snRNP. In addition, we show that *Lea1p* interacts directly with Yib9p. *In vivo* association of *Lea1p* with U2 snRNA requires Yib9p. Reciprocally, Yib9p binds to the U2 snRNA only in the presence of *Lea1p* *in vivo*, even though it has been previously shown to associate on its own with the U2 snRNA stem-loop IV *in vitro*. Strains lacking *LEA1* and/or *YIB9* grow slowly, are temperature sensitive and contain reduced levels of U2 snRNA. Pre-mRNA splicing is strongly impaired in these cells. *In vitro* studies demonstrate that spliceosome assembly is blocked prior to addition of U2 snRNP. This phenotype can be rescued partially, but specifically, by addition of the corresponding recombinant protein(s). This demonstrates a specific role for the yeast U2 snRNP specific proteins during formation of the pre-spliceosome.

Keywords: commitment complexes/*LEA1*/pre-mRNA splicing/*Saccharomyces cerevisiae*/U2 snRNP

Introduction

Pre-mRNA splicing occurs through two transesterification reactions. For nuclear pre-mRNA splicing these reactions take place in a large RNA-protein complex named the spliceosome (Moore *et al.*, 1993). The spliceosome is assembled onto the pre-mRNA by the stepwise addition of small nuclear ribonucleoproteins (snRNPs), which consist of a small nuclear RNA (snRNA) and proteins. In addition, several non-snRNP splicing factors are required for spliceosome assembly and interact transiently with the spliceosome (reviewed in Moore *et al.*, 1993; Krämer, 1996; Will and Lührmann, 1997; Staley and Guthrie, 1998). The ordered pathway of spliceosome assembly has been dissected by following the fate of synthetic RNA substrates *in vitro*. This process appears to be essentially conserved in different species. First, the U1 snRNP recognizes the 5' splice site through base pairing with the 5' end of the U1 snRNA (Rosbash and Séraphin, 1991) leading to the formation of commitment complex 1 in

yeast (CC1) (Séraphin and Rosbash, 1989, 1991). A second form of yeast commitment complex (CC2) is then formed through joining of at least two non-snRNP splicing factors, BBP/SF1 (branchpoint binding protein/SF1; Krämer, 1992; Abovich and Rosbash, 1997) and Mud2p/U2AF65 (Abovich *et al.*, 1994), that recognize the 3' part of the intron. The E complex is likely to be the counterpart of the yeast commitment complexes in metazoans (Michaud and Reed, 1991). These early complexes are the substrates for the ATP-dependent addition of U2 snRNP that leads to formation of the pre-spliceosome. U4/U6-U5 tri-snRNP joins this complex to form the mature spliceosome.

The U2 snRNP plays an essential role in spliceosome assembly with U2 snRNA base-pairing with both the pre-mRNA branchpoint sequence and U6 snRNA (Moore *et al.*, 1993). The human U2 snRNP contains two sets of proteins. A group of seven or eight Sm proteins is shared with other snRNPs including U1, U4 and U5. In addition, the U2 snRNP contains two snRNP specific proteins: U2A' and U2B'' (Lührmann *et al.*, 1990). Biochemical fractionation of HeLa nuclear extracts revealed the presence of an additional form of U2 snRNP. This species, named 17S U2 snRNP, contains nine additional specific proteins and appears to be unstable at high salt concentration (Lührmann *et al.*, 1990; Behrens *et al.*, 1993a,b; Brosi *et al.*, 1993a). Some of these snRNP specific proteins were shown to associate in two subcomplexes: SF3a and SF3b (Brosi *et al.*, 1993b). SF3a is required for pre-spliceosome formation and is conserved from yeast to man (Behrens *et al.*, 1993a; Bennett and Reed, 1993; Brosi *et al.*, 1993a; Legrain and Chapon, 1993; reviewed in Krämer, 1996). SF3b has been only partly characterized; however, some of its subunits have been shown to play specific and essential roles in spliceosome assembly and splicing (Champion-Arnaud and Reed, 1994; Gozani *et al.*, 1996; Wells *et al.*, 1996; Igel *et al.*, 1998; Wang *et al.*, 1998). Surprisingly, the function of the long-known U2A' and U2B'' has remained unclear.

Cloning and sequencing of the cDNA encoding the human U2B'' protein revealed that its sequence is closely related to the U1A protein, a component of the U1 snRNP (Sillekens *et al.*, 1987). Both proteins contain two RNA recognition motifs (RRM) (reviewed by Burd and Dreyfuss, 1994). The N-terminal RRM motif, which is 75% identical between the two proteins is, together with a small number of flanking amino acids, required for binding their cognate snRNAs (Scherly *et al.*, 1990; Bentley and Keene, 1991; Oubridge *et al.*, 1994). The function of the C-terminal RRM, which is the most conserved (86% identity), is still not well understood (Tang *et al.*, 1996). U1A binds to stem-loop II of U1 snRNA while U2B'' interacts with stem-loop IV of U2 snRNA. These two RNA sequences are similar in structure

and sequence. However, whereas U1A binds to its RNA target on its own, U2B'' requires the presence of U2A' for RNA binding (Scherly *et al.*, 1990; Bentley and Keene, 1991). Orthologues of the human U1A and U2B'' proteins have been identified in several eukaryotic species (Polycarpou-Schwarz *et al.*, 1996). Interestingly, the D25/SNF protein of *Drosophila melanogaster* fulfills the function of both U1A and U2B'', and appears to be a hybrid protein. D25/SNF binds to U1 snRNA without an additional factor, but it still requires co-factors (e.g., human U2A') to interact with U2 snRNA *in vitro* (Polycarpou-Schwarz *et al.*, 1996).

The human U2A', which is unrelated in sequence to U1A or U2B'' (Sillekens *et al.*, 1989), is a member of the leucine-rich protein family (Hofsteenge *et al.*, 1988). Members of this family contain tandem repetitions of a motif rich in leucine residues folding in a regular structure that has been proposed to be involved in protein-protein interactions (Kobe and Deisenhofer, 1993). There are six such repeats in human U2A', located at the N-terminus of the protein, followed by a region with no obvious sequence characteristics. To date, a single orthologue of the human U2A' protein has been characterized in *Trypanosoma brucei*. It shows 31% identity and 57% similarity to its human counterpart, the homology being concentrated to its leucine-rich repeats which are located in the N-terminal region (Cross *et al.*, 1993). Although the *Trypanosoma* U2B'' homologue has not yet been described, it is known that interaction of the *Trypanosoma* U2A' with loop IV sequence of U2 snRNA requires additional proteins (Cross *et al.*, 1993).

With the completion of the yeast genome sequence, it has become possible to identify (putative) homologues of human splicing factors using sensitive database searches. This allowed for the identification of yeast Sm proteins (e.g. Séraphin, 1995) as well as some snRNP specific subunits (e.g. U1-70K; Smith and Barrell, 1991). Yib9p, the yeast homologue of human U2B'', was identified in this way as well as through a genetic screen (Voss *et al.*, 1995; Polycarpou-Schwarz *et al.*, 1996; Tang *et al.*, 1996). Yib9p harbors only one RRM. Interestingly, it binds to the yeast U2 snRNA stem-loop IV and the human U1 snRNA stem-loop II *in vitro* (the target of U1A) but interacts only weakly with human U2 snRNA stem-loop IV in the same assay (Polycarpou-Schwarz *et al.*, 1996; Tang *et al.*, 1996). This might result from the higher similarity of yeast U2 snRNA stem-loop IV with human U1 snRNA stem loop II than with its human counterpart. These *in vitro* studies also revealed that Yib9p is able to bind yeast U2 snRNA in a sequence-specific manner in the absence of other protein factors (Polycarpou-Schwarz *et al.*, 1996). The addition of human U2A' and yeast extract did not influence this binding (Tang *et al.*, 1996) suggesting that the Yib9p function is more related to human U1A than human U2B''. These results suggested that the U2A' function might be missing in yeast. Consistent with this, a large-scale two-hybrid screen using Yib9p as a bait failed to uncover a yeast U2A' homologue (Fromont-Racine *et al.*, 1997).

We have used a database search approach to look for a putative homologue of the human U2A' in *Saccharomyces cerevisiae*. This identified an open reading frame coding for a protein with striking sequence similarity to human

U2A' which was therefore named *LEA1* (looks exceptionally like U2A'). Here, we demonstrate that the encoded protein, Lea1p, associates with U2 snRNA in the presence of Yib9p to which it binds directly. Lea1p is therefore a true homologue of the human U2A' protein. Characterization of a *LEA1*-disruption mutant demonstrates that Lea1p is required for splicing *in vivo* and suggests that Lea1p and Yib9p have no independent functions. *In vitro* analyses revealed that these two proteins are specifically required for the transition from CC2 to pre-spliceosome during the splicing process.

Results

Database search for a yeast homologue of human U2A'

To look for a counterpart of human U2A' in *S.cerevisiae*, we searched nucleic acid and protein sequence databases (see Materials and methods) using either the human U2A' or its *Trypanosoma* homologue as a probe. This identified an open reading frame located on chromosome XVI as the best candidate for a U2A' homologue. Pairwise alignment indicated that the yeast protein was 52% similar and 29% identical to human U2A' (data not shown). The similar sizes of the two proteins (238 and 255 amino acids for yeast and human, respectively) further suggested that they might be counterparts. Detailed analysis of the protein alignment revealed that the sequence homology is mainly located in the N-terminal region of the two proteins and corresponds to six leucine-rich repeats (Figure 1). Similar results were obtained with the *Trypanosoma* homologue of human U2A' (Figure 1). It is noteworthy that in database searches with either the human or the *Trypanosoma* protein, the yeast protein gave a much stronger score than any other yeast polypeptide containing leucine-rich repeats. This indicated that sequence similarity was not solely due to the presence of these repeats. As this putative yeast U2A' homologue looks exceptionally like U2A', it was named *LEA1*. Database searches revealed several other putative human U2A' homologues in *Salmo salar* (81% identical to human U2A') and *Arabidopsis thaliana*, and two partial, but highly related sequences in the worms *Onchocera volvulus* and *Brugia malayi*. Multiple sequence alignment of divergent members of this protein family (i.e. excluding *S.salar* and *B.malayi* which are highly related to human and *O.volvulus* proteins, respectively; Figure 1) indicated that several amino acids were conserved in all proteins. Some of those corresponded to highly conserved residues of the leucine-rich repeats. However, others were found in variable positions of the leucine-rich repeats or upstream of the repeats. The presence of conserved residues in the latter subset of positions strongly suggest that these proteins are homologues rather than unrelated members of the leucine-rich protein family.

Lea1p is specifically associated with the U2 snRNA

To test whether Lea1p is a component of yeast U2 snRNP, we inserted a cassette coding for two IgG-binding units of the *Staphylococcus aureus* protein A (Puig *et al.*, 1998) downstream of, and in-frame with, the *LEA1*-coding sequence. Splicing extracts were prepared from the resultant strain and used in an immunoprecipitation experiment.

Fig. 1. Sequence alignment of the N-terminal regions of human and trypanosoma U2A' with putative homologues. Amino acid identities and conserved substitutions are highlighted. The six tandem leucine-rich repeats, defined according to Cross *et al.* (1993), are indicated by numbered boxes.

Extracts from isogenic wild-type and SmB-ProtA tagged strains were used as controls. Western blot analysis revealed that the Leal-ProtA and SmB-ProtA were present at similar levels in the corresponding input and pellet fractions while they were undetectable in the supernatant fractions (data not shown). This indicated that the proteins were stable for the duration of the experiment and efficiently immunoprecipitated. RNAs extracted from the various fractions were analyzed by primer extension for the presence of the five spliceosomal snRNAs (Figure 2). All snRNAs were efficiently immunoprecipitated by the SmB-ProtA protein (Figure 2, lane 8), consistent with other observations (J.Salgado-Garrido, E.Bragado-Nilsson, S.Kandels-Lewis and B.Séráphin, unpublished results). The Leal-ProtA fusion specifically coprecipitated the U2 snRNA (Figure 2, lane 9, compare with background in lane 7). Trace amounts of U5 and U6 snRNAs could be detected in the Leal-ProtA IgG-beads pellet after prolonged exposure. This could be due to the presence of low amounts of splicing complexes in the extract. Approximately 50% of the U2 snRNA present in the starting extract was recovered in the pellet (Figure 2, lanes 6 and 9). This suggests that only a fraction of U2 snRNA is associated with Lealp. Alternatively, dissociation of the Leal-ProtA fusion from a fraction of the U2 snRNP might have occurred during extract preparation and/or immunoprecipitation. In any case, these results demonstrate that Lealp associates directly or indirectly, and specifically, with the U2 snRNA.

LEA1 disruptants are temperature-sensitive

The *LEA1* gene was disrupted by replacing its coding sequence with a *Kluyveromyces lactis* *URA3* gene in a diploid strain. After sporulation, tetrads were dissected. All intact tetrads gave rise to four viable spores at 30°C (data not shown). However, we noticed that two spores per tetrad grew slower (data not shown). These spores carried the *URA3* marker indicating that strains lacking *LEA1* ($\Delta lea1$) were viable but impaired for vegetative growth at 30°C. This result was somewhat surprising because disruption of the *YIB9* gene encoding the yeast U2B" protein was reported to confer no growth phenotype (Tang *et al.*, 1996). This situation might be explained by functional differences between *YIB9* and *LEA1* or by a

Fig. 2. Coimmunoprecipitation of snRNAs by Leal-ProtA. Extracts from a control wild-type yeast strain (WT), a strain expressing SmB-ProtA and a strain harboring Leal-ProtA were immunoprecipitated with IgG-agarose beads. The extracted RNAs of input (lane 1–3), supernatant (lane 4–6) and pellet (lane 7–9) fractions were analyzed by primer extension with specific primers for U1, U2, U4, U5 and U6 snRNAs. Position of the corresponding signals are shown on the left. RNA from five times more extract was used for the pellets relative to the input and supernatant fractions.

difference in the strain backgrounds. We therefore repeated the disruption of the *YIB9* gene in our standard strain ($\Delta yib9$) and also constructed an isogenic strain carrying both gene disruptions ($\Delta lea1 \Delta yib9$). All strains were viable, allowing us to compare their growth behavior. The strains were grown in liquid media at different temperatures and cell density was measured at various

Fig. 3. Phenotype of *LEA1*-disrupted yeast strains. (A) Growth behavior of *LEA1*- (Δ *lea1*), *YIB9*- (Δ *yib9*) or *YIB9*- and *LEA1*- (Δ *lea1* Δ *yib9*) disrupted yeast strains compared with a wild-type control strain (WT) at 37°C. The arrow indicates the transfer from 30 to 37°C. (B) U2 snRNA levels in *LEA1*-disrupted yeast strains. U2 and U4 snRNA from extracts derived from Δ *lea1*, Δ *yib9* and Δ *lea1* Δ *yib9* strains were analysed by primer extension.

time points. Compared with an isogenic wild-type strain, the three mutant strains grew with the same reduced rate at 30°C (1.3-fold reduction; data not shown). This phenotype was more pronounced at 16°C, where the mutant strains grew at half the rate of the wild-type strain (data not shown). At 37°C the three mutant strains stopped growing after ~23 h of incubation (Figure 3A). Similar phenotypes were observed on solid media (data not shown). These results indicate that disruption of the *LEA1* gene is not lethal but confers a slow growth phenotype, particularly at low temperatures, as well as a thermosensitive lethal phenotype. Similar phenotypes were observed following disruption of the *YIB9* gene. Interestingly, the growth of the strain disrupted for both the *LEA1* and *YIB9* gene was identical to the growth of the two single disruptants (Figure 3A) suggesting that the two proteins have no independent function.

***Lea1p* is required for normal accumulation of the U2 snRNA**

As *Lea1p* is associated with the U2 snRNA (see above), we next analyzed the effect of *LEA1* and/or the *YIB9*

disruptions on the level of U2 snRNA. Total RNA was extracted from the various disrupted strains grown at 30°C and analyzed by primer extension. This revealed reduced levels of U2 snRNA in the mutant strains compared with an isogenic wild-type control (Figure 3B). In contrast, the levels of U4 snRNA (Figure 3B) and of the other spliceosomal snRNAs (data not shown) were not affected by the disruption of the *LEA1* and/or *YIB9* gene. The three mutant strains had a 2.5-fold lower level of U2 snRNA compared with the isogenic wild-type strain. A comparable reduction of U2 snRNA has been reported previously for *YIB9* (Tang *et al.*, 1996). These data demonstrate that *Lea1p* and *Yib9p* are specifically required for U2 snRNA accumulation, as expected for snRNP proteins. *Lea1p* and *Yib9p* have identical effects on U2 snRNA level when disrupted alone or in combination, suggesting again that the two proteins have no independent function.

***Lea1p* association with U2 snRNA is *Yib9p*-dependent**

The results presented above indicate that *Lea1p* is a component of the U2 snRNP. In the mammalian system, the simultaneous presence of U2B'' and U2A' (the homologues of *Yib9p* and *Lea1p*, respectively) is required for interaction with U2 snRNA *in vitro*. In yeast, however, *Yib9p* was shown to interact with U2 snRNA on its own *in vitro* (Polycarpou-Schwarz *et al.*, 1996; Tang *et al.*, 1996). We therefore decided to test the requirement for *Yib9p* and *Lea1p* interaction with U2 snRNA *in vivo*. To examine whether *Lea1p* can interact with U2 snRNA in the absence of *Yib9p*, the *YIB9* gene was disrupted in the strain harboring the *Lea1*-ProtA fusion (Δ *yib9* *Lea1*-ProtA). Conversely, to test whether *Yib9p* can interact with U2 snRNA in the absence of *Lea1p*, a plasmid carrying a ProtA-*Yib9* fusion (Polycarpou-Schwarz *et al.*, 1996) was introduced in the strain carrying the *LEA1* gene disruption. Extracts were prepared from these two strains. Strains expressing the same tagged proteins in a non-mutated background and an isogenic wild-type were used as controls. Aliquots of these extracts were mixed with a similar volume of an extract containing the ProtA-Pop1 fusion, and the mixtures were incubated with IgG-agarose beads. Pellets of material bound to the beads were recovered and analyzed for their protein and RNA contents. The level of ProtA-Pop1 detected by Western blotting was similar in all lanes, indicating that all pellets had been efficiently recovered. Similar amounts of *Lea1*-ProtA were detected in the immunoprecipitates from the *Lea1*-ProtA and Δ *yib9* *Lea1*-ProtA extracts (Figure 4A, lanes 2 and 4). This result indicates that *Lea1p* is stable in the absence of *Yib9p*. Likewise, the similar levels of ProtA-*Yib9* in pellets derived from extracts carrying or lacking *Lea1p* indicate that *Yib9p* was stable in the absence of *Lea1p*. A comparison of the levels of proteins present in input and pellet fractions also indicated that all proteins were efficiently precipitated (data not shown). Primer extension analysis revealed that U2 snRNA was efficiently precipitated by the tagged *Lea1p* only in the presence of *Yib9p* (Figure 4B, compare lanes 2 and 4). Surprisingly, *Yib9p* could also only coprecipitate U2 snRNA in the presence of *Lea1p* (Figure 4B, compare lanes 3 and 5). The lack of U2 snRNA signal in immunoprecipitates from

Fig. 4. Lea1p interacts with Yib9p and this interaction is required for association with U2 snRNA. (A) Extracts from a wild-type strain (WT) (lane 1), a strain with the integrated LEA1-ProtA construct (lane 2), a wild-type strain with the plasmid coding for the YIB9-ProtA fusion (lane 4), the YIB9-disrupted strain with the integrated LEA1-ProtA construct (lane 3) ($\Delta yib9$ LEA1-ProtA) or the LEA1-disrupted strain transformed with the plasmid harboring the YIB9-ProtA fusion ($\Delta lea1$ ProtA-Yib9) (lane 5) mixed with an extract harboring ProtA-Pop1 (all lanes), were immunoprecipitated with IgG-agarose beads. Proteins present in the pellet were analyzed by Western blotting. The signals corresponding to the different ProtA-tagged proteins are shown on the left while a molecular size marker is depicted on the right. (B) RNA was extracted from pellets described in (A) (lanes 1–5) and the presence of U2 snRNA and RNase MRP RNA (labeled MRP RNA; loading control) was assayed by primer extension. The lane numbers correspond to those in (A). (C) Interaction of Lea1p and Yib9p *in vitro*. Coprecipitation experiments were performed with Lea1p and Yib9p fused to either a His or GST using glutathione-agarose beads. GST alone and His-SmX₆ served as negative controls. In lanes 1–6 the different proteins used were loaded according to their molecular size. Lanes 8–14 show the different combinations tested. The proteins were visualized by Coomassie staining.

the cells carrying the *LEA1* or *YIB9* disruption cannot be explained by the loss of the corresponding RNA pellets. Indeed, similar levels of the MRP RNA that associate with the Pop1-ProtA (Lygerou *et al.*, 1994) were recovered in all samples. We therefore conclude that Lea1p and Yib9p need to be present simultaneously to allow stable interaction with U2 snRNA *in vivo*.

Lea1p interacts specifically and directly with Yib9p

The data presented above suggest a significant similarity between the yeast Lea1p and Yib9p proteins and human U2A' and U2B''. These latter two proteins have been shown to interact directly. To test whether this is also the case for Yib9p and Lea1p, we inserted the corresponding coding sequences in vectors suitable to test for protein-protein interaction in the yeast two-hybrid assay (Fields and Song, 1989). High levels of β -galactosidase activity indicated that Lea1p and Yib9p are interacting in this assay (Table I). This interaction was specific, as no homotypic binding of Lea1p or Yib9p or interaction with other snRNP proteins used as controls (SmE and SmG) could be detected (Table I). To confirm this result and show direct physical interactions between Lea1p and Yib9p, we conducted an *in vitro* binding study. We independently expressed recombinant Lea1p and Yib9p proteins fused to either a hexa-histidine (His) or glutathione S-transferase tag (GST). Aliquots of *E. coli* lysates containing the GST-Lea1p and GST-Yib9p, as well as a control lysate harboring only GST, were mixed with purified His-Lea1p, His-Yib9p or His-SmX₆ proteins. After incubation with glutathione-agarose beads, the proteins specifically bound to the support were released, fractionated on a denaturing gel and detected by Coomassie staining (Figure 4C). The His-Lea1p protein was specifically coprecipitated by the GST-Yib9p fusion (Figure 4C, lane 11) but not by GST alone or GST-Lea1p (Figure 4C, lanes 8 and 14). Conversely, the His-Yib9p fusion coprecipitated with GST-Lea1p (Figure 4C, lane 13) but not with GST or GST-Yib9p (Figure 4C, lanes 7 and 10). Further evidence for the specificity of these coprecipitations comes from the analysis of the His-SmX₆ protein that was not recovered with either of the two GST fusion proteins (Figure 4C, lanes 9 and 12). These results demonstrate that Lea1p and Yib9p are interacting directly and specifically with each other in the absence of other factors including U2 snRNA.

LEA1 disruption affects splicing *in vivo*

Next, we analyzed whether Lea1p or Yib9p affected splicing *in vivo*, because the role of their human counterparts, U2A' and U2B'', in splicing is unknown. For this purpose, strains deleted for the *LEA1* gene, the *YIB9* gene or an isogenic wild-type control were transformed with a reporter plasmid containing the RP51A intron inserted into the *lacZ* coding sequence or an empty control vector (Teem and Rosbash, 1983). Transformants were grown at 30°C, the reporter gene was induced and total RNA was extracted. The levels of pre-mRNA, mRNA and lariat intermediate were determined by primer extension (Figure 5). The wild-type strain contained high levels of mRNA and low levels of pre-mRNA (Figure 5, lane 4). In contrast, cells lacking Lea1p or Yib9p harbored reduced levels of mRNA and accumulated pre-mRNA (Figure 5,

Table I. Yeast two-hybrid assay for Lea1p and Yib9p protein interaction

	pAS2 ^{ΔΔa}	Lea1p ^a	Yib9p ^a	SmE ^a
pACTII ^b	0.025 ± 0.004	0.018 ± 0.004	0.012 ± 0.002	0.032 ± 0.015
Lea1p ^b	0.023 ± 0.001	0.026 ± 0.006	55.8 ± 48.3	0.11 ± 0.007
Yib9p ^b	0.030 ± 0.004	12.7 ± 4.4	0.030 ± 0.005	0.027 ± 0.008
SmG ^b	0.027 ± 0.001	0.024 ± 0.001	0.020 ± 0.003	10.0 ± 9.1

^aConstructs with a Gal4p DNA-binding domain (pAS2^{ΔΔ}-derivatives).
^bConstructs with a transactivation domain (pACTII-derivatives).
The standard error was calculated from two independent experiments. Significant activities are shown in bold.

Fig. 5. *LEA1* disruption affects splicing *in vivo*. A reporter construct (reporter) or a plasmid lacking the reporter construct (vector) were introduced into a wild-type (WT), a *Δlea1* or a *Δyib9* strain. RNA was extracted and splicing was analyzed by primer extension using the exon 2 specific EM38 primer. The reporter construct contains the wild-type RP51A intron inserted into the β-galactosidase coding sequence. The different extension products are labeled on the right of the figure. Signals were quantified using a PhosphorImager. The ratio between mRNA and pre-mRNA was calculated and is indicated below the figure (M/P). Signals in control lanes 1–3 were not quantified.

lanes 5 and 6). Assaying for the β-galactosidase produced by these reporters confirmed this result (data not shown). Quantification of the extension products revealed that the ratio of mRNA to pre-mRNA (a measurement of splicing efficiency; Pikielny and Rosbash, 1985) was reduced ~20-fold in the mutant strains (M/P; Figure 5, bottom). The ratio of lariat intermediate to pre-mRNA was reduced by the same proportion while the ratio of mRNA to lariat intermediate was similar for the three strains, indicating that the first splicing step was specifically affected (Fouser and Friesen, 1986). We conclude that *Lea1p* and *Yib9p* are required for an efficient first step of splicing *in vivo*.

Lea1p is required for spliceosome assembly

To define the nature of the splicing block conferred by the absence of *Lea1p* (and *Yib9p*), we analyzed spliceosome

Fig. 6. Analysis of spliceosome assembly. Splicing extracts from wild-type, *Δlea1*, *Δyib9* and *Δlea1 Δyib9* strains were tested for splicing complex formation. Complexes were assembled on a radioactively labeled wild-type pre-mRNA and fractionated by native gel electrophoresis. In lanes 1–4 ATP was depleted by addition of glucose and in lanes 5–8 additional exogenous ATP was added. S indicates the position of migration of the pre-spliceosome and spliceosome in this system; CC1 and CC2 indicates the commitment complexes 1 and 2; U indicates the free probe and unspecific protein–pre-mRNA complexes (Séraphin and Rosbash, 1989).

assembly *in vitro*. Splicing extracts prepared from wild-type, *Δlea1*, *Δyib9* and *Δlea1 Δyib9* strains were incubated with a radioactively labeled pre-mRNA in either the presence or absence of ATP, and splicing complex formation was assayed by native gel electrophoresis (Figure 6). In the absence of ATP, CC2 accumulated in a wild-type extract (Figure 6, lane 1) as reported previously (Séraphin and Rosbash, 1989). In these conditions, CC2 also formed efficiently in extracts prepared from the mutant strains, indicating that the absence of *Lea1p* or *Yib9p* did not affect early splicing complex formation (Figure 6, lane 2–4). This is consistent with the observation that U2 snRNP is not required for commitment complex assembly (Séraphin and Rosbash, 1989). In the presence of ATP, spliceosome assembled efficiently in the extract prepared from a wild-type strain (Figure 6, lane 5). Interestingly, extracts prepared from the mutant strains were consistently unable to form spliceosome under these conditions (Figure 6, lanes 6–8) and accumulated CC2. A similar observation was made independently following the disruption of *YIB9* (Tang *et al.*, 1996). The absence of spliceosome in the mutant extracts suggested that *Lea1p* and

Fig. 7. Rescue of pre-spliceosome formation. Splicing extracts prepared from the *LEA1*-disrupted yeast strain ($\Delta lea1$) (lane 5) were complemented with either recombinant Lea1p (lane 6), Yib9p (lane 7), both recombinant proteins (lane 8) or with a splicing extract derived from a *YIB9*-disrupted yeast strain ($\Delta yib9$) (lane 9). In lanes 1 and 2, control reactions using wild-type extracts, without and with ATP, respectively, were loaded to indicate the position of migration of the various splicing complexes. Complex formation by the recombinant Lea1p or Lea1p plus Yib9p was tested in lanes 3 and 4, respectively. Complexes are labeled as in Figure 6.

Yib9p affect addition of U2 snRNP to CC2. An early block in spliceosome assembly is consistent with our *in vivo* observation that Lea1p is required for the first splicing step. However, the *in vitro* effect could be indirect (e.g. due to the reduced level of some factors in the mutant extracts) or directly related to the function of Lea1p or Yib9p.

Lea1p and Yib9p have a specific role during spliceosome assembly

To test these possibilities, we assessed whether addition of recombinant Lea1p to an extract prepared from the corresponding mutant strain restored spliceosome formation (Figure 7). As described above, in an extract lacking Lea1p CC2 accumulated in the presence of ATP (Figure 7, lane 5, compare with wild-type extract in the presence or absence of ATP, lanes 1 and 2). Addition of recombinant Lea1p to this extract restored spliceosome assembly (Figure 7, lane 7). Lea1p did not affect RNA mobility by itself (Figure 7, lane 3) indicating that we observed bona fide spliceosomes. Restoration was specific as addition of Yib9p instead of Lea1p was unable to restore spliceosome assembly (Figure 7, lane 6). The restoration was partial, however, as a significant level of CC2 was still present (Figure 7, lane 7, compare with lane 2) at all concentrations of recombinant protein added (His-Lea1p or GST-Lea1p; data not shown). We therefore tested whether addition of Yib9p together with Lea1p would improve the spliceosome assembly. This was not the case (Figure 7, lane 8). We noted that mixing extracts lacking either Lea1p or Yib9p also resulted in a similar low level of spliceosome formation (Figure 7, lane 9). This suggests that essential splicing factors, beside Lea1p and Yib9p, are present at reduced levels in these extracts and account for the low level of spliceosome assembly that we observe. The missing activ-

ity is not associated with Lea1p nor Yib9p as addition of excess of one or both of these proteins to the mutant extracts did not restore full spliceosome formation. Furthermore, our experiments have shown that Lea1p is stable in the absence of Yib9p and vice versa (Figure 4). It is likely that the missing factor(s) is unstable in the strains lacking Lea1p and/or Yib9p. Candidates for the missing factor(s) include U2 snRNA (Figure 3) and/or a U2 snRNP protein(s) (see Discussion). In any case, our observation that Lea1p partially restores the spliceosome assembly activity of an extract lacking this factor demonstrates that Lea1p has a specific role during the CC2 to pre-spliceosome transition. We have reached a similar conclusion for Yib9p as the recombinant protein can restore spliceosome formation when added in an extract prepared from the corresponding disruption strain (data not shown). Lea1p was inactive in this situation, demonstrating again the specificity of these complementations. Additionally, in splicing extracts lacking both proteins, we could only restore spliceosome formation by the addition of the two proteins (data not shown). We therefore conclude that the two U2 snRNP specific proteins Lea1p and Yib9p are required for the efficient addition of U2 snRNP onto the pre-mRNA.

Discussion

We have identified a yeast protein that is similar to human and *Trypanosoma* U2A'. Our studies demonstrate that this protein, Lea1p, is indeed the yeast homologue of U2A'. We have investigated the function of this protein and its interaction partner Yib9p *in vivo* and could demonstrate a specific role for these snRNP proteins during spliceosome assembly *in vitro*.

Our database search revealed that Lea1p was highly related to human U2A' and that the sequence similarity was stronger than expected had only the leucine-rich repeats been conserved. This strongly suggested that Lea1p was the yeast U2A' homologue. This hypothesis was confirmed experimentally by showing that Lea1p is associated specifically with the U2 snRNA. Lea1p also interacts specifically and directly with Yib9p, in the same way as human U2A' interacts with U2B'. It was somewhat surprising to find a yeast U2A' homologue as previous studies had shown that Yib9p was able, unlike its mammalian counterpart, to bind specifically to its cognate RNA by itself *in vitro*. Furthermore, Lea1p was not recovered in a saturating two-hybrid screen using Yib9p as the bait (Fromont-Racine *et al.*, 1997), even though the two proteins do interact in this assay (see above). Therefore, one could have anticipated that yeast did not require a U2A' function. However, our studies demonstrate that Yib9p is unable to interact with yeast U2 snRNA *in vivo* in the absence of Lea1p. It is probable that the apparently contradictory results of the *in vitro* and *in vivo* assays reflect differences in the two systems such as protein concentrations or the presence of competing RNA and proteins *in vivo*. Lea1p probably increases the affinity of Yib9p for RNA. This observation reinforces the similarity between the yeast and mammalian proteins and confirms that Lea1p is the true yeast U2A' homologue.

Disruption of the *LEA1* gene conferred a slow growth phenotype $\leq 30^\circ\text{C}$ and a lethal phenotype at 37°C . This

was unexpected because disruption of the *YIB9* gene was reported to have no associated phenotype. We have repeated the *YIB9* disruption in our strain. This resulted in growth phenotypes identical to those observed for the *LEA1* disruption. It is possible that these results can be explained by differences in the strain backgrounds. However, this is highly unlikely as the original disruption was made in a strain closely related to ours (Tang *et al.*, 1996; see Material and methods). It is therefore more likely that the growth phenotype of the *YIB9* disruption was not noticed during the original study. *YIB9* is synthetic lethal with *MUD2* (Tang *et al.*, 1996). We have not tested whether this is also the case for *LEA1*, but it is likely given the similar phenotypes of the *LEA1* and *YIB9* disruptants. Indeed, in all experiments that we performed, Yib9p and Lea1p behaved identically and produced indistinguishable phenotypes. Furthermore, the double mutant was not more severe than each single mutant. It is noteworthy that the U2B''/Yib9p-binding site of U2 snRNA is not essential for splicing in *Xenopus* oocytes or yeast extracts (Hamm *et al.*, 1989; McPheeters *et al.*, 1989; Pan and Prives, 1989). *In vivo* yeast U2 snRNA lacking this region generates a slow growth phenotype at 18, 23, 30 and 37°C (Shuster and Guthrie, 1990). These results suggest that the functional unit is the Lea1p–Yib9p complex bound to U2 snRNA and that Lea1p and Yib9p have no additional independent functions. If this is truly the case, the conservation of Lea1p suggests that it is not required only for tightening U2B'' binding to U2 RNA, since evolution would probably have selected U2 snRNA or U2B'' variants with high affinity for each other. Lea1p is probably widely evolutionarily conserved because it interacts, together with Yib9p, with other factors. It is unclear why the *YIB9* and *LEA1* genes did not become fused during evolution if the functional unit is a heterodimer. One possibility is that topological constraints prevent such an event from occurring. One will be able to assess this possibility once the structure of the U2A'/U2B'' complex is known. However, it is also possible that the two genes did not fuse because the formation of heterodimers provide a target step for the control of snRNP assembly, as it is highly sensitive to changes in the concentrations of the two subunits. Consistent with this hypothesis, our results support a role for Lea1p and Yib9p in snRNP stability. Indeed, the level of U2 snRNA was reduced 2.5-fold in the disruption mutant. Similar results had been obtained previously for Yib9p (Tang *et al.*, 1996; also see above) suggesting that the binding of these two proteins stabilizes the U2 snRNA *in vivo*. This process could therefore be important to control snRNP levels *in vivo*. However, at this stage, we cannot rule out the possibility that Lea1p and Yib9p are involved in transcriptional control of the U2 snRNA gene rather than, or in addition to, controlling its stability. Apart from their effect on U2 snRNA levels, we have shown that Lea1p and Yib9p are required for efficient splicing *in vivo* (Figure 5; data not shown). It is unclear which of these two phenotypes cause the growth defect of the mutant strains. *In vivo* depletion experiments have demonstrated that yeast cell growth was not affected by an ~10-fold reduction in U2 snRNA (Sraphin and Rosbash, 1989). In addition, we were unable to suppress the thermosensitive phenotype conferred by the *LEA1* or *YIB9* disruption by

U2 snRNA overexpression (data not shown). It is therefore likely that reduced splicing of some essential transcript rather than the lower U2 snRNA level causes the slow growth phenotype. Under the additional stress of high temperature, the reduced splicing activity may not be sufficient to support growth.

Further evidence for an important role of Lea1p and Yib9p in splicing came from the analysis of *in vitro* spliceosome assembly. In extracts lacking Lea1p or Yib9p, commitment complexes assembled efficiently, but the formation of pre-spliceosome was impaired (Figure 6; Tang *et al.*, 1996). We demonstrate that these effects reflect the activity of Lea1p and Yib9p because spliceosome assembly can be rescued by recombinant proteins (Figure 7; data not shown). It is noteworthy that the homologous U2A' and U2B'' proteins from higher eukaryotes have been known for years but no specific function for these proteins has yet been described. While we were able to demonstrate a specific role for Lea1p and Yib9p during spliceosome assembly using a complementation assay, we were never able to fully restore spliceosome formation in the depleted extracts. This is unlikely to be due to the level or the quality of the protein added as this result was obtained with an excess of protein over the endogenous protein concentration and using several preparations of four different recombinant proteins (Figure 7; data not shown). The presence of an inhibitor in the depleted extracts is also unlikely because wild-type extract could efficiently restore spliceosome formation (data not shown). One possibility is that Lea1p and Yib9p do not easily form heterodimers under these conditions. Another is that an additional factor(s) besides Lea1p and/or Yib9p is present at reduced concentration in extracts prepared from the disrupted strains. This interpretation is supported by the low level of spliceosome assembling in a reaction where both a Lea1p- and a Yib9p-depleted extract were mixed together (Figure 7, lane 9). Although one could argue that this partial complementation is due to the halved levels of Lea1p and Yib9p in the final reaction, this is unlikely because full spliceosome assembly in each extract cannot be restored by addition of both recombinant proteins (e.g. Figure 7, lane 8) suggesting that one or more factors, beside Lea1p and Yib9p, is likely to be present at reduced levels in these extracts. Since *in vivo* the U2 snRNA level is reduced 2.5-fold compared with extracts prepared from a wild-type strain (Figure 3), U2 snRNA might be the key factor. To test whether this was the case, we artificially decreased the U2 snRNA concentration in extracts by adding various amounts of a DNA oligonucleotide complementary to U2 snRNA. This oligonucleotide promotes the specific degradation of U2 snRNA with the help of an RNase H activity present in the extracts (McPheeters *et al.*, 1989). We assayed U2 snRNA levels (by primer extension) and spliceosome assembly in these various situations. This revealed that a 2.5-fold reduction of U2 snRNA had only a slight negative effect on the level of spliceosome formed (data not shown). From this result we conclude that U2 snRNA could contribute to, but is probably not the only factor limiting and essential for spliceosome assembly in extracts lacking Lea1p. There is likely to be still another splicing factor besides U2 snRNA involved in this process. The reduced level of this factor would become limiting for spliceosome formation in

reactions containing a *Lea1p*- and/or *Yib9p*-deprived extract complemented with the cognate recombinant protein. It is likely that this factor would normally interact with *Lea1p* and or *Yib9p*. Further study will be aimed at the identification of this factor.

Materials and methods

Strains

For the database searches, WU-BLAST2 (W.Gish, unpublished) and the GCG package (Devereux *et al.*, 1984) were used. The multiple sequence alignment was done with Clustal_X (Thompson *et al.*, 1997). The DDBJ/EMBL/GenBank accession numbers of the corresponding nucleic acid sequences are as follows: X13482; X69137; AA618819; X69934; Z73569; AA228180; AJ004824.

The wild-type strains used in all experiments are either the haploid strain MGD453-13D (Séraphin *et al.*, 1988) or the isogenic diploid strain BSY320. All yeast transformations were performed as described previously (Soni and Carmichael, 1993).

To tag *Lea1p* (*Lea1*-ProtA) and *SmB* (*SmB*-ProtA), the sequence coding for two IgG-binding domains of the *S.aureus* protein A (ProtA) together with the *Kluyveromyces lactis* *URA3* marker (*KL-URA3*; Langle-Rouault and Jacobs, 1995) were inserted in the genome downstream of, and in-frame with, the *LEA1* and *SmB* open reading frames by transformation with PCR fragments (Baudin *et al.*, 1993; Puig *et al.*, 1998). For *LEA1*, this fragment was generated by amplification from plasmid pBS1365 with oligonucleotides: FC1-1 (5'-CTTCTTTAGAA-GAGATTGCCAGGCTGGAAAACACTACTCTCTGGTGGTGTAAAGC-TGGAGCTCAAAAC-3') and FC1-2 (5'-TTTCGTTTATATATTATT-ATATAATGGCACAGAATAATTATAATTCTTACGACTCACTAT-AGGG-3'). The PCR product was transformed into the strain MGD453-13D generating strain BSY624. The same strategy was followed for *SmB* generating strain BSY677. The strain BSY414 containing the protein A tagged POP1 (ProtA-Pop1) was described previously (Lygerou *et al.*, 1994).

LEA1 and *YIB9* were disrupted according to Puig *et al.* (1998). For *LEA1*, this fragment was generated by amplification from plasmid pBS1365 with oligonucleotides: FC4-1 (5'-GAAGTGAAACTACAGG-ACCTGGAAAATATCAGTTTTTATAAGCAATAATGAAGCTGGAG-CTCAAAAC-3') and FC4-2 (5'-TTTATAATTCTTTTTTTTAAGTCA-TTGAACAGTCGCACTAACCAGGATACGACTCACTATAGGG-3'). This PCR fragment was transformed into the diploid strain BSY320. The disruption was analyzed by PCR using one primer OF1 (5'-TATCAACGGTACCCTTAG-3') annealing inside *KL-URA3* and a second primer FC5-2 (5'-CTAAGGCATTGACATTGAAATTGA-3') annealing downstream of *LEA1*. The diploid strain was sporulated, the tetrads obtained were dissected and a spore containing the disruption was selected for further experiments ($\Delta lea1$; BSY650).

For the *YIB9* disruption, a fragment was generated by amplifying a *KL-TRP1*-containing fragment from plasmid pBS1408 (E.Bragado-Nilson and B.Séraphin, unpublished) with oligonucleotides FC6-1 (5'-GAGCAACTGTTTCGAAACTACGCATCATTCACACGTTAGGATA-TAGTAATGTTTGATACATGATTG-3') and FC6-2 (5'-TCGGTATA-TACGTGCTGCTATGAATAGAGCGTTAAGCCAGGAACGATCATT-CACTA-3'). The PCR fragment obtained was transformed in BSY320. The disruption was analyzed by PCR using the primer FC3-1 (5'-gactctcgagttctgaagcatcgtaata-3') which anneals upstream of *YIB9*, and a second primer EM157 (5'-GGTGGATCCTTTGTATTATTTAAGCAA-3') annealing downstream of *YIB9*. After sporulation and dissection of the tetrads a spore harboring the disruption was isolated for further experiments ($\Delta yib9$; BSY660).

To construct the double disruption of *YIB9* and *LEA1*, the PCR fragment used to disrupt the *YIB9* gene (see above) was transformed into the diploid cell already disrupted for *LEA1* (see above). Transformants were tested as above. After sporulation, tetrads were dissected and a strain harboring both disruptions was conserved (BSY661). To construct the strain containing *YIB9* disruption and *LEA1*-ProtA (BSY730), a PCR fragment containing the protein A cassette and the *KL-URA3* marker (see above) was integrated in strain BSY660 (see above). The N-terminal ProtA-tagged *YIB9*, encoded by plasmid pBS956 was described previously (Polycarpou-Schwarz *et al.*, 1996). For the coimmunoprecipitation experiment this plasmid was transformed in MGD453-13D and BSY650 (see above).

Immunoprecipitation, primer extension and Western blotting

Extract preparation from strains described above, immunoprecipitation and primer extension were as described previously (Séraphin, 1995). For Western blot analysis, proteins were resolved on 10% SDS-polyacrylamide gels and detected using peroxidase-antiperoxidase complex (Sigma) and the ECL detection system (Amersham).

Cloning, expression and purification of recombinant proteins

Plasmids were constructed using standard methods (Sambrook *et al.*, 1989). Enzymes were purchased from New England Biolabs. *LEA1* was cloned by PCR from total yeast genomic DNA. The primers used were FC10-1 (5'-AGGAATTCACCATGGAACATATGAAGTTCACCCCTA-3') and FC10-2 (5'-CGTAAGCTTAGTCATTGAACAGTCGC-3'). The PCR product was subcloned as an *EcoRI*-*HindIII* fragment into pBlueScriptKS(+/-), the insert was sequenced and the resulting plasmid was named pBS1497. To express *Lea1p* in *Escherichia coli*, *LEA1* was subcloned as a *NcoI*-*HindIII* fragment from pBS1497 into pBS1380, which contains a T7 promoter followed by a N-terminal His₆-tag. The His₆-tagged *Yib9p* has been described previously (pBS956; Polycarpou-Schwarz *et al.*, 1996). Expression and purification of these proteins were carried out according to Polycarpou-Schwarz *et al.* (1996).

To generate GST fused proteins, *YIB9* was subcloned as an *NdeI*-*BamHI* fragment from pBS1495 (see below) into pGEX-2T'6 generating pBS1501. To generate the GST-*LEA1* fusion, we followed the strategy of Zabarovsky and Allikmets (1986) to insert the *LEA1* coding region from pBS1497 digested with *XhoI* and *NdeI* into pGEX-2T'6 digested with *BamHI* and *NdeI*. The resulting construct was named pBS1520. Lysates from *E.coli* expressing these proteins were obtained by sonication of bacterial pellets in 1× PBS (Sambrook *et al.*, 1989). Lysates were cleared by centrifugation and proteins purified using glutathione-agarose beads and following the protocol provided by Sigma.

Protein-protein interaction

GST fusion protein binding assay. For GST-fusion binding assays, 300 μ l of lysates containing GST-*Lea1p*, GST-*Yib9p* and GST alone were mixed with 20 μ l glutathione-agarose beads and ~30 μ g of purified His-*Lea1p*, His-*Yib9p* and His-*SmX6* (kindly provided by J.Salgado-Garrido). After 1 h of rotation at room temperature, the beads were washed three times with buffer B (800 mM NaCl, 2.7 mM KCL, 0.1% NP-40, 8.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) for 2–5 min, followed by elution with 20 mM reduced glutathione in 50 mM Tris-HCl pH 8.0. The eluates were mixed with SDS-sample buffer, boiled for 5 min and loaded on a 14% SDS-polyacrylamide protein gel. Proteins were visualized on the gel by Coomassie staining.

Yeast-two-hybrid assay. *LEA1* and *YIB9* were subcloned as *NcoI*-*XhoI* or *NcoI*-*BamHI* fragments from pBS1497 and pBS956 into pACTII (Clontech). The resulting plasmids were named pBS1515 and pBS1496, respectively. To construct the Gal4-DNA-binding domain fusions, *LEA1* and *YIB9* were subcloned as *NcoI*-*SalI* or *NcoI*-*BamHI* fragments from pBS1497 and pBS956 into pAS2^{ΔΔ} (Fromont-Racine *et al.*, 1997). The resulting plasmids were named pBS1518 and pBS1495, respectively. pBS1105 harboring the SmG-activation domain fusion and pBS1487 harboring the SmE-DNA-binding domain fusion (Camasses *et al.*, 1998) served as positive controls. Empty vectors were used as negative controls. Yeast strain Y190 (Clontech) was first transformed with the pAS2^{ΔΔ}-derived constructs. These first generation transformants were then transformed with pACTII-derived constructs and β -galactosidase assays (see below) were performed on the resulting transformants.

RNA analysis and β -galactosidase assay

Total RNA was extracted as described previously (Pikielny and Rosbash, 1985) from cells grown in minimal medium containing 2% lactate-glycerol to an OD₆₀₀ of 0.5–1.0 and induced with 2% galactose for 2 h. Primer extension analysis using the exon 2-specific EM38 oligonucleotide (Luukkonen and Séraphin, 1997) has been described previously (Pikielny and Rosbash, 1985). Quantification of primer extension was made with a PhosphorImager (Molecular Dynamics). β -galactosidase assays were performed as described previously (Kandels-Lewis and Séraphin, 1993), and activities expressed in arbitrary units.

Native gel mobility shift assay

Yeast splicing extracts were prepared as described previously (Séraphin and Rosbash, 1989). Pre-mRNA was generated by *in vitro* transcription of the RP51A intron derivative contained in plasmid pBS195 (Séraphin

and Rosbash, 1991) after digestion with *Dde*I. Native gels were according to Séraphin and Rosbash (1989) but using 3% (37.5:1) acrylamide-mix (Protogel; National Diagnostic). To deplete ATP, the reactions were incubated for 10 min at room temperature with 2 mM glucose prior to addition of labeled pre-mRNA (Liao *et al.*, 1992). For the complementation experiments with recombinant proteins, 3 µl of extract or buffer D were used with 1 µl of either recombinant Lea1p or Yib9p (25 ng) or the corresponding dialysis buffer (negative control). To test for complementation between two extracts 1.5 µl of each extract was used.

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