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Requirement for SLU7 in yeast pre-mRNA splicing is dictated by the distance between the branchpoint and the 3′ splice site

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ABSTRACT

Yeast pre-mRNA splicing factors SLU7 and PRP16 are required for cleavage of the 3′ splice site and exon ligation in vitro. Using natural and model precursor RNAs, we found that SLU7 is dispensable for splicing of RNAs in which the 3′ splice site is in close proximity to the branchpoint. SLU7 is only required when the interval between the branchpoint and the 3′ splice site is greater than 7 nt. In contrast, PRP16 is essential for splicing of all pre-mRNAs tested. Immunoprecipitation of the products of step 1 by anti-SLU7 antibodies demonstrates that SLU7 is a component of the spliceosome. Recruitment of SLU7 to the spliceosome is greatly enhanced by prior addition of PRP16. PRP16 is liberated from the spliceosome after completion of step 2, whereas SLU7 remains bound to the excised intron and spliced mature RNA until the spliceosome disassembles, in a reaction that requires ATP.

Keywords: ATP; PRP16; *Saccharomyces cerevisiae*; second step; spliceosome

INTRODUCTION

Yeast pre-mRNAs are spliced by two consecutive transesterification reactions: (1) cleavage at the 5′ splice site and formation of the 2′-5′ branched lariat intermediate, and (2) cleavage at the 3′ splice site and ligation of the exons. Conserved sequence elements within the introns of yeast precursor RNAs, at the 5′ and 3′ splice sites (↓GUAUGU and PyAG↓, respectively), and at the branchpoint (UACUAACA) are required for accurate splicing. Spliceosomal snRNPs U1, U2, U4/6, and U5 recognize these elements and assemble onto the pre-mRNA in an ordered, ATP-dependent fashion (reviewed by Guthrie, 1991; Ruby & Abelson, 1991). Execution of the first catalytic step requires base pairing of U1 snRNA with the 5′ splice site sequence and base pairing of U2 snRNA with the branchpoint region (Parker et al., 1987; Séraphin et al., 1988; Siliciano & Guthrie, 1988). U5 snRNA, which plays a role in both steps of the splicing reaction (Patterson & Guthrie, 1987; Séraphin et al., 1991), may base pair with the precursor at the 5′ and 3′ splice sites (Newman & Norman, 1991, 1992). Mutations in the 3′ splice site of yeast pre-mRNA specifically block the second transesterification step without affecting either spliceosome assembly or 5′ exon cleavage/lariat formation. This indicates that recognition of the 3′ splice site is not required for step 1 (Rymond & Rosbash, 1985; Vijayraghavan et al., 1986). How the 3′ splice site is chosen during step 2 is not well understood. Structural features of the yeast RNA precursor, such as the distance from the branchpoint to the PyAG↓ splice site, or the nucleotide sequence context upstream of the 3′ splice site, can impact 3′ splice site utilization, particularly in experimental situations that offer a choice between competing 3′ splice sites (Cellini et al., 1986; Fouser & Friesen, 1987; Patterson & Guthrie, 1991). *Trans*-acting protein factors also play a role; genetic and biochemical studies implicate the PRP8, PRP16, and SLU7 proteins in 3′ splice site selection (reviewed in Umen & Guthrie, 1995c).

PRP8, a component of the U5 snRNP (Lossky et al., 1987), is necessary for step 1, but is also involved in 3′ splice site recognition. For example, a mutant allele

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prp8-101 was identified in a phenotypic screen for impaired recognition of a distant 3′ splice site containing a flanking polypyrimidine tract (Umen & Guthrie, 1995a). Two non-snRNP proteins, PRP16 and SLU7, which are essential in vivo, are required specifically for the second catalytic step of splicing in vitro (Schwer & Guthrie, 1991; Frank & Guthrie, 1992; Frank et al., 1992; Ansari & Schwer, 1995; Jones et al., 1995). PRP16 is an RNA-dependent NTPase that binds to the spliceosome after completion of step 1. NTP hydrolysis by PRP16 is then required for step 2 (Schwer & Guthrie, 1991). Concomitant with hydrolysis of ATP is a change in the accessibility of the 3′ splice site to oligonucleotide-directed RNase H cleavage (Schwer & Guthrie, 1992). The previously accessible splice site becomes protected, suggesting that it is masked by protein and/or RNP interactions. The association of PRP16 with the spliceosome is destabilized after ATP hydrolysis, as assessed by the inability to immunoprecipitate the mature RNA or released lariat intron after completion of the second step (Schwer & Guthrie, 1991). SLU7, which interacts genetically with the U5 snRNA (Frank & Guthrie, 1992; Frank et al., 1992), functions after PRP16 in an ordered second-step pathway in vitro (Ansari & Schwer, 1995; Jones et al., 1995). SSF1, a biochemically defined factor, is necessary for completion of the second step in vitro. Gradient-purified spliceosomes blocked at the PRP16-dependent step require the addition of SSF1 along with PRP16 and SLU7 to allow the formation of mature RNA (Ansari & Schwer, 1995). It is likely that PRP8 (in the context of the U5 snRNP) and PRP16 are situated near the 3′ splice site during the second step, because these proteins can be UV crosslinked to this region of the precursor (Teigelkamp et al., 1995a, 1995b; Umen & Guthrie, 1995a, 1995b). SLU7 can also be crosslinked to the RNA (Umen & Guthrie, 1995b). Although the requirement for these factors has been established, the question of how the spliceosome, assembled at the branchpoint sequence, recognizes the 3′ splice site which, in an average intron is 20–60 nt away, has yet to be answered.

Here we investigate the role of SLU7 in 3′ splice site selection in vitro by comparing the requirement for SLU7 during the second step of splicing of different precursor RNAs. We demonstrate that SLU7 function is only required for the second step when the interval between the branchpoint sequence and 3′ splice is greater than 7 nt. This suggests that SLU7 acts as a molecular bridge that facilitates positioning of the 3′ splice site at the catalytic center of the spliceosome. We show by immunoprecipitation that SLU7 is associated with the spliceosome prior to execution of step 2 and that this interaction occurs even in a truncated pre-mRNA, lacking the 3′ splice site region. SLU7 remains associated with the spliceosome after catalysis is complete and becomes released when the spliceosome disassembles in an ATP-dependent step.

RESULTS

Splicing of the U3 precursor RNA is independent of SLU7

Using a reporter gene with two competing 3′ splice sites, Frank and Guthrie (1992) showed that the *slu7-1* mutation reduced splicing to the branch-distal site without affecting use of the branch-proximal site. This effect may be unique to (1) the mutant *slu7-1*; (2) the situation when two competing 3′ splice sites are present in the pre-mRNA; or (3) SLU7 may simply not be required for splicing of precursors in which the branchpoint and 3′ splice site are in close proximity. We analyzed the effect of SLU7 inhibition on the splicing of several different precursor RNAs in vitro. Yeast whole-cell extract prepared from a wild-type strain was treated with increasing amounts of anti-SLU7 serum prior to addition of radiolabeled RNA precursors. SLU7 antibodies inhibited splicing of yeast actin pre-mRNA by blocking the second transesterification step; this resulted in the accumulation of lariat intermediate at the expense of mature product (Fig. 1B, right panel). However, splicing of the pre-U3 RNA was refractory to anti-SLU7 serum, even at antibody concentrations fivefold greater than that needed to block splicing of actin pre-mRNA (Fig. 1B, left panel). Mature RNA was clearly produced. Control experiments showed that splicing of both actin and U3 precursors was inhibited in a dose-dependent fashion by anti-PRP16 serum (Fig. 1A). PRP16 antibody caused accumulation of the products of step 1—lariat intermediate and exon 1—with both the actin and U3 precursor, although the extent of intermediate accumulation was less with the U3 precursor RNAs (Fig. 1A).

Distance between the branchpoint and 3′ splice site confers SLU7 dependence

Our initial experiment demonstrated that SLU7 was not required for splicing of the U3 precursor, but was necessary for splicing the actin pre-mRNA in vitro. This differential requirement for SLU7 could be a consequence of: (1) the different nucleotide sequences of the exons and introns of the U3 and actin precursors; (2) the difference in spacing between the 3′ splice site and branchpoint; or (3) the branchsite sequence itself, which is UACUAACA in actin versus GACUAACA in U3 (Fig. 2). The third possibility was ruled out by introducing the conserved U nucleotide into the pre-U3 branchpoint sequence in place of G. Splicing of the G and U containing precursors was identical insofar as both required PRP16, but not SLU7 (not shown).

In the actin precursor, the branchpoint is separated from the 3′ splice site by an interval of 38 nt, whereas this distance is only 7 nt in the U3 precursor (Fig. 2). We examined the requirements for SLU7 during splic-

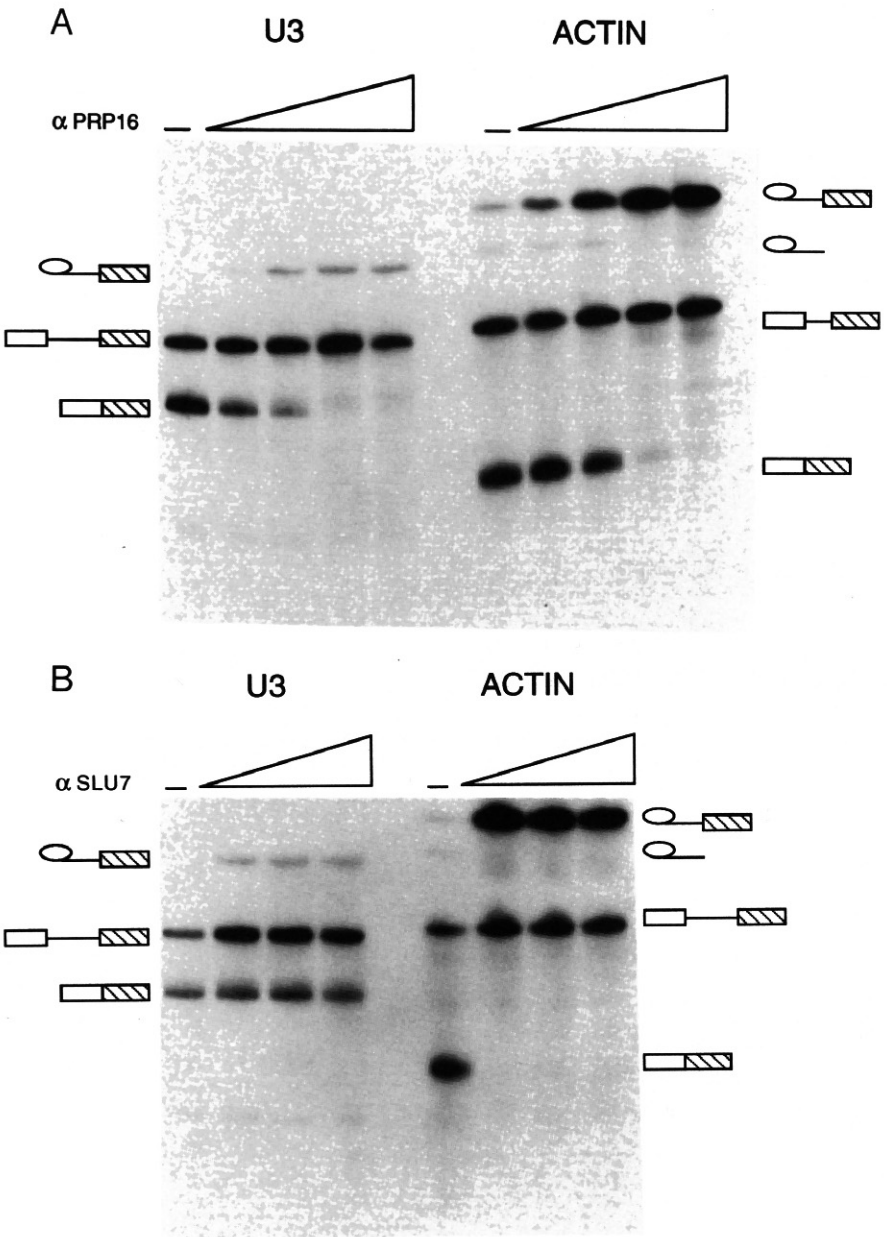

FIGURE 1. SLU7 is dispensable for splicing of U3 precursor RNA. **A:** Splicing of uniformly labeled U3 (left panel) and actin precursor (right panel) was carried out for 20 min in the absence (−) and presence of increasing amounts of anti-PRP16 serum from 0.5 μg to 5 μg. **B:** Same as in A, except affinity-purified SLU7 antibodies were used from 0.1 μg to 0.5 μg. Splicing products were analyzed by denaturing PAGE and autoradiography. Symbols depict the precursor and products of the splicing reaction; exon 1 is represented by the open box, exon 2 by the hatched box, and the solid line represents the intron.

ing of two other pre-mRNAs: pre-RP51A, in which the branchpoint and 3′ splice site are separated by 53 nt; and RP28A, in which this distance is 19 nt (Teem & Rosbash, 1983; Molenaar et al., 1984). In both cases, step 2 was blocked by SLU7 antibody (not shown). This suggested that SLU7 dependence might be determined by the distance between the branchpoint and 3′ splice site, with a threshold value between 7 and 19 nt.

In order to test this hypothesis, we varied the length of the RNA between the branchpoint and 3′ splice site in the actin precursor from 38 nt to 7 nt (Fig. 2) and assayed for splicing of these precursors in extract that had been immunodepleted of either SLU7 (Δ7) or PRP16 (Δ16). All precursors required PRP16 for completion of the second step of splicing, as seen by the accumulation of lariat intermediate and exon 1 and the absence of mature RNA in reactions performed using Δ16 extract (Fig. 3, lanes 3, 6, 9, and 12). The ACT_{12} precursor, in which the distance was decreased to 12 nt, required SLU7 for completion of the second step (lane 5). The ACT_9 precursor showed a partial dependence on SLU7. Mature RNA was formed; however, not to levels observed with wild-type extract (compare lanes 7 and 8). This inhibition was caused by a partial second-step block, because lariat intermediate and exon 1 accumulated (lane 8). In contrast, splicing of the ACT_7 precursor was unaffected by depletion of SLU7 (lane 11), arguing that SLU7 independence was determined by

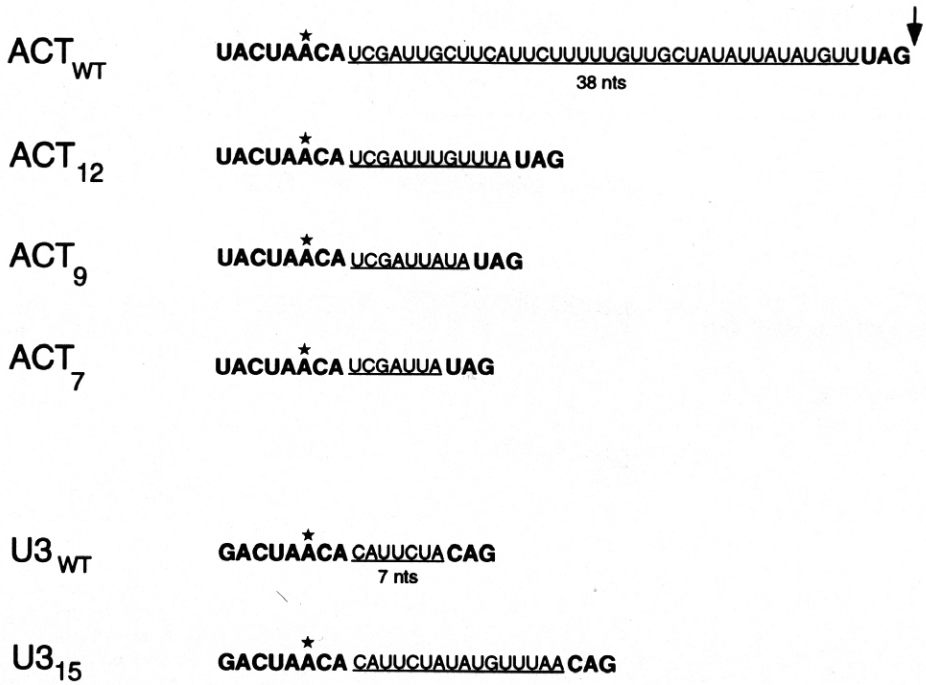

FIGURE 2. Partial intron sequences of the precursor RNAs used in this study. The sequence between the branchpoint a 3′ splice site is underlined. The branch nucleotide is denoted with an asterisk and the branchpoint sequence and 3′ sp site are in bold. The arrow indicates the site of cleavage.

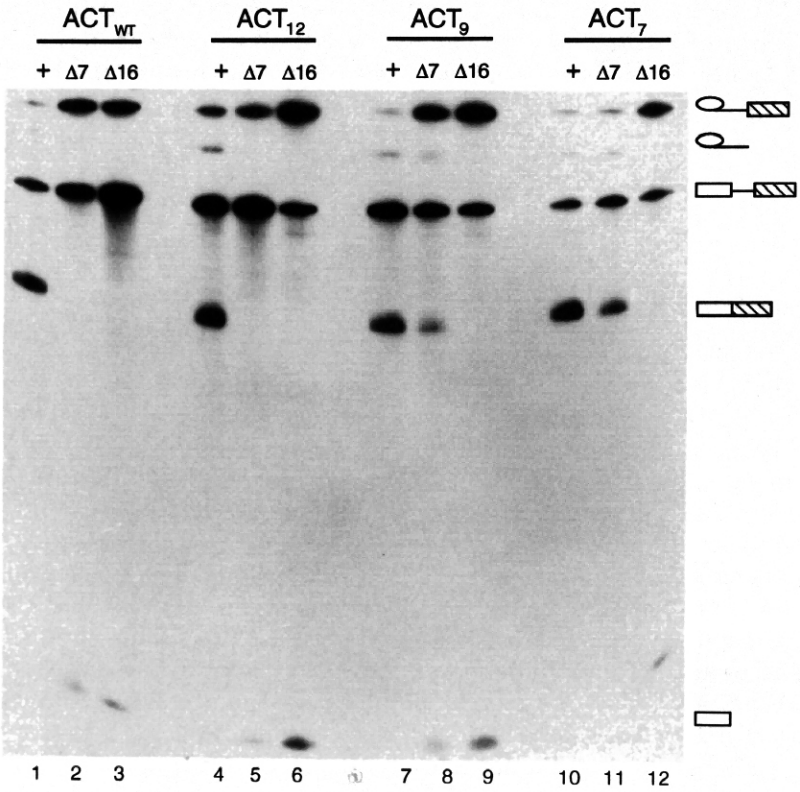

FIGURE 3. Interval between the branch splice site determines SLU7 depende ing of actin pre-mRNA. Splicing reactio formed in wild-type extract (+), or ext been immunodepleted of either SLU7 ((Δ16). Splicing substrates are indicate lanes. The ACT_{12}, ACT_9, and ACT_7 a in which the distance between the bra quence and 3′ splice site has been redu or 7 nt, respectively. After 20-min i room temperature, RNA was extract lyzed by denaturing PAGE and auto

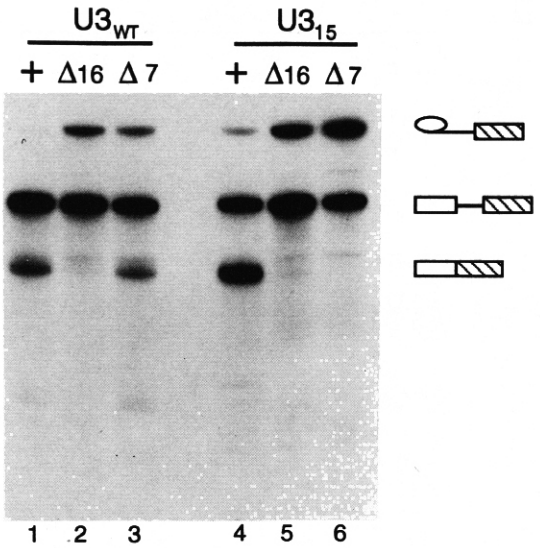

FIGURE 4. Increasing the number of nucleotides between the branchpoint and 3′ splice site from 7 to 15 in the U3 precursor RNA confers SLU7 dependence. Splicing of the $U3_{wt}$ (lanes 1–3) or $U3_{15}$ precursor (lanes 4–6) in wild-type (+), Δ16, and Δ7 extract. RNA was analyzed by denaturing PAGE and autoradiography.

the distance between the branchpoint sequence and 3′ splice site rather than by any feature unique to the U3 precursor.

If only distance matters, then splicing of U3 should become dependent on SLU7 if the interval between the branchpoint and the 3′ splice site were lengthened. Indeed, increasing the spacing in the U3 precursor RNA from 7 to 15 nt conferred SLU7 dependence (Fig. 4). No mature RNA was produced with the $U3_{15}$ precursor in extract that had been immunodepleted of SLU7 (lane 6). However, the first step was unaffected and lariat intermediate accumulated. In contrast, wild-type U3 was spliced in the Δ7 extract (lane 3). Lariat intermediate accumulated for both wild-type U3 and $U3_{15}$ in extract that had been immunodepleted of PRP16 (lanes 2 and 5). Taken together, these results demonstrate that SLU7 is not required for the second step of splicing in vitro of precursor RNAs with a distance between the branchpoint and 3′ splice site of less than 9 nt, independent of the sequence context.

SLU7 associates with the spliceosome

Our data suggest that SLU7 may function by bridging the distance between the branchpoint sequence and the 3′ splice site, thus bringing the 3′ cleavage site into close proximity of the 3′ OH of the 5′ exon at the catalytic center of the spliceosome assembled on the branchpoint. Using immunoprecipitation assays, we asked if SLU7 could bind to the spliceosome and if so, what the requirements for this association were. Spliceosomes were formed on actin precursor RNA in extract that had been immunodepleted of PRP16 (Fig. 5A). After the addition of purified PRP16 protein, with or without ATP, the reaction mixtures were incubated with SLU7 antibodies bound to protein A-Sepharose. The precipitates were analyzed for the labeled precursor RNA, precipitated indirectly by anti-SLU7 serum. Low amounts of pre-mRNA and intermediates were precipitated by anti-SLU7 serum in the absence of PRP16 (Fig. 5A, lanes 5 and 6). In the presence of PRP16, precipitation of intermediates was specifically increased (lane 7), whereas immunoprecipitation of pre-mRNA remained at background levels. Interestingly, SLU7 remained bound after the addition of ATP and the completion of step 2, resulting in immunoprecipitation of the products of splicing—lariat-intron and mature RNA (lane 8). These interactions appeared specific, because precursor was not immunoprecipitated above the background levels in any of the reactions. Two main conclusions can be drawn from these results: (1) PRP16 strongly enhances the association of SLU7 with the spliceosome; and (2) unlike PRP16, SLU7 remains associated with the spliced RNA products after completion of step 2 in the reconstituted system.

Similar experiments were performed with the ACT_7 precursor using extract that had been immunodepleted of SLU7. Mature RNA was formed in this case, because SLU7 is not required for step 2 of splicing of the ACT_7 precursor. Supplementation of the extract with purified SLU7 did not affect the extent of splicing (Fig. 5B, lanes 1–4). Although the reaction products formed in the SLU7-depleted extract were not precipitated by anti-SLU7 antibody (as expected), the mature mRNA and excised lariat intron were both precipitated by anti-SLU7 antibody in SLU7-reconstituted extracts (lanes 7 and 8). Thus, although SLU7 was not required for splicing of the ACT_7 precursor, it appeared to have bound to the spliceosomes and remained associated with the splicing reaction products.

The immunoprecipitation results suggest that SLU7 interacts with the spliceosome independent of the 3′ splice site sequences in the RNA. A more rigorous test for the importance of the pre-mRNA and in particular, the 3′ splice site region, in the association of SLU7 and PRP16 with the spliceosome (assayed by immunoprecipitation), was to physically remove this segment of the RNA. To do this, spliceosomes containing exon 1 and lariat intermediate were formed using wild-type pre-mRNA and extract immunodepleted of PRP16. To aliquots of this reaction mixture, we added either buffer (Fig. 6A, lanes 1–3) or a DNA oligonucleotide complementary to the pre-mRNA (Fig. 6A, lanes 4–6; Fig. 6B, lanes 1–3). The oligonucleotide hybridized to a 16-nt region of the precursor, the upstream margin of which was located 24 nt 3′ of the branchpoint. Targeted cleavage of the RNA-DNA hybrid near the 3′ splice site by endogenous RNaseH converted the lariat intermediates into products that migrated during electrophoresis like excised lariat-intron. The products

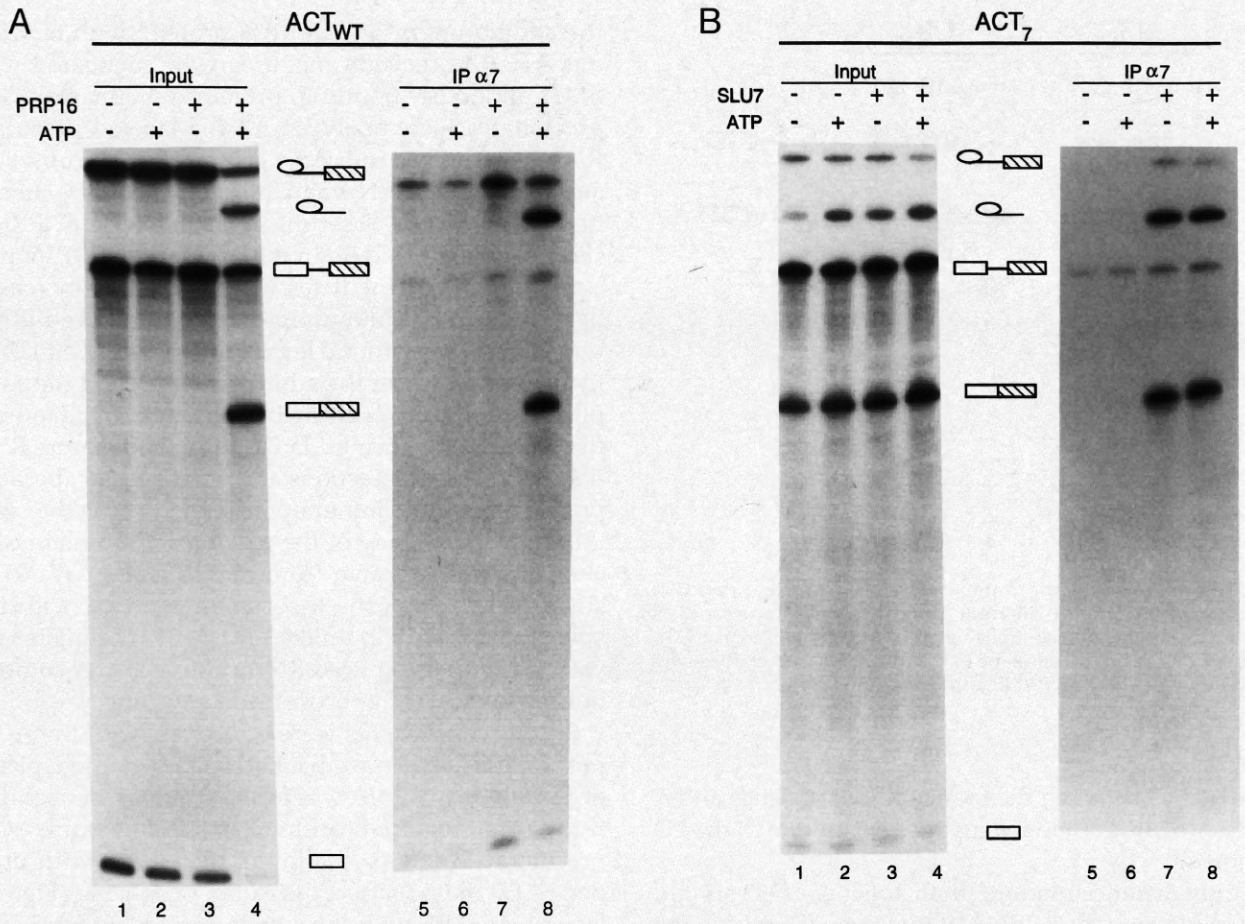

FIGURE 5. SLU7 associates with the spliceosome. Splicing was performed in either Δ16 or Δ7 extract for 20 min at 24 °C in a 40-μL reaction. ATP was depleted by hexokinase present in the extract upon addition of 6 mM glucose. After a 10-min incubation, aliquots of these reactions were supplemented with ATP (6 mM) and purified proteins as indicated above the lanes. From each reaction, a 10-μL aliquot was analyzed (left panels) whereas the rest of the reaction (30 μL) was immunoprecipitated (right panels) with SLU7 antibodies. **A:** Splicing of ACT_{wt} precursor in Δ16 extract in the presence or absence of purified PRP16 (lanes 1–4). Immunoprecipitation with αSLU7 antibodies (lanes 5–8). **B:** Splicing of ACT_7 precursor in Δ7 extract in the presence or absence of recombinant His-SLU7 (lanes 1–4). Reaction mixtures were immunoprecipitated with αSLU7 antibodies (lanes 5–8). Exposure time of the autoradiographs was identical for each panel.

of the cleavage by RNase H are indicated with an asterisk in Figure 6; the 3′ segments of the cleaved RNA molecules were susceptible to exonuclease activity present in the extract as previously reported, whereas the 5′ segments were stable (Rymond & Rosbash, 1985; Schwer & Guthrie, 1992). Precursor RNA served as an internal control for RNase H activity, because it was cleaved specifically in the presence of oligonucleotide (Fig. 6A, lanes 4–6; Fig. 6B, lanes 1–3). As reported previously (Schwer & Guthrie, 1991), PRP16 bound to the spliceosomes in the absence of ATP, resulting in immunoprecipitation of lariat intermediate and exon 1 (Fig. 6A, lane 8). This interaction was destabilized upon addition of ATP (lane 9). The physical removal of the 3′ splice site segment of the lariat intermediates did not affect the interaction of either PRP16 or SLU7 with the spliceosome as assayed by immunoprecipitation of spliceosomes containing these truncated RNAs (Fig. 6A, lanes 10–12; Fig. 6B, lanes 4–6). In fact, the pattern of RNA, co-precipitated with either PRP16 or SLU7, was identical to that of full-size RNA (Fig. 6A, compare lanes 7–9 with lanes 10–12; compare Fig. 5A, lanes 5–8 with Fig. 6B, lanes 4–6). These data showed that the 3′ splice site region of the RNA was not necessary for SLU7 or PRP16 to bind to the spliceosome. Note that the efficiency of immunoprecipitation was between 25 and 30% of input RNA (quantitated by scanning the gels using a phosphorimager).

SLU7 is released when the spliceosome disassembles

When splicing reactions were performed using depleted extracts reconstituted with purified protein factors, SLU7 remained bound to the products after completion of step 2 (Figs. 5, 6). In contrast, SLU7 antibodies did not co-precipitate mature RNA and lariat-intron in unmanipulated extracts (not shown). The reason for this

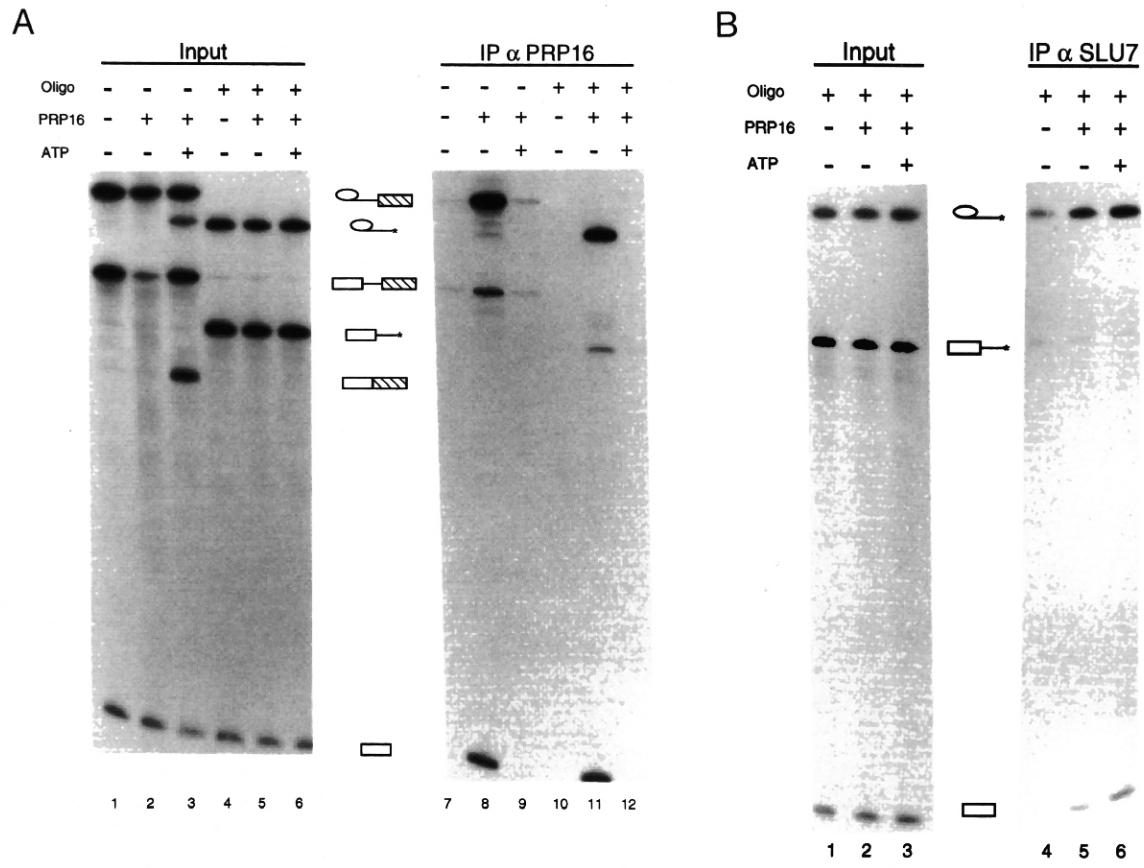

FIGURE 6. SLU7 and PRP16 associate with the spliceosome independent of a 3′ splice site. Splicing was performed in Δ16 extract for 20 min at 24 °C. ATP was depleted by hexokinase present in the extract upon addition of 2.5 mM glucose. After a 10-min incubation, an oligonucleotide, complementary to the precursor RNA 16 nt upstream of the 3′ splice site (5′-TAAACATATAATATAG-3′), was then added as indicated above the lanes and the incubation continued for 10 min. Aliquots of these reactions were supplemented with ATP (5 mM) and purified PRP16 as indicated. From each reaction, a 10-μL aliquot was analyzed, and the remainder (30 μL) was immunoprecipitated with either anti-PRP16 serum or SLU7 antibodies. Exposure time of the autoradiographs was identical for each panel. **A:** Splicing in the absence (lanes 1–3) or presence (lanes 4–6) of oligonucleotide. Immunoprecipitation with αPRP16 serum (lanes 7–12). **B:** Splicing after addition of oligonucleotide in the presence or absence of PRP16 (lanes 1–3). Immunoprecipitation with αSLU7 antibodies (lanes 4–6).

difference may be that the spliceosomes did not disassemble in the reconstituted system. It has been suggested that disassembly of spliceosomes is an energy-requiring process. We tested the effect of increasing ATP concentration on completion of step 2, immunoprecipitation of spliceosomes by anti-SLU7 serum and release of mature RNA from the spliceosome.

Lariat intermediates were formed in a standard splicing reaction containing 2 mM ATP and extract that had been depleted of PRP16. ATP was then depleted by endogenous hexokinase upon the addition of glucose (2.5 mM) during 10 min of incubation. PRP16 and increasing amounts of ATP were added and the incubation continued for 10 min. Aliquots were then analyzed for the production of mature RNA (Fig. 7A, Input) and for immunoprecipitation of spliceosomes by anti-SLU7 serum (IPαSLU7). Mature RNA was formed upon addition ≥ 2.5 mM ATP (Fig. 7A, lanes 3–5). Immunoprecipitations with anti-SLU7 serum demonstrated that SLU7 co-precipitated the products of the splicing reaction only at concentrations of 2.5 mM of ATP (Fig. 7A, lane 8). Lower concentrations of ATP (0.5 mM) were sufficient to promote formation of mature RNA and immunoprecipitation of splicing products by SLU7 antibodies (not shown). In several experiments in which the amounts of glucose and ATP varied, we determined that SLU7 co-precipitated mature RNA and intron whenever the concentration of ATP was equal to or lower than the concentration of glucose added (not shown). (Note that the Mg^{2+} concentrations were adjusted to be equimolar with ATP in all experiments).

In order to test whether mature RNA was released from the spliceosomes, we employed separation of splicing complexes by native gel electrophoresis (Company et al., 1991). In extract depleted of PRP16, a discrete complex was formed, referred to as complex A (Fig. 7B, lane 1). The RNA was eluted from this complex and analyzed by denaturing gel electrophoresis.

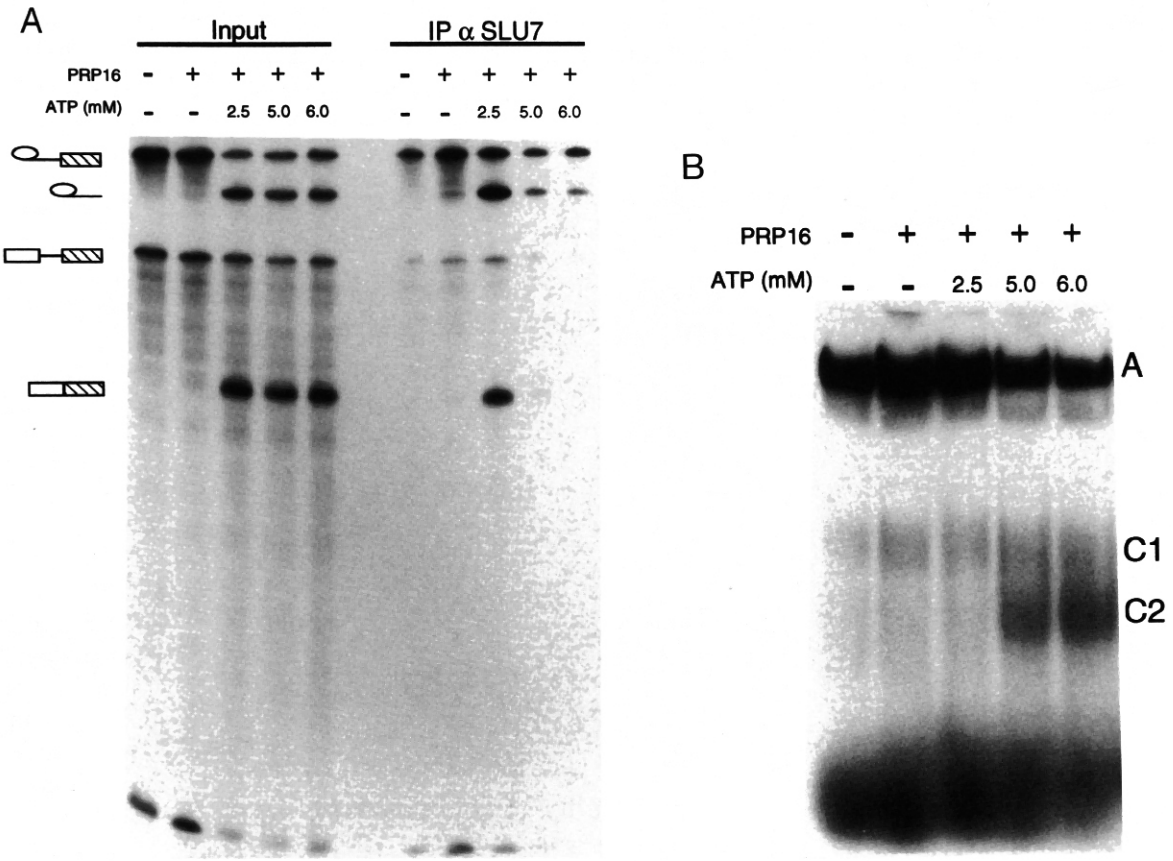

FIGURE 7. SLU7 remains associated with the spliceosome until mature RNA is released. Splicing was performed in Δ16 extract for 20 min at 24 °C. After depletion of ATP by the addition of 2.5 mM glucose and incubation for 10 min, aliquots of these reactions were supplemented with ATP (from 2.5 to 6.0 mM) and purified PRP16 as indicated. **A:** 10-μL aliquot from each reaction was analyzed (lanes 1–5) and 30 μL were immunoprecipitated with αSLU7 antibodies (lanes 6–10). **B:** 10- μL aliquots were adjusted to 0.4 μg/mL of heparin and analyzed on a native agarose–acrylamide composite gel. Positions of complexes A, C1, and C2 are indicated.

This verified that complex A contained predominantly the products of the first catalytic reaction, lariat intermediate and exon 1, as well as small amounts of pre-mRNA (not shown). Addition of PRP16 did not change this (lane 2). When ATP (2.5 mM) was added together with PRP16, complex A persisted (lane 3), however, the RNA contents of complex A now included mature RNA and lariat intron. When the ATP concentration was increased (≥5 mM), a new complex formed, referred to as complex C2 (Fig. 7B, lanes 4–5). Analysis of the RNA species in this complex verified that it was mature RNA (not shown). The transition from complex A to complex C2 is indicative of spliceosome disassembly.

Several conclusions could be drawn from these results: (1) the depletion/reconstitution assays uncover an ATP requirement that could not be detected in a standard splicing reaction; (2) release of mature RNA from the spliceosome requires ATP; (3) ATP concentrations required for release are higher than needed for production of mature RNA; and (4) SLU7 associates specifically with the spliceosome and not the products of the splicing reaction.

DISCUSSION

The first transesterification step of yeast pre-mRNA splicing entails attack by the branchpoint nucleotide sugar at the 5′ splice site, resulting in the formation of a 2′-5′ branched lariat intermediate. This step can occur in the absence of an intact 3′ splice site (Rymond & Rosbash, 1985; Vijayraghavan et al., 1986). In order for the second catalytic step to occur, the 3′ splice site must be brought into proximity of the 3′-OH nucleophile of the upstream exon at the "active site" of the spliceosome. The active site is presumably situated at the branchpoint after completion of step 1. The distance between the branchpoint and 3′ splice site can vary from 5 nt, as in the case in *MATa1* (Miller, 1984), to 134 nt in *TUB3* (Schatz et al., 1986), with an average between 20 and 60 nt (Parker & Patterson, 1987; Ry-

mond & Rosbash, 1992). How is this distance bridged? Are changes elicited in the spliceosome to accomplish this and, if so, which factors are responsible? The present findings provide biochemical insights into these questions.

Using precursor RNAs in which the interval between the branchpoint and 3′ splice site varies, we tested the requirement for two second-step splicing factors: PRP16 and SLU7. We found that, whereas PRP16 is essential for splicing of all precursor RNAs tested, SLU7 is needed for splicing of actin, RP28A, and RP51A precursors, but is dispensable for splicing of the U3 precursor RNA. To show that this differential requirement was solely due to the short spacing between the branchsite sequence and the 3′ splice site of 7 nt in the U3 precursor, we shortened this interval in the actin pre-mRNA. The results demonstrated that splicing of the actin pre-mRNA in which the length between branchpoint and 3′ splice site was reduced to 7 nt proceeds in the absence of SLU7. In a reverse experiment, when the length of the U3 branchpoint to 3′ splice site region was increased to 15 nt, the presence of SLU7 became critical for completion of the second cleavage/ligation reaction.

Our in vitro results are in agreement with in vivo studies. In a reporter gene containing two competing 3′ splice sites, the *slu7-1* mutation decreased splicing to the distal 3′ splice site, located 39 nt downstream of the branchpoint, without affecting splicing to a proximal 3′ splice site situated 5 nt from the branchpoint (Frank & Guthrie, 1992). Our findings argue that this is not unique to the *slu7-1* mutation or the competition construct, but rather that the need for SLU7 in vivo and in vitro is dictated by the distance between the branchpoint and the 3′ splice site.

SLU7 acts after PRP16 during the second step of splicing in vitro; whereas PRP16 function entails NTP hydrolysis, SLU7 does not require a nucleotide cofactor (Ansari & Schwer, 1995; Jones et al., 1995). NTP hydrolysis by PRP16 elicits a change in the spliceosome that renders the previously accessible 3′ splice site inaccessible to targeted RNase H cleavage (Schwer & Guthrie, 1992). In fact, protection of the 3′ splice site region of the lariat intermediate from oligonucleotide-directed RNaseH cleavage requires both PRP16 and SLU7 (data not shown).

Does SLU7 associate with the spliceosome and, if so, what are the requirements for the association? This question was addressed by immunoprecipitation experiments using anti-SLU7 serum. We showed that SLU7 antibodies specifically co-precipitate the products of step 1—lariat intermediate and exon 1—in the presence of PRP16. In the absence of PRP16, only trace amounts of pre-mRNA and intermediates are precipitated. The interaction of SLU7 with the spliceosome is independent of ATP. This result suggests that SLU7 may associate with the spliceosome through direct contact with PRP16. When ATP is added, mature mRNA is formed. Interestingly, SLU7 remains bound to the products of the splicing reaction—lariat-intron and mRNA in the reconstituted reaction. This can be explained by positing that the spliceosomes in our assay system do not disassemble after splicing is complete.

PRP22, a putative NTPase, is required for release of mature mRNA from spliceosomes in vitro (Company et al., 1991). It has not been determined whether release is actually dependent on NTP hydrolysis by PRP22. We now demonstrate that release of mRNA from the spliceosome requires ATP; in fact, the ATP concentrations needed for disassembly are higher than those required to promote the second cleavage/ligation reaction catalyzed by PRP16. The different ATP requirement observed for splicing in whole-cell extract and splicing in the reconstituted system may be explained if high concentrations of ADP (a product of phosphorylation of glucose by hexokinase) inhibit release of mature RNA. However, an answer to this question has to await the development of a more purified system in which all components can be controlled.

We have shown that SLU7 is required for splicing of precursors in which the 3′ splice site is distant from the branchpoint. How could SLU7 perform this function? Does SLU7 recognize the segment in the pre-mRNA upstream of the 3′ splice site? SLU7 may interact with the spliceosome, in particular with the U5 snRNP, to position the 3′ splice site close to the 3′ hydroxyl group of exon 1 at the active site. Many lines of evidence point to the importance of U5 snRNA and its associated proteins in 3′ splice site choice (Patterson & Guthrie, 1987; Newman & Norman, 1991, 1992). *slu7-1* was identified on the basis of synthetic lethality with a U5 snRNA mutant (Frank et al., 1992). The U5 snRNP protein PRP8, in addition to its role in spliceosome assembly, is important for recognition of a polypyrimidine tract upstream of a distant 3′ splice site (Jackson et al., 1988; Brown & Beggs, 1992; Umen & Guthrie, 1995a). In support of the functional data, UV crosslinking studies show that PRP8 is near the 3′ splice site (Teigelkamp et al., 1995a, 1995b; Umen & Guthrie, 1995a, 1995b). In addition, SLU7 and PRP16 can be crosslinked to the 3′ splice site, suggesting direct contact of these proteins with this region in the RNA (Umen & Guthrie, 1995b). In particular, a 15-nt T1 fragment (containing a single ^{32}P) was immunoprecipitated by SLU7 and PRP16 antisera upon UV crosslinking (Umen & Guthrie, 1995b). The present study shows that elimination of the corresponding segment in the actin pre-mRNA (16-nt upstream of the 3′ cleavage site) does not impact on the association of either PRP16 or SLU7 with the spliceosome. Consequently, we conclude that the contacts (established by crosslinking) are not essential, at least for the initial interaction of SLU7 and PRP16 with the

spliceosome, and we suggest instead that protein/protein interactions are involved in recruitment of these splicing factors.

When the 3′ splice site is situated only 7 nt away from the branchpoint, the physical distance between the exon-hydroxyl and the 3′ splice site may not be a limiting factor. In this case, positioning of U5, the exon, and the 3′ splice site would not depend on SLU7, even though SLU7 is present in the spliceosome. Only when the physical distance is greater would recruitment of the 3′ splice site become limiting and the SLU7 requirement be made evident.

MATERIALS AND METHODS

Splicing extract preparation and in vitro splicing assays

Whole-cell extract was prepared from yeast strain BJ2168 (Jones, 1991) as described (Ansari & Schwer, 1995). Splicing assays were performed in 10-μL reactions containing 40% whole-cell extract, 50,000 cpm (1 fmol) of labeled precursor RNA, 60 mM potassium phosphate, 2.5 mM $MgCl_2$, and 2 mM ATP (Lin et al., 1985). Precursor RNAs were synthesized by run-off transcription using T7 RNA polymerase (Boehringer).

Immunodepletion of splicing extracts

Splicing extract (120 μL) was immunodepleted of either PRP16 or SLU7 by incubation with 30 μL of protein A–Sepharose-purified anti-PRP16 serum (10 mg/mL) or 15 μL of affinity-purified SLU7 antibodies (1 mg/mL). After incubation for 50 min at 0 °C, the extract-antiserum mixture was added along with 30 μL of PBS (10 mM Na_3PO_4, pH 7.2, 150 mM NaCl) to 150 μL of protein A–Sepharose suspension (0.1 g/mL) that had been washed previously three times with 1 mL of PBS. After gentle shaking at 4 °C for 50 min on a nutator, the suspension was centrifuged briefly, and the extract removed. Six microliters of the immunodepleted extract was used in a 10-μL splicing reaction as described above. Complementation of immunodepleted extract was tested using either purified PRP16 (20 ng) (Schwer & Guthrie, 1991) or purified His-SLU7 (40 ng) (Ansari & Schwer, 1995).

Immunoprecipitation

Immunoprecipitations were performed by pre-incubating 5 μL of anti-PRP16 serum or 5 μL of SLU7 antibodies with 40 μL of protein A–Sepharose (0.1 g/mL) in 400 μL of IPP_{500} (10 mM Tris, pH 8, 500 mM NaCl, 0.1% NP40) for 1 h at 4 °C. The beads were washed three times in IPP_{150} (10 mM Tris, pH 8, 150 mM NaCl, 0.1% NP40). Splicing reactions (40 μL total volume) were performed under the conditions indicated. Ten-microliter aliquots of the splicing reactions were halted by the addition of 200 μL stop solution (50 mM sodium acetate, 1 mM EDTA, and 3 μg/mL *Escherichia coli* tRNA), phenol-extracted, precipitated, and the RNA analyzed by PAGE. The additional 30 μL of the splicing reactions were added to the prepared beads and incubated at 4 °C on a nutator for 1 h. After the beads were washed three times with 400 μL of IPP_{150}, 200 μL of stop solution was added, the reactions were phenol/chloroform extracted, and the RNA analyzed by denaturing PAGE.

Agarose-acrylamide composite gels

Splicing reactions (10 μL) were stopped by addition of an equal volume of heparin buffer (60 mM KPO_4, pH 7.0, 2.5 mM $MgCl_2$, 3% PEG 8000, and 0.8 μg/mL heparin) and incubated at 0 °C for 15 min prior to loading on a 0.5% agarose, 2.5% acrylamide-bis (60:1) gel. Electrophoresis was carried out at 4 °C in 25 mM Tris-acetate, pH 7.6, 6 mM potassium acetate, and 2 mM magnesium acetate (Goodwin & Dahlberg, 1982).

Plasmids and mutagenesis

Plasmid pT7Actin has been described previously (Lin et al., 1985). A 1.2-kb *Sac* II–*Xho* I fragment from a Bluescript (Stratagene) plasmid containing the U3 gene (Hughes et al., 1987; Myslinski et al., 1990) was ligated into the *Sac* II/*Sal* I-digested pGEM-3 (Promega) vector to create pT7U3. The original U3 plasmid was kindly provided by Josette Banroques. The templates for the ACT_{12}, ACT_9, ACT_7, and $U3_{15}$ precursors were generated by PCR-mediated site-directed mutagenesis of the pT7Actin and pT7U3 plasmids. The mutagenic primers, ACT_{12}, 5′-TAACATCGATTTGTTTATAGG TTGCTGC; ACT_9, 5′-TAACATCGATTATATAGGTTGCGCT TGG; ACT_7, 5′-TAACATCGATTATAGGTTGCTGCTTTGG; and $U3_{15}$, 5′-TAACACATTCTATATGTTTACAGTAGGATC, were designed to delete sequences between the actin branchpoint and 3′ splice site to 12, 9, or 7 nt or to insert 8 nt between the U3 branchpoint and 3′ splice site. For the ACT_7 and $U3_{15}$ templates, PCR was performed using the mutagenic primer and a downstream primer, ACT-3′, [5′- CCCCAAGCTTGGG CTGCAGG] complementary to pT7Actin polylinker sequences or U3-3′, [5′-CAAGCTTGCATGCCTGCAG] (complementary to pT7U3 polylinker nt 6–24). A second PCR reaction was performed using a primer with complementarity to the mutagenic primer at the branchpoint sequence, [5′-GAAGCAATCGATGTTAGTAC] (actin) or [5′-GAATGTG TTAGTCAAAAGCTG] (U3), and a second primer complementary to intron sequences, ACT-5′, [5′-CCCAATTGCTCG AGAGATTTC] (actin nt 736–756) or U3-5′, [5′-GGCGCTCTA GAACTAGCG] (pT7U3 polylinker sequences). PCR products from the two reactions were mixed and used in a subsequent PCR reaction with the two outside primers (ACT-5′ and ACT-3′ or U3-5′ and U3-3′). The mutant actin PCR products were digested with *Xho* I and *Hin*d III and inserted into the *Xho* I/*Hin*d III-digested pT7actin vector to produce the ACT_7 template. The PCR product from the U3 reaction was digested with *Pst* I and *Xba* I and inserted into the *Pst* I/*Xba* I-digested pT7U3 vector to create the $U3_{15}$ template.

For the ACT_{12} and ACT_9 templates, PCR was performed using the appropriate mutagenic primer, and the ACT-3′ primer (described above). The resultant PCR products were digested with *Cla* I and *Hin*d III and inserted into the *Cla* I/*Hin*d III-digested pT7Actin vector. The actin and U3 templates were digested with *Hin*d III or *Hpa* I, respectively, prior

to use in the in vitro transcription reactions. All mutant clones were sequenced throughout the PCR-amplified region to ensure that no extraneous mutations were introduced during amplification.

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