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SLU7 and a novel activity, SSF1, act during the PRP16-dependent step of yeast pre-mRNA splicing

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Understanding the mechanism of pre-mRNA splicing requires the characterization of all components involved. In the present study, we used the genetically and biochemically defined yeast PRP16 protein as a point of departure for the identification of additional factors required for the second catalytic step *in vitro*. We isolated by glycerol gradient sedimentation spliceosomes that were formed in yeast extracts depleted of PRP16. This procedure separated the spliceosomal complexes containing lariat intermediate and exon 1 from free proteins present in the whole-cell yeast extract. We then supplemented these spliceosomes with purified proteins or yeast extract fractions as a functional assay for second-step splicing factors. We show that SLU7 protein and a novel activity that we named SSF1 (second-step factor 1) were required in concert with PRP16 to promote progression through the second catalytic step of splicing. Taking advantage of a differential ATP requirement for PRP16 and SLU7 function, we show that SLU7 can act after PRP16 in the splicing pathway.

Keywords: pre-mRNA splicing/PRP16/SLU7/spliceosome/yeast

Introduction

Pre-mRNA splicing occurs within a large (40–60S) ribonucleoprotein complex, the spliceosome. Assembly of this large, dynamic structure entails the ordered recruitment of multiple components onto the mRNA precursor (Brody and Abelson, 1985; Frendewey and Keller, 1985; Grabowski *et al.*, 1985; Bindereif and Green, 1987; Cheng and Abelson, 1987). Biochemical fractionation of mammalian cell extracts and genetic studies in yeast have led to the identification of many of the spliceosome components and the genes that encode them. These include snRNPs and many non-snRNP proteins (for reviews, see Guthrie and Patterson, 1988; Lührmann, 1988; Guthrie, 1991; Ruby and Abelson, 1991; Krämer, 1993; Lamm and Lamond, 1993; Moore *et al.*, 1993). The most extensively studied constituents of the spliceosome are the snRNAs (U1, U2, U4, U5 and U6). Elegant genetic and biochemical studies revealed a network of RNA–RNA interactions. Conformational rearrangements (i.e. changes in snRNA–pre-mRNA and snRNA–snRNA contacts) accompany, and are most likely required for, the juxtaposition and the catalytic activation of the splice sites (for reviews, see Madhani and Guthrie, 1994; Nilsen, 1994). Protein components of the splicing apparatus likely play a role in coordinating these rearrangements. In particular, proteins with associated enzymatic activities, such as the yeast splicing factors PRP2 and PRP16, which are RNA-dependent NTPases (Schwer and Guthrie, 1991; Kim *et al.*, 1992; Plumpton *et al.*, 1994), play a crucial role in this process. PRP2 is required to promote the first step of the reaction, leading to the production of lariat intermediate and exon 1 (Lin *et al.*, 1987).

PRP16, a 121 kDa protein of the DExH family of RNA-dependent NTPases, is required for the second catalytic step of splicing—cleavage at the 3′ splice site and exon ligation (Schwer and Guthrie, 1991). The ATPase is essential for the function of PRP16 in the splicing reaction, as there is a correlation between reduced ATPase activity and impaired splicing *in vitro* and *in vivo* (Schwer and Guthrie, 1992a). PRP16 binds to pre-assembled spliceosomes that contain the products of the first catalytic step; this binding is NTP independent. NTP hydrolysis by PRP16 is then required to convert the splicing intermediates into mature mRNA (Schwer and Guthrie, 1991). A conformational change occurs in the spliceosome concomitant with hydrolysis of ATP by PRP16, manifest as protection of the previously accessible 3′ splice site from oligonucleotide-directed RNaseH cleavage (Schwer and Guthrie, 1992b). Detection of this ATP-dependent rearrangement was facilitated by the use of a mutated pre-mRNA that precluded the second transesterification reaction. In a standard splicing reaction that proceeds to completion, PRP16 is apparently neither associated with the spliced product nor with the released lariat intron after the ATP-dependent step has been accomplished. This suggests that PRP16 dissociates from the spliceosome after ATP hydrolysis, but before the second cleavage–ligation reaction has occurred, or that it simply recycles along with other spliceosome components after splicing is complete.

A key question is how spliceosome binding and nucleotide hydrolysis by PRP16 affect or recruit other components involved in the second step of splicing. Needless to say, these questions cannot be adequately addressed until all the factors required for the second step have been identified and their order of action has been defined. Yeast genetic studies implicate the PRP18, PRP17 and SLU7 gene products in the second step of splicing *in vivo*. Mutant alleles of *PRP17* and *PRP18* were isolated in a screen for temperature-sensitive mutants with a defect in pre-mRNA processing (Vijayraghavan *et al.*, 1989). Conditional lethal alleles of *PRP17* (*slu4-1*) and *SLU7* (*slu7-1*) were isolated in an independent screen for mutants that exacerbate the phenotype of a mutation in the conserved loop of U5 snRNA (Frank *et al.*, 1992). The SLU7 gene product is essential for yeast growth, unlike PRP18 and PRP17, for which gene disruption elicits a temperature-sensitive phenotype (Frank and Guthrie, 1992;

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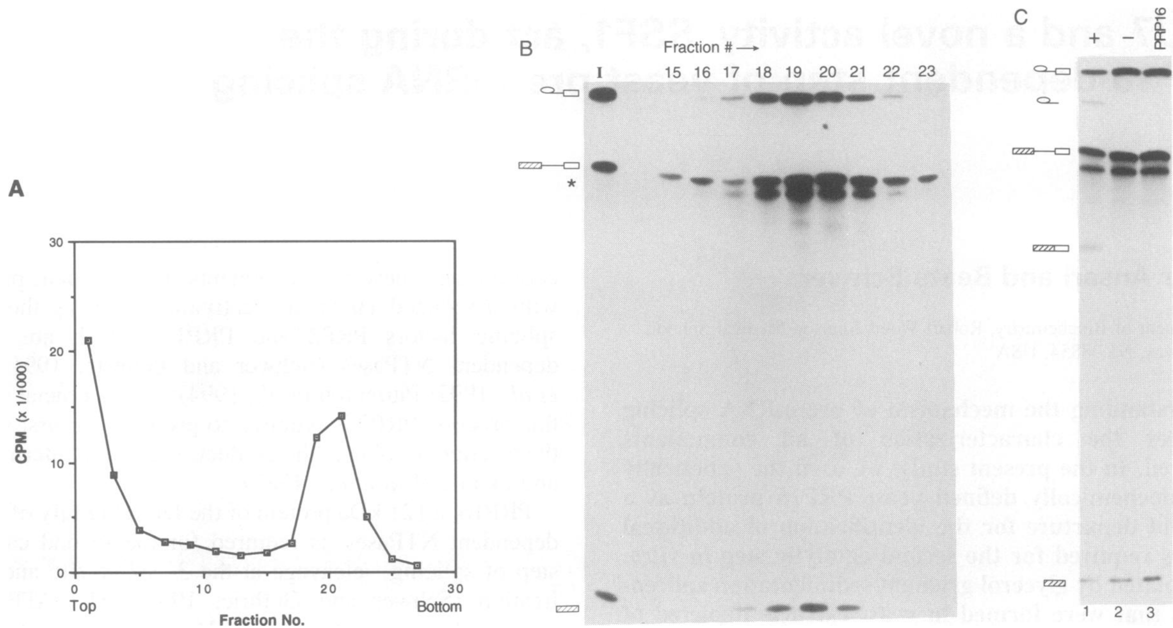

Fig. 1. Purification of ΔPRP16 spliceosomes. Uniformly labeled actin pre-mRNA was incubated for 20 min at room temperature under splicing conditions in extract that had been immunodepleted of PRP16. The reaction mixture was layered onto a 11.5 ml 15–40% glycerol gradient and centrifuged in a Sorvall TH641 rotor at 35 000 r.p.m. for 12 h at 4°C. Fractions of 400 μl each were collected from the top of the gradient. (**A**) Radioactivity profile of a typical gradient. A 20 μl aliquot of the odd-numbered fractions was used to determine the radioactivity in c.p.m. (**B**) RNA (40 μl aliquots) from fractions 15–23 was extracted and analyzed on denaturing acrylamide gels, followed by autoradiography. An aliquot of the reaction mixture that was layered onto the gradient is shown (I). The star indicates a band that only appears after centrifugation. Its size is consistent with that of debranched lariat intermediate (see Results). (**C**) Complementation assay. Peak fractions (18–21) were pooled and incubated with a 0–40% ammonium sulfate fraction of splicing extract (lane 1), buffer D (lane 2) and purified PRP16 protein (lane 3) at room temperature. After 30 min incubation, the reactions were stopped, RNA extracted and analyzed by denaturing PAGE. The symbols depict precursor and products of the splicing reaction; exon 1 is represented by a hatched box, exon 2 by an empty box and intron by a line.

Horowitz and Abelson, 1993b; M.Haltiner Jones and C.Guthrie, personal communication).

The goal of this study was to identify splicing factors that are essential for the second step of splicing *in vitro*. To accomplish this, we blocked splicing at the PRP16-dependent step, purified the spliceosomes by glycerol gradient sedimentation, and then analyzed the requirements of the arrested spliceosomes to progress through the second cleavage–ligation reaction. We provide evidence for the essential role of the SLU7 protein during the second step *in vitro*. Furthermore, we describe a novel factor extrinsic to gradient-purified spliceosomes (arrested at the PRP16-dependent step) that is required, along with PRP16 and SLU7, to complete the second step. We name this activity SSF1 (second-step factor 1).

Results

Complementation of gradient-purified ΔPPR16 spliceosomes requires PRP16 and other factors

In extracts that have been immunodepleted of PRP16 (ΔPRP16), the second transesterification step does not occur and lariat intermediates and exon 1 accumulate. In the presence of ATP, these splicing intermediates can be chased into mRNA by the addition of purified PRP16 protein (Schwer and Guthrie, 1991). In order to determine whether PRP16 was the only extrinsic factor required, we isolated ΔPRP16 spliceosomes by glycerol gradient sedimentation. Using ΔPRP16 extract, spliceosomes were formed on uniformly labeled actin pre-mRNA (Lin *et al.* 1985) during incubation for 20 min at room temperature. The reaction mixture was layered onto a 15–40% glycerol gradient and centrifuged for 12 h at 35 000 r.p.m.. Fractions were collected from the top of the gradient and analyzed for the distribution of the labeled RNA (Figure 1A). Two peaks of radioactivity were resolved. Aliquots of the input reaction mixture (I) and the peak gradient fractions (15–23) were analyzed for their RNA content by denaturing gel electrophoresis (Figure 1B). Spliceosomes, containing pre-mRNA or the products of the first catalytic step, peaked in fractions 18–21. Fractions at the top of the gradient (1–4) contained degraded pre-mRNA (not shown). RNA extracted from the gradient-purified spliceosomes included a species (denoted by a 'star' in Figure 1B) that was not present in the input mixture (Figure 1B, I). The mobility of this species was consistent with it being a debranched lariat intermediate (i.e. intron–exon 2). This was supported by the following observations: (i) when lariat intermediates were incubated with HeLa S100 fraction containing debranching activity (Ruskin and Green, 1985), the debranched RNA product co-migrated with the 'star' RNA species; and (ii) gel-purified 'star' RNA was cleaved by RNaseH in the presence of complementary oligonucleotides to yield fragments of the size expected for debranched lariat (not shown).

Gradient fractions 18–21 (ΔPRP16 spliceosomes) were pooled and analyzed for complementation of the second catalytic step by yeast extract or by purified PRP16 protein. As shown in Figure 1C, all the factors required to chase the products of step 1 into mature mRNA were present in a 0–40% ammonium sulfate fraction of a yeast whole-cell extract (Figure 1C, lane 1). However, the addition of purified PRP16 protein alone did not suffice (Figure 1C, lane 3). This suggests that other factor(s)

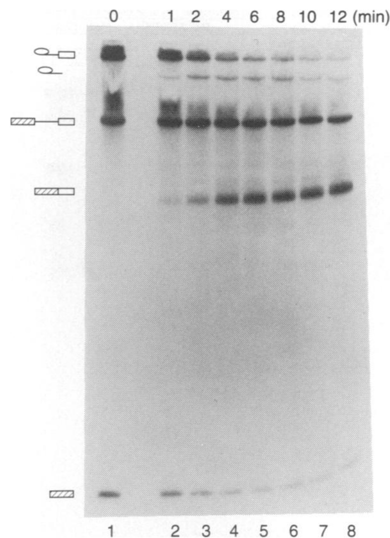

Fig. 2. Purified SLU7 protein complements the second-step block in ΔSLU7 extracts. Splicing of uniformly labeled actin pre-mRNA was assayed in an extract immunodepleted of SLU7 (ΔSLU7 extract) for 20 min at room temperature. Purified His-SLU7 protein was then added and aliquots removed after the times indicated (lanes 2–8). The RNA was extracted and analyzed by denaturing PAGE, followed by autoradiography.

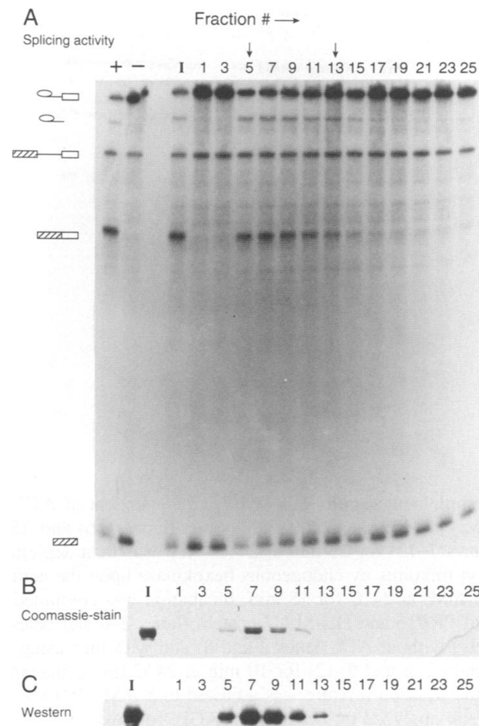

Fig. 3. Co-fractionation of the splicing complementation activity (A) and the His-SLU7 polypeptide (B and C) in a 10–30% glycerol gradient. An aliquot (50 µl) of the Ni-NTA eluate was applied to a 4.7 ml 10–30% glycerol gradient in buffer (20 mM HEPES–KOH, pH 7.2, 2 mM $MgCl_2$, 0.1% Triton X-100) containing 200 mM KCl. The gradient was centrifuged for 18 h at 4°C in a Beckman SW55 rotor at 55 000 r.p.m. Fractions (0.2 ml) were collected from the top of the tube. Positions of marker proteins sedimented in a parallel gradient are indicated by arrows (left, ovalbumin; right, bovine serum albumin). (**A**) Splicing extract was immunodepleted of SLU7 and incubated with actin pre-mRNA at 24°C for 20 min to allow formation of intermediates. (+) denotes the products after the addition of a 0–40% fraction of splicing extract; in the lane (−), buffer was added. Intermediates formed in the ΔSLU7 extracts were further incubated with the input fraction (I) or an aliquot of glycerol gradient fractions (1–25) at 24°C for 10 min. Splicing products were analyzed by denaturing gel electrophoresis and autoradiography. (**B**) Coomassie stain. Aliquots (40 µl) of the gradient fractions were separated by SDS–PAGE and the proteins were visualized by Coomassie blue staining. (**C**) Western blot analysis. Aliquots (12 µl) were separated by SDS–PAGE, transferred to nitrocellulose, and incubated with affinity-purified anti-SLU7 antibodies. His-SLU7 protein was visualized with chemiluminescence.

besides PRP16 are required for completion of step 2. Such factors are either not associated with spliceosomes formed in PRP16-depleted extracts, or else they dissociate during glycerol gradient sedimentation.

SLU7 is essential for step 2 in vitro

SLU7 is a strong candidate for one of the ancillary second step factors. Initial experiments were aimed at documenting the role of SLU7 during splicing *in vitro*. We produced the SLU7 protein in bacteria using an inducible T7 RNA polymerase-based expression system (Studier and Moffat, 1986). To facilitate purification, the 382 amino acid SLU7 polypeptide was fused to an N-terminal leader peptide containing six tandem His residues. Because the *His-SLU7* gene complemented the temperature-sensitive growth phenotype of the slu7-1 mutant (Frank *et al.*, 1992), we infer that the His tag did not impair the biological activity of the SLU7 protein (not shown). His-SLU7 was purified to homogeneity from soluble bacterial extracts by ammonium sulfate fractionation and Ni-NTA affinity chromatography (not shown).

Purified His-SLU7 protein was used as antigen to produce polyclonal antibodies in rabbits. The antiserum was affinity purified for all subsequent experiments. Yeast extract immunodepleted of SLU7 was tested for splicing of actin pre-mRNA; exon 1 and lariat intermediate accumulated, but no mature mRNA was apparent (Figure 2, lane 1). Upon addition of purified recombinant His-SLU7 protein, mature mRNA was produced (Figure 2, lanes 2–8). Mature mRNA was detected within 1 min and increased up to 6 min of incubation. Concomitant with the appearance of mRNA, the products of step 1 decreased, suggesting that the intermediates were chased into mRNA. The second-step complementing activity (Figure 3A) co-sedimented in a glycerol gradient with the 56 kDa His-SLU7 polypeptide, detected either by Coomassie blue staining (Figure 3B) or by immunoblotting (Figure 3C). His-SLU7 sedimented at a position intermediate to BSA (68 kDa) and ovalbumin (43 kDa) markers that were centrifuged in a parallel gradient, suggesting that His-SLU7 was a monomer. These experiments demonstrated that SLU7, like PRP16, is required for the second step of splicing *in vitro*.

SLU7 can function after PRP16

The PRP16-dependent step involves the hydrolysis of ATP (Schwer and Guthrie, 1991). Does SLU7 action also require ATP? To address this question, spliceosomes were formed in extracts depleted of PRP16 (Figure 4, lanes 1–6) or SLU7 (Figure 4, lanes 7–12). ATP was then depleted from the reaction mixtures by the action of endogenous hexokinase upon addition of glucose (Horowitz and Abelson, 1993a). The ATP-depleted samples were supple-

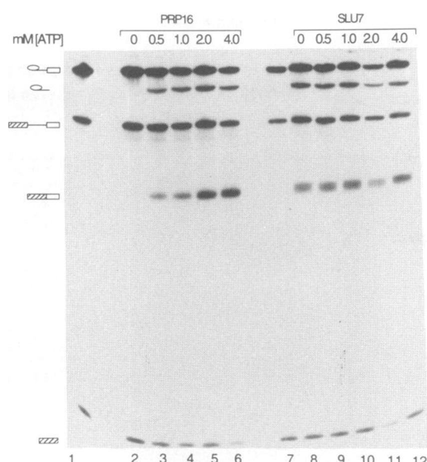

Fig. 4. Complementation by His-SLU7 is independent of ATP. Splicing was carried out in ΔPRP16 extract (lanes 1–6) and ΔSLU7 extract (lanes 7–12) for 20 min at 24°C. ATP was then depleted from the reaction mixtures by endogenous hexokinase upon the addition of glucose (5 mM) at 24°C for 15 min. Incubation was continued in the presence of PRP16 and His-SLU7 protein (lanes 2–6 and lanes 8–12, respectively) without ATP (lanes 2 and 8) and with increasing amounts of ATP (lanes 3–6 and 9–12) for 10 min at 24°C (the concentration of Mg^{2+} in the reaction mixture was adjusted to 5 mM). RNA was extracted and analyzed on denaturing PAGE, followed by autoradiography. In lanes 7 and 11, the recovery of the RNA was lower than in the other samples. The symbols represent precursor, lariat intermediates and mature mRNA; exon 1 is depicted by a hatched box, exon 2 by an empty box and intron by a line.

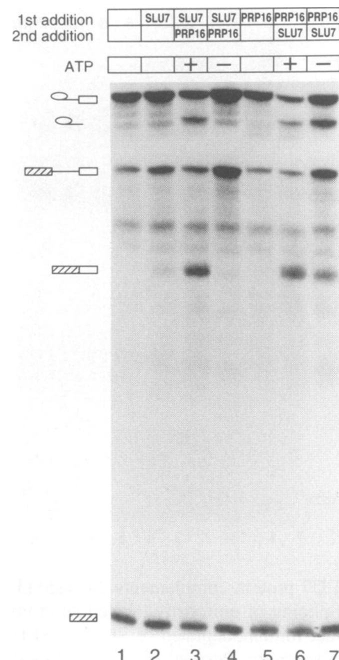

Fig. 5. PRP16 function precedes that of SLU7. Splicing extract was simultaneously immunodepleted of both PRP16 and SLU7 (Δ16Δ7 extract). Actin pre-mRNA was incubated under splicing conditions in Δ16Δ7 extract at 24°C for 20 min. An aliquot was removed and analyzed (lane 1). To aliquots of the reaction mixture, either His-SLU7 or PRP16 protein (1st addition, lanes 2–4 and 5–7, respectively) was added and the incubation continued for 10 min at 24°C. ATP was then depleted for 15 min. To aliquots of the reaction mixtures we added PRP16 or His-SLU7 protein (2nd addition, lanes 3 and 4 and 6 and 7, respectively) either in the presence or absence of ATP (lanes 3 and 6 and 4 and 7, respectively). After 10 min, the reactions were stopped, RNA extracted and analyzed. The products of the reaction are indicated by symbols; exon 1 is represented by a hatched box, exon 2 by an empty box and intron by a line.

mented with purified PRP16 or His-SLU7 proteins in the presence of ATP (0–4 mM) (Figure 4, lanes 2–6 and lanes 8–12, respectively). Whereas second-step complementation of ΔPRP16 extract by purified PRP16 required ATP (lane 2), the ΔSLU7 extract was complemented by His-SLU7 without added ATP (lane 8). This result clearly demonstrates a difference in the ATP requirement of PRP16 and SLU7. The simplest interpretation is that the SLU7 step is ATP independent; however, we cannot exclude the possibility that low levels of residual ATP satisfy an NTP requirement of SLU7.

Because it is possible to distinguish the SLU7- and PRP16-dependent steps based on the differential ATP requirement, we sought to determine the temporal order of their action. Extract was immunodepleted of both SLU7 and PRP16 (Δ16Δ7). As expected, when actin pre-mRNA was incubated in this extract under splicing conditions, lariat intermediate and exon 1 accumulated (Figure 5, lane 1). To aliquots of the reaction mixture, we either added (1st addition) His-SLU7 protein (Figure 5, lanes 2–4) or PRP16 protein (Figure 5, lanes 5–7). The formation of mRNA required the addition of both proteins; neither one alone could complement double-depleted extract (Figure 5, lanes 2 and 5). After 10 min of incubation, ATP was depleted by addition of glucose. During the next incubation (2nd addition), PRP16 or His-SLU7 protein was added in the presence or absence of ATP. When PRP16 protein was added first, the complementation by His-SLU7 was independent of ATP. This result strongly suggests that the ATP-dependent function of PRP16 occurs before the action of SLU7.

Complementation of gradient-purified ΔSLU7 spliceosomes

Was SLU7 the only factor required to chase lariat intermediate and exon 1, present in gradient-purified ΔSLU7 spliceosomes into mature mRNA? Spliceosomes were assembled on actin precursor RNA in extracts that had been immunodepleted of SLU7. ΔSLU7 spliceosomes were then purified by glycerol gradient sedimentation. Second-step complementation (seen as production of mature mRNA) was achieved by addition of an ammonium sulfate fraction of yeast whole-cell extract (Figure 6, lane 1) but not by purified His-SLU7 protein (Figure 6, lane 3). Other factors were obviously required. PRP16 appeared not to be one of these factors, because addition of purified PRP16 along with His-SLU7 failed to complement the ΔSLU7 spliceosomes (Figure 6, lane 6), whereas extract depleted of both SLU7 and PRP16 (Δ16Δ7) did complement the second step (Figure 6, lane 8) in concert with His-SLU7. Control experiments verified that the Δ16Δ7 extract was truly depleted of both proteins, insofar as splicing by the double-depleted extract was arrested after the first step (Figure 6, lane 9), and completion of step 2 depended absolutely on addition of both PRP16 and His-SLU7 (Figure 6, lanes 10–12). The finding that progression through the SLU7 step was independent of added PRP16 further supported our inference from the ATP depletion experiments that PRP16 acts before SLU7 in the splicing

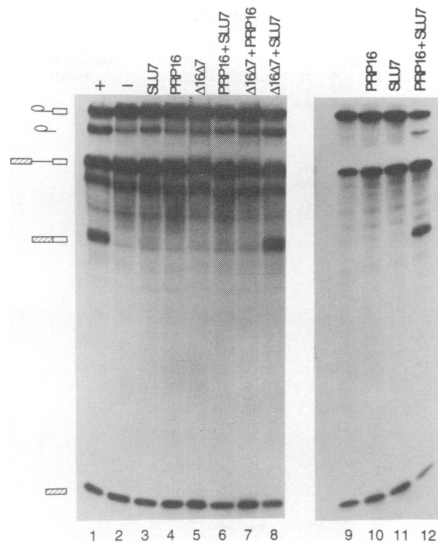

Fig. 6. Complementation of gradient-purified ΔSLU7 spliceosomes. Splicing reactions were carried out in extract that had been immunodepleted of SLU7. The reaction mixture (200 μl) was layered onto a 15–40% glycerol gradient and centrifuged for 12 h in a Sorvall TH-641 rotor at 4°C and the peak fractions containing spliceosomes were pooled. Gradient-purified ΔSLU7 spliceosomes were incubated with a 0–40% ammonium sulfate fraction of extract (lane 1), buffer (lane 2), His-SLU7 protein (lane 3), PRP16 protein (lane 4), extract that has been immunodepleted of both PRP16 and SLU7 (Δ16Δ7, lane 5) or various combinations of PRP16 protein, His-SLU7 protein and Δ16Δ7 extract as indicated above each lane. All incubations were carried out at 24°C for 30 min. The right panel (lanes 9–12) shows that the double-depleted extract (Δ16Δ7) used in these assays was free of PRP16 and SLU7, since formation of mature mRNA required the addition of both proteins (lane 12) to the intermediates that formed in the Δ16Δ7 extract (lane 9).

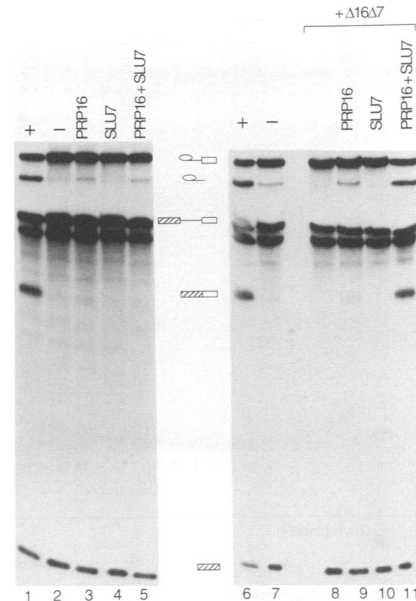

Fig. 7. Complementation of gradient-purified ΔPRP16 spliceosomes requires PRP16, SLU7 and the additional factor, SSF1. Gradient-purified ΔPRP16 spliceosomes were incubated with 10 μl of a 0–40% ammonium sulfate fraction from splicing extract (lanes 1 and 6), buffer (lanes 2 and 7), PRP16 protein (lane 3), His-SLU7 protein (lane 4) and both, PRP16 and His-SLU7 protein (lane 5) at 24°C. In a parallel experiment, 5 μl of splicing extract immunodepleted of both PRP16 and SLU7 (Δ16Δ7) was added to the gradient-purified ΔPRP16 spliceosomes either alone (lane 8), or together with PRP16 protein (lane 9), with His-SLU7 protein (lane 10) or with both PRP16 and His-SLU7 (lane 11). All the incubations were performed at 24°C for 30 min. RNA was extracted and analyzed by denaturing PAGE, followed by autoradiography.

pathway. This experiment also demonstrates that complementation of gradient-purified ΔSLU7 spliceosomes requires factor(s) other than SLU7.

Requirement for a novel second-step factor, SSF1

The data presented above indicate that: (i) SLU7 acts *in trans* on pre-assembled spliceosomes arrested after the first transesterification; (ii) SLU7 functions after the ATP-dependent step of PRP16; and (iii) SLU7 function is either ATP independent or requires much lower levels of NTP than PRP16. Therefore, SLU7 was a plausible candidate for the accessory factor needed, along with PRP16, to complement gradient-purified ΔPRP16 spliceosomes (Figure 1). However, we found that addition of purified PRP16 and His-SLU7 proteins to gradient-purified ΔPRP16 spliceosomes did not suffice to complement the second step, and no mature mRNA was formed (Figure 7, lane 5). Complementation was achieved by addition of a 0–40% ammonium sulfate fraction from yeast extract (Figure 7, lane 1). Based on this result (and the observation that gradient-purified ΔSLU7 spliceosomes could be complemented by crude extracts but not by purified His-SLU7), we surmised that at least one other factor, in addition to PRP16 and SLU7, was necessary to catalyze the second step. This factor was present in extracts that had been immunodepleted of both SLU7 and PRP16 (Figure 7, lane 11). We refer to this activity as SSF1 (second-step factor 1).

The SSF1 activity was partially purified from a 0–40% ammonium sulfate fraction of yeast whole-cell extract, based on functional complementation of gradient-purified ΔPRP16 spliceosomes in the presence of purified PRP16 and His-SLU7 proteins. SSF1 activity was adsorbed to DEAE–Sepharose and eluted at 0.5 M KCl (not shown). Active fractions were pooled and further purified by gel filtration through Sephacryl S-300. SSF1 activity chromatographed as a discrete peak during gel filtration (Figure 8A). The complementing activity (gauged by the yield of mature mRNA) was dependent on the amount of the Sephacryl S-300 fraction added (Figure 9). A native molecular weight of 200 kDa for SSF1 was estimated by comparing its K_{av} during gel filtration with the values obtained for several globular protein calibration standards (Figure 8B). SSF1 activity was abrogated by treatment with proteinase K beads, suggesting that the active principle was a protein (not shown). A 20-min pre-incubation at 50°C or 70°C completely abolished SSF1 activity, while incubation for the same time at 25°C and 37°C had no effect (not shown).

Complementation of gradient-purified ΔSLU7 spliceosomes required additional factor(s) besides SLU7 protein (Figure 6). Interestingly, when we tested whether the partially purified SSF1 activity, together with His-SLU7, was sufficient to complement gradient-purified ΔSLU7 spliceosomes, we found that no mature mRNA was produced (not shown). This finding suggests that in addition to SLU7 and the SSF1 activity, yet another factor has to act at this step. This factor must be present in ΔPRP16 spliceosomes, but not in ΔSLU7 spliceosomes,

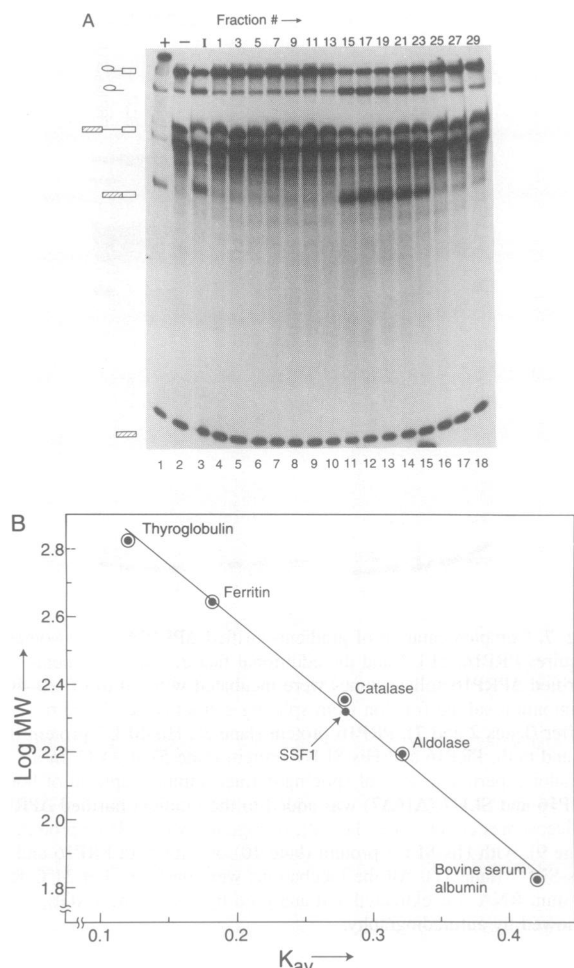

Fig. 8. Fractionation of the SSF1 activity by gel filtration. (**A**) The concentrated eluate (19.2 mg protein in 2 ml) from a DEAE column was fractionated on a Sephacryl S-300 column (30×1.8 cm). Gradient-purified ΔPRP16 spliceosomes were incubated with a 0–40% ammonium sulfate fraction from splicing extract (lane 1) which contains all the factors required. In order to monitor the gel filtration column, aliquots of the gradient-purified ΔPRP16 spliceosomes were incubated in the presence of PRP16 and His-SLU7 proteins (lanes 2–18) without (lane 2) or with an aliquot of the Sephacryl S-300 fraction to be tested (lanes 3–18). Input (I, lane 3) shows the products of the reaction when 5 μl of the eluate from the DEAE column was added. After 30 min incubation at room temperature, the reactions were stopped, the RNA extracted and analyzed on urea–acrylamide gels. (**B**) The marker proteins: thyroglobulin (mol. wt 669 000), ferritin (mol. wt 440 000), catalase (mol. wt 232 000), aldolase (158 000) and bovine serum albumin (mol. wt 67 000) were fractionated on the same Sephacryl S-300 column. On the basis of their elution volumes (V_e), the partition coefficient (K_{av}) was calculated for each of the proteins according to the formula $K_{av} = V_e - V_0/V_t - V_0$ (V_0 is the void volume and V_t is the total bed volume). Log mol. wt of each marker protein was plotted as a function of its K_{av} value. The relative molecular weight of SSF1 was estimated from this calibration plot.

suggesting that the factor is lost during the transition from the PRP16 to the SLU7 step.

Discussion

PRP16 associates with spliceosomes that have completed the first cleavage–ligation reaction and promotes the second catalytic step in an ATP-dependent fashion (Schwer and Guthrie, 1991). Concomitant with the action of PRP16 is a conformational rearrangement in the spliceosome,

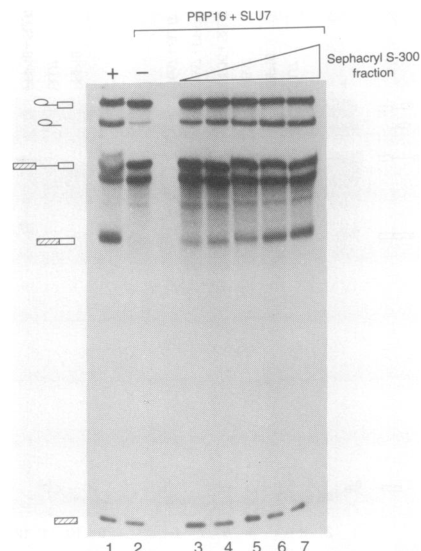

Fig. 9. Titration of the SSF1 activity. Gradient-purified ΔPRP16 spliceosomes were incubated with a 0–40% ammonium sulfate fraction of splicing extract (lane 1), with PRP16 and His-SLU7 proteins (lanes 2–7) and increasing amounts (1–12 μl) of pooled Sephacryl S-300 fraction (lanes 3–7) for 30 min at 24°C. The RNA was extracted and analyzed on urea–acrylamide gels. The positions of the precursor and splicing products are indicated by symbols.

resulting in protection of the previously accessible 3′ splice site against oligonucleotide-mediated RNaseH cleavage (Schwer and Guthrie, 1993b). In order to identify factors that were essential for this reaction, we separated spliceosomes, blocked at the PRP16-dependent step, from free or loosely associated proteins present in the whole-cell yeast extract and assayed for functional complementation of the splicing intermediates. We demonstrated that PRP16 is not the only extrinsic factor necessary to promote the second cleavage–ligation reaction. Immunodepletion and complementation experiments establish that SLU7 is a true second-step splicing factor *in vitro*. SLU7 can perform its function after depletion of ATP, unlike PRP16, which is absolutely dependent on nucleotide hydrolysis for its function. We show that the ATP-dependent role of PRP16 precedes the SLU7 function by depleting the extract of SLU7 and PRP16 and then sequentially adding back the two purified proteins. When PRP16 was added first (in the presence of ATP) and then SLU7 was added after ATP removal, the second incubation was independent of ATP and mature mRNA was produced. These results are in agreement with our finding that purified spliceosomes, blocked at the SLU7-dependent step, do not require the addition of PRP16 protein in order to produce mature mRNA. Three plausible models can explain these results: (i) ATP hydrolysis by PRP16 elicits a rearrangement in the spliceosome which allows SLU7 to act; (ii) PRP16 can bind ATP stably (insensitive to hexokinase and sedimentation in glycerol gradient) once it is associated with the spliceosome and ATP hydrolysis is triggered by SLU7; or (iii) PRP16 hydrolyses ATP to ADP, and SLU7 promotes the release of ADP and/or PRP16 from the spliceosome. The second and third model would predict that PRP16 is associated with spliceosomes formed in ΔSLU7 extract. Preliminary data, however, indicate that splicing intermediates formed in ΔSLU7

extract cannot be co-immunoprecipitated with anti-PRP16 antibodies. More functional assays will be necessary to distinguish between these possibilities.

SLU7 is not the only splicing factor besides PRP16 that is missing from ΔPRP16 spliceosomes. At least one other component, which we have named second-step factor 1 (SSF1), is absolutely required for splicing of actin pre-mRNA *in vitro*. We partially purified the SSF1 activity by functional complementation of gradient-purified ΔPRP16 spliceosomes supplemented with purified His-SLU7 and PRP16 proteins. Gel filtration revealed a native molecular weight of ~200 kDa. The SSF1 activity was heat-labile and sensitive to proteinase K, suggesting that the active component in the preparation is a protein. Does the SSF1 activity correspond to one of the genetically identified, step 2 factors, PRP17 or PRP18? Kinetic studies revealed that PRP18 is not absolutely required for splicing of actin precursor RNA *in vitro* (at 23°C), but rather enhances the rate of step 2 in an ATP-independent manner (Horowitz and Abelson, 1993a). Thus, PRP18 is not absolutely essential for splicing at low temperature, both *in vivo* and *in vitro* (Horowitz and Abelson, 1993a,b). To date, there have been no reports on the function of PRP17 *in vitro*; however, given that the gene product is not essential at low temperatures *in vivo* (M.Haltiner Jones and C.Guthrie, personal communication), it may, like PRP18, serve to increase the rate of the second step of splicing. In contrast, the SSF1 activity is absolutely required for splicing of actin pre-mRNA at room temperature, suggesting that SSF1 represents a novel splicing factor (with a native molecular weight of ~200 kDa) which is defined by its biochemical activity to function after PRP16 in step 2 of the splicing reaction. Only the purification and the identification of the gene encoding SSF1 will ultimately resolve the question of whether SSF1 activity can be attributed to a known splicing factor.

Studies in the mammalian system suggest that several protein factors associate with the spliceosome after step 1 has occurred; analysis of the protein composition of purified spliceosomes blocked before the second cleavage–ligation reaction (using a mutant precursor RNA) revealed the presence of 14 novel spliceosome-associated proteins (SAPs) which are not associated with spliceosomes blocked at an earlier stage (Gozani *et al.*, 1994). Although this study provides a static view of the spliceosome it underscores the complexity of the splicing machinery. Using mild heat treatment to specifically inactivate step 2 of the reaction, two activities have been identified in chromatographic fractions of HeLa extracts that are only required for cleavage at the 3′ splice site and exon ligation (Krainer and Maniatis, 1985; Perkins *et al.*, 1986). Employing heat-treated HeLa extracts in solid phase splicing assays revealed two stages of step 2—the ATP-independent binding of protein factors to the spliceosome followed by the production of mature mRNA upon addition of ATP (Sawa and Shimura, 1991). More studies and the identification of the gene encoding the SSF1 activity will be necessary to determine whether SSF1 corresponds to one of the described mammalian second-step factors.

It is interesting that while complementation of gradient-purified ΔPRP16 spliceosomes requires the addition of PRP16, SLU7 and SSF1, these components do not satisfy the requirement of isolated ΔSLU7 spliceosomes to produce mature mRNA (an activity that can complement ΔSLU7 spliceosomes is present in crude extract). This finding strongly suggests that upon action of PRP16, a factor that is associated with gradient-purified ΔPRP16 spliceosomes (stable enough to withstand sedimentation in glycerol gradient) is lost during centrifugation of ΔSLU7 spliceosomes. This factor may either act at multiple steps in the reaction, or it may be an intrinsic component of the spliceosome early in the splicing pathway but only function at a later stage. An example of a protein factor that plays roles at multiple steps in the splicing pathway is the U5 snRNP-specific PRP8 protein (Brown and Beggs, 1992; Teigelkamp *et al.*, 1995; Umen and Guthrie, 1995). In a genetic screen for mutants that eliminate the normal preference for a uridine-rich 3′ splice site, an allele of the *PRP8* was isolated (Umen and Guthrie, 1995). This finding demonstrates a function of PRP8 protein in 3′ splice site selection, in addition to its well-documented role in spliceosome assembly (Brown and Beggs, 1992; Umen and Guthrie, 1995). PRP8 protein can be cross-linked to the substrate RNA in regions encompassing the 5′ and 3′ splice sites. While the interaction with the 5′ splice site occurs before step 1, cross-linking of PRP8 to the 3′ splice site region is contingent on the first catalytic step (Teigelkamp *et al.*, 1995).

PRP16 is an essential splicing factor that elicits a conformational rearrangement in the spliceosome involving the 3′ splice site. We have now identified additional components that function downstream of PRP16. It will be interesting to determine whether SLU7 and SSF1 bind directly to the 3′ splice site or whether they elicit additional conformational changes.

Materials and methods

Preparation of splicing extract

This was modified from Umen and Guthrie (1995). Yeast cells, strain BJ2168 (Jones, 1991), were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose) at 30°C to late logarithmic phase and harvested by spinning at 3000 r.p.m. for 5 min in a Sorvall GS-3 rotor. Cell pellets from 2 l of culture were first washed with 50 ml of cold, sterile double-distilled water and then with 50 ml of AGK buffer (10 mM HEPES–KOH, pH 7.9, 1.5 mM $MgCl_2$, 200 mM KCl, 0.5 mM DTT and 10% glycerol). The cell pellets were then suspended in 7.5 ml (per 2 l culture) of AGK buffer. The cell suspension was frozen in liquid nitrogen and stored at –70°C. The frozen cell pellets were homogenized to a very fine powder, using a Waring blender or a mortar and pestle. The powder was allowed to thaw slowly at 4°C, stirred for 30 min and then centrifuged at 18 000 r.p.m. for 30 min in a Sorvall SS-34 rotor. The supernatant from this spin was centrifuged at 37 000 r.p.m. for 1 h in a Sorvall TH-865 rotor. After the spin, the pale yellow aqueous phase was carefully removed and dialyzed twice against 2 l of buffer D (20 mM HEPES–KOH, pH 7.9, 0.2 mM EDTA, 50 mM KCl, 0.5 mM DTT and 20% glycerol) for 1.5 h each.

Preparation of precursor mRNA

The template for actin precursor mRNA contained the actin gene under the control of the T7 promoter (Lin *et al.*, 1985). Transcription was carried out using T7 polymerase (Boehringer) in the presence of 125 μCi α-^{32}P-labeled GTP (NEN), 25 μM GTP and 500 μM GpppG (Pharmacia) under the conditions described by the manufacturer (Boehringer). After extraction with phenol/chloroform, the run-off transcripts were separated from unincorporated nucleotides by gel filtration in a 1 ml spin column of Sepharose CL-6B. 50 000 c.p.m. (~1 fmol) of the precursor were used per splicing assay of 10 μl.

Expression of SLU7 in bacteria

The *SLU7* gene (kindly provided by Dan Frank) was ligated into the phage T7-based expression vector pET14b fusing the open reading

frame to a sequence encoding six histidine residues and a thrombin cleavage site (Novagen). The resultant plasmid was pET14b–SLU7. The His-SLU7 polypeptide contains an additional 34 amino acids at the N-terminus. The sequence, N-terminal of SLU7 is as follows: MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGAGS.

Purification of His-SLU7 protein

Escherichia coli BL21 (DE3) cells were transformed with pET14b–SLU7, grown in LB medium with ampicillin (0.1 mg/ml) at 37°C to an optical density of 0.5 to 0.7 (Studier and Moffat, 1986). Expression was then induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM for 3 h. Cells were harvested by centrifugation. The bacteria from 2 l of cell culture were suspended in 70 ml of lysis buffer (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1 mM β-mercaptoethanol and 10% sucrose). Freshly prepared lysozyme was added to the cell suspension to the final concentration of 0.25 mg/ml and incubated on ice for 30 min. Suspensions were adjusted to 0.1% Triton X-100, and the incubation continued for 30 min. The cell lysate was centrifuged at 18 000 r.p.m. for 30 min in a Sorvall SS-34 rotor to obtain the soluble fraction. Initial fractionation was performed by gradual addition of solid ammonium sulfate to the extract. The His-SLU7 protein was detected in the fractions by analyzing aliquots on SDS–PAGE followed by Coomassie blue staining. The protein preparation from 20–40% saturation with ammonium sulfate contained His-SLU7. The 20–40% ammonium sulfate fraction (30 mg protein) was dialyzed against buffer NB (20 mM Tris–HCl, pH 7.6, 1 mM β-mercaptoethanol, 10% glycerol) for 2 h and then applied directly to a 2 ml column of Ni-NTA agarose (Quiagen). The column was washed with buffer NB containing 10 mM imidazole and 250 mM NaCl until no protein was detected in the eluate. Adsorbed His-SLU7 protein was specifically eluted by a linear gradient of 10–125 mM imidazole. Fractions of 1.5 ml each were collected, an aliquot was tested for SLU7 activity in a SLU7-dependent splicing assay (see below) and also analyzed on 10% SDS–PAGE. The electrophoretically homogeneous, active fractions were pooled and dialyzed against buffer D (20 mM HEPES–KOH, pH 7.9, 0.2 mM EDTA, 50 mM KCl, 0.5 mM DTT and 20% glycerol) for 6 h.

Immunodepletion of splicing extracts

Splicing extract was immunodepleted either of PRP16 or SLU7 or both by incubating with the appropriate antibodies for 45 min at 4°C. For immunodepletion of PRP16, 120 μl of splicing extract were incubated with 30 μl of protein A–Sepharose (Pharmacia) purified anti-PRP16 antiserum (10 mg/ml); SLU7 was immunodepleted by incubating 120 μl extract with 15 μl of affinity purified anti-SLU7 antibodies (1 mg/ml); and depletion of both PRP16 and SLU7 was carried out by incubating 120 μl of splicing extract with both anti-PRP16 antiserum and anti-SLU7 antiserum simultaneously. 150 μl of protein A–Sepharose suspension (0.1 g/ml) was washed three times with 1 ml each of PBS (10 mM Na_3PO_4, pH 7.2, 150 mM NaCl) and briefly centrifuged. The supernatant was carefully removed and the extract–antiserum mixture was added to the beads with an additional 30 μl of PBS and gently shaken at 4°C on a nutator. After 45 min the beads were removed by brief centrifugation and the supernatant (ΔPRP16 extract, ΔSLU7 extract or Δ16Δ7 extract) was used in the *in vitro* splicing assays. 6 μl of immunodepleted extract and ~1 fmol (50 000 c.p.m.) of actin pre-mRNA was used per 10 μl splicing assay (Lin *et al.*, 1985).

Gradient purification of ΔPRP16 and ΔSLU7 spliceosomes

Splicing was carried out using ΔPRP16 or ΔSLU7 extract in 200 μl reactions (20-fold) for 20 min at 24°C. A 10 μl aliquot of the reaction mixture was saved for analysis (I, in Figure 1B) and 190 μl were layered onto a 11.5 ml 15–40% glycerol gradient at 4°C. The glycerol gradient was formed in 20 mM HEPES–KOH, pH 7.2, 2 mM $MgCl_2$, 100 mM KCl. After spinning at 35 000 r.p.m. for 12 h in a Sorvall TH-641 rotor at 4°C, fractions of 2×200 μl each were collected from the top. A 20 μl aliquot of each odd numbered gradient fraction was used for direct scintillation counting and 40 μl each was used for analysis on denaturing–PAGE. The fractions representing the peak of spliceosomes were pooled and stored at –70°C until used further.

SSF1 assay

SSF1 activity was monitored in terms of its ability to complement 120 μl of gradient-purified ΔPRP16 spliceosomes in the presence of PRP16 (200 ng), purified as described (Schwer and Guthrie, 1991) and His-SLU7 (400 ng) under splicing conditions in a final volume of 200 μl. An aliquot (5 μl) of the fraction to be tested for SSF1 activity was added and incubation was carried out for 30 min at 24°C.

Partial purification of SSF1

Splicing extract was prepared from 15 l of yeast cell culture as described. The extract (50 ml) was subjected to ammonium sulfate fractionation. The 0–40% fraction (85 mg protein) containing the SSF1 activity was dialyzed against buffer D for 6 h and then loaded onto a 30 ml column of DEAE–Sepharose pre-equilibrated with buffer D. After binding, the column was washed with buffer D until no protein was detected in the eluate. Elution was performed with 500 mM KCl and 30 fractions of 2 ml each were collected. Alternate fractions were tested for SSF1 activity. The active fractions were pooled, concentrated by 0–65% ammonium sulfate precipitation and dialyzed against 4 l of buffer D for 3 h. Approximately 20 mg dialyzed protein in 2 ml was subjected to gel filtration chromatography on a Sephacryl S-300 column pre-equilibrated with buffer D containing 500 mM KCl. After the void volume (V_0 = 35 ml), 40 fractions of 1 ml each were collected. Alternate fractions were tested for SSF1 activity; fractions exhibiting peak SSF1 activity were pooled and used for further studies.

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