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Functional and physical interaction between the yeast splicing factors Slu7 and Prp18

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ABSTRACT

We show that the requirement for Prp18 during the second step of actin pre-mRNA splicing *in vitro* is dictated by the distance between the branch point and the 3′ splice site. Prp18 is dispensable for splicing of precursor RNAs in which the interval between the branch point and 3′ splice site is <12 nt. This resembles the requirement for another second step factor, Slu7. Excess Slu7 protein can bypass the need for Prp18 *in vitro*, suggesting that Slu7 and Prp18 function in a concerted manner. Physical interaction between Slu7 and Prp18 was demonstrated by using the two-hybrid assay. Deletion mutants of *SLU7* were tested for their ability to support growth of a *slu7* null strain. Removal of 199 amino acids from the N-terminus of the 382 amino acid Slu7 protein did not affect cell viability at 25°C. A more extensive N-terminal deletion of 221 amino acids was lethal, as was a C-terminal deletion of 47 amino acids. Deleted versions of Slu7 were also tested for interaction with Prp18 in the two-hybrid system. We define a segment of Slu7 from residue 200 to 224 that is necessary for interaction with Prp18.

INTRODUCTION

Splicing of yeast pre-mRNAs occurs in a large complex, the spliceosome, composed of snRNPs (U1, U2, U4, U5 and U6) and protein factors that assemble onto the precursor RNA in a coordinated fashion. The precursor RNA is spliced by two consecutive transesterification reactions. In the first step, the 5′ splice site is cleaved and a branched lariat intermediate is formed; in the second step the 3′ splice site is cleaved and ligated to the 5′ exon (1–3). Accurate recognition and selection of splice sites involves numerous RNA–RNA and protein–RNA interactions (4,5).

Events specific to the second step include identification of the 3′ splice site, its juxtaposition with the 3′ hydroxyl of exon 1 and the transesterification reaction joining the exons. The yeast splicing proteins Prp16, Prp17, Prp18, Slu7 and Ssf1 are required for the second catalytic step (6–10). The earliest defined stage during step 2 is the binding of Prp16 to spliceosomes containing the 5′ exon and lariat intermediate. Prp16, a 121 kDa RNA-dependent NTPase, then hydrolyzes ATP (6). This elicits a conformational change in the spliceosome, seen as protection of the previously accessible 3′ splice site from targeted RNase H digestion (11). The 44 kDa Slu7 protein is also required for this conformational change (12). Slu7 binds to the Prp16-containing spliceosome, as demonstrated by immunoprecipitation of the products of step 1 by Slu7-specific antibodies (12). Slu7 acts subsequent to NTP hydrolysis by Prp16, i.e. the Slu7-dependent reaction during step 2 is ATP independent (7,10). Prp16 is liberated from the spliceosome after completion of step 2, whereas Slu7 remains bound until the spliceosome is disassembled (12).

Biochemical and genetic data implicate Slu7 in 3′ splice site selection (12,13). The distance between the branch point and the 3′ splice site can impact on 3′ splice site utilization (14–16). Slu7 is essential for splicing *in vitro* of precursor RNAs in which the distance between the branch point and 3′ splice site is >9 nt (12). In an *in vivo* situation where there is a choice between two competing 3′ splice sites, the *slu7-1* mutant affects only the use of the branch-distal site (13). Although the initial binding of Slu7 to the spliceosome is not via the 3′ splice site segment in the RNA (12), Slu7 can be UV crosslinked to this region (17).

PRP18 is a non-essential gene encoding a 29 kDa protein; cells that are disrupted for PRP18 grow slowly and have a temperature-sensitive growth phenotype (9). Horowitz and Abelson (9) have shown that the second step of splicing is inhibited, but not abolished, in extracts immunodepleted of Prp18. The low efficiency of splicing in these extracts could be restored by adding back recombinant Prp18 protein (18). Genetic interactions between Prp18, Slu7 and U5 snRNA have been reported (7,19). The *prp18-1* and *slu7-1* mutant alleles each show synergistic lethal effects with mutant versions of U5 snRNA and with one another (19). Overexpression of *SLU7* can suppress the temperature-sensitive growth defect of *prp18-1* (7).

We now show that Prp18, like Slu7, is dispensable for splicing *in vitro* of precursor RNAs in which the 3′ splice site is in close proximity to the branch point. Prp18 is required for splicing of pre-mRNAs in which the distance is >9 nt. The requirement for Prp18 in the second step *in vitro* can be bypassed by excess Slu7 protein. A model for Prp18 function will be discussed, taking into account our findings that Prp18 can physically interact with Slu7 in the two-hybrid assay.

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MATERIALS AND METHODS

Expression of Prp18 in bacteria

The *PRP18* gene was isolated by PCR amplification of total genomic DNA from a wild-type *Saccharomyces cerevisiae* strain, using oligonucleotide primers corresponding to the 5′- and 3′-ends of *PRP18* (9). These oligonucleotides introduced an *Nde*I restriction site at the start codon and a *Xho*I cleavage site 3′ of the stop codon. The amplified DNA fragment was digested with *Nde*I and *Xho*I and ligated into the T7 RNA polymerase-based expression vector pET14b, so as to fuse the open reading frame of *PRP18* to an N-terminal leader peptide containing six histidines. pET14-PRP18 was transformed into *Escherichia coli* strain BL21(DE3). A 1 l culture of BL21(DE3) pet14-PRP18 was grown at 37°C in LB medium containing 0.1 mg/ml ampicillin to an A_{600} of 0.7. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to 0.4 mM. Cells were harvested 3 h later by centrifugation. The cell pellet was stored at –80°C.

Purification of Prp18

All operations were performed at 4°C. The cell pellet was suspended in 70 ml lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 10% sucrose, 0.2 mg/ml lysozyme). The suspension was incubated for 30 min, then adjusted to 0.1% Triton X-100 and incubated for an additional 30 min. Soluble and insoluble fractions were separated by centrifugation. The soluble fraction (100 mg protein) was adjusted to 40% saturation with ammonium sulfate. After stirring for 30 min, the precipitate was collected by centrifugation. The pellet was suspended in 7 ml buffer A (20 mM Tris, pH 7.6, 50 mM KCl, 1 mM β-mercaptoethanol, 20% glycerol) and then dialyzed against the same buffer. The dialysate was incubated with 2 ml Ni–NTA–agarose resin (Quiagen) on a nutator for 1 h. The resin was recovered by centrifugation and then subjected to repeated cycles of washing with buffer W (10 mM imidazole, 20 mM Tris, pH 7.6, 250 mM NaCl, 1 mM β-mercaptoethanol, 10% glycerol). Adsorbed material was eluted step-wise with 50 and 250 mM imidazole in buffer E (20 mM Tris, pH 7.6, 1 mM β-mercaptoethanol, 10% glycerol). Elution of the Prp18 polypeptide was monitored by SDS–PAGE analysis of the column fractions. The fractions containing Prp18 were pooled (4 mg protein) and dialyzed against buffer D (20 mM HEPES, pH 7.9, 0.2 mM EDTA, 50 mM KCl, 0.5 mM DTT, 20% glycerol). Protein concentrations were determined using the Bradford dye reagent (BioRad), with bovine serum albumin as the standard. The recombinant Prp18 preparation was essentially homogeneous with respect to the His-tagged Prp18 polypeptide, which migrated with an apparent size of 35 kDa during SDS–PAGE (not shown).

Anti-Prp18 serum

Antiserum was raised against recombinant Prp18 that had been purified by preparative SDS–PAGE and concentrated by ultrafiltration with a Centricon filter to 2.5 mg/ml in 50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS. Immunization was performed at Pocono Hill Rabbit Farm and Laboratory (Canadensis, PA). Polyclonal anti-Prp18 serum was purified with protein A–Sepharose and concentrated to 15.6 mg/ml in phosphate-buffered saline (PBS; 10 mM Na_3PO_4, pH 7.2, 150 mM NaCl).

Immunodepletion

Whole-cell yeast splicing extract was prepared using the liquid nitrogen method (10). To deplete the extract of Prp18, 45 µl purified IgG were incubated with 120 µl extract for 45 min on ice. Protein A–Sepharose (15 mg, washed with PBS) was then mixed with the extract/antibody mixture for 1 h. The slurry was centrifuged and the supernatant (Δ18 extract) used for *in vitro* splicing assays. Slu7 depletions were performed as described previously (10) using affinity purified anti-Slu7 antibodies. Control mock-depleted extracts were prepared using PBS in lieu of antiserum.

mRNA splicing *in vitro*

Radiolabeled intron-containing precursor RNAs were transcribed from plasmid templates by T7 RNA polymerase and purified by gel filtration through Sepharose CL-6B (10). Splicing reaction mixtures (10 µl) containing 8 mM HEPES, pH 7.0, 60 mM potassium phosphate, pH 7, 20 mM KCl, 80 µM EDTA, 0.2 mM DTT, 3% (w/v) PEG 8000, 8% (v/v) glycerol, 2.5 mM $MgCl_2$, 2 mM ATP, 40% (v/v) yeast whole cell extract and 2 fmol precursor RNA (100 000 c.p.m.) were incubated at room temperature (20). The reaction products were analyzed by electrophoresis through a 6% polyacrylamide gel containing 7 M urea in TBE. The labeled RNAs were visualized by autoradiography.

Deletion mutants of *SLU7*

A 1.8 kb restriction fragment (*Eco*RI–*Xba*I) containing the entire *SLU7* gene (13) was inserted into pBluescript (KS–) to yield pKS-SLU7. Uracil-substituted single-stranded DNA was prepared for use as a template for oligonucleotide-directed mutagenesis. A DNA primer was designed to create an *Nde*I cleavage site at the translational initiation codon of *SLU7*. The expression cassette was then inserted into pSE360 (*URA3*, *CEN*) and pSE358 (*TRP1*, *CEN*) to yield p360-SLU7 and p358-SLU7. N-Terminal deletion variants of *SLU7* were generated by PCR amplification using oligonucleotide primers designed to introduce *Nde*I restriction sites at the codons for Gln40, Lys81, Cys125, Thr163, Glu184, Lys200 and Glu222, while substituting the respective amino acids by methionine. The PCR products were digested with *Nde*I and *Eco*RI (+1 to +1289) and inserted into p358-SLU7. In order to create C-terminal deletions, a *Bam*HI restriction site was introduced at position +1150 by site-directed mutagenesis, yielding p358-SLU7(B). The C-terminal deletions were engineered using primers that introduced a stop codon at residues 362 and 336. The PCR fragments were restricted with *Nde*I and *Bam*HI and inserted into p358-SLU7(B), so as to replace the wild-type sequence. Slu7(1–307) was created by inserting an *Nde*I–*Bgl*II fragment from p358-SLU7 into the p358-SLU7(B) cassette.

SLU7 gene disruption

The SLU7 gene was disrupted by insertion of a *hisG-URA3-hisG* cassette (21). We first constructed pΔslu7, a Bluescript-based plasmid in which the region of *SLU7* from nucleotide +104 to +1048 had been replaced by a *hisG-URA3-hisG* cassette. The haploid wild-type strain A364A (MAT**a**, *leu2*, *ura3*, *trp1*, *his7*) was transformed with p358-SLU7 to obtain strain YXP1 (MAT**a**, *leu2*, *ura3*, *his7*, *p358-SLU7*). YXP1 was then transformed with linearized pΔslu7 and Ura^+ transformants were selected. Insertion

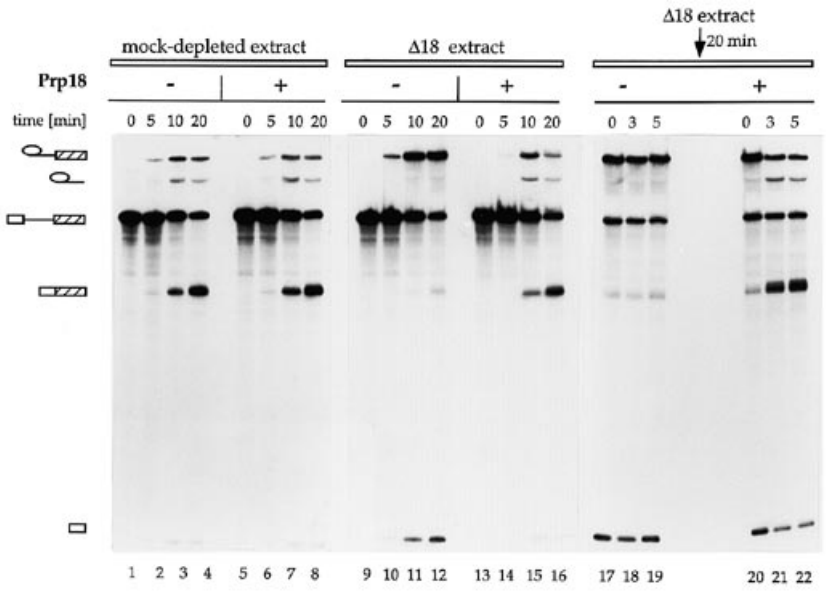

Figure 1. Requirement for Prp18 during the second step of pre-mRNA splicing *in vitro*. Yeast whole cell extracts were mock-depleted (mock-depleted extract) or immunodepleted of Prp18 (Δ18 extract) and incubated with ^{32}P-labeled actin pre-mRNA. The reaction mixtures (10 µl) were supplemented with 0.5 µg purified Prp18 where indicated by (+). The reactions were halted after incubation for 5, 10 or 20 min at 22°C. In the experiment shown in the right panel (lanes 17–22), ^{32}P-labeled actin pre-mRNA was incubated for 20 min in Δ18 extract. The mixtures were then supplemented with 0.5 µg Prp18 (+) or an equal vloume of buffer (–). The reactions were terminated immediately prior to adding Prp18 (time 0) and 3 and 5 min after adding Prp18. The RNA reaction products were analyzed by PAGE. The positions of the pre-mRNA and the products of steps 1 and 2 are indicated by symbols on the left; exon 1 is depicted by an open rectangle, the intron as a straight line and exon 2 as a hatched box.

at the correct position was confirmed by Southern blot analysis. Selection in the presence of 5-fluoroorotic acid (FOA) yielded a strain that had lost the *URA3* gene via excisive recombination between flanking *hisG* direct repeats (21). This strain, YXP2 (MAT**a**, *leu2*, *ura3*, *his7*, *slu7*::*hisG*, *p358-SLU7*), was then transformed with p360-SLU7 (*CEN*, *URA3*). The cells were grown in liquid culture in SD-ura to maintain p360-SLU7 but allow for loss of p358-SLU7 (*CEN*, *TRP1*). Strain YXP3 (MAT**a**, *leu2*, *his7*, *trp1*, *slu7*::*hisG*, *p360-SLU7*) was used for testing the ability of the various deletion mutants of *SLU7* (*TRP1*, *CEN*) to support growth using the plasmid shuffle technique (22).

Two-hybrid assay system

Matchmaker System 2 (Clontech) was used in these studies. p358-SLU7 was digested with *Nde*I and *Eco*RI and inserted into pAS2-1 to create a fusion between *SLU7* and the *GAL4* binding domain. The resultant plasmid is pGBD-SLU7. pGBD-SLU7 can complement a *slu7* null strain. The N-terminal deletions were engineered in the same way by inserting the *Nde*I–*Eco*RI fragments from the corresponding p358-SLU7 deletion mutation into pAS2-1. To create fusions with the C-terminal deletion versions, the *Nde*I–*Bam*HI fragment from p358-SLU7(B) was inserted into pAS2-1 restricted with *Nde*I and *Bam*HI. pGBD-PRP18 was created by insertion of the *Nde*I–*Xho*I fragment from pET14-PRP18 into pAS2-1. The fusion of *SLU7* to the *GAL4* activation domain was constructed by inserting the *Nde*I (filled in by T4 DNA polymerase)–*Eco*RI fragment from p358-SLU7 into pACT2 (*Sma*I–*Eco*RI), yielding pGAD-SLU7. To construct pGAD-PRP18, the *Nde*I (blunted with T4 DNA polymerase)–*Xho*I fragment from pET14-PRP18 was ligated into pACT2 digested with *Sma*I and *Xho*I.

Yeast strains Y187 (with the *lacZ* reporter gene) and Y190 (with the *lacZ* and *HIS3* reporter genes) (23) were each co-transformed with two plasmids and transformants were selected on medium lacking Trp and Leu. To test for expression of the reporter genes, cells were patched onto SD-trp-leu plates and grown overnight. A filter paper (Whatman 50) was placed on the patches. The filter paper was removed and frozen in liquid nitrogen in order to permeabilize the cells. The filter was then placed onto a second filter paper that had been soaked in X-gal solution. After incubating at 30°C for 2–4 h, possitive patches turned blue. Transformants of Y190, a strain that contained the *lacZ* and *HIS3* reporter genes (23), were grown on SD-trp-leu-his containing 25 mM 3-amino-1,2,4-triazole (3-AT; Sigma). Expression of *lacZ* was assayed as described above.

RESULTS

Prp18-dependent splicing *in vitro*

We established an *in vitro* depletion/complementation assay to assess the requirement for Prp18 during step 2 of splicing. Anti-Prp18 IgG was used to deplete Prp18 from a yeast whole cell extract (Δ18 extract). Control extracts were mock-depleted in parallel. We compared the ability of the two extracts to catalyze splicing of ^{32}P-labeled actin pre-mRNA. A kinetic analysis is shown in Figure 1. In the control extract, lariat intermediate (the step 1 product) was formed within 5 min. Mature mRNA appeared at 10 min and increased at 20 min (Fig. 1, lanes 1–4). Addition of pure recombinant Prp18 protein to the control extract had no effect on reaction kinetics (Fig. 1, lanes 5–8).

The second catalytic step was blocked in extracts that had been depleted of Prp18. The products of step 1 (lariat intermediate and

5′ exon) accumulated to high levels during a 20 min incubation and almost no mature mRNA was formed (Fig. 1, lanes 9–12). However, when the Δ18 extract was supplemented with purified Prp18, the rate and extent of splicing was restored to a level comparable with that of the control extract (Fig. 1, lanes 13–16). By varying the order of addition, we demonstrated that Prp18 specifically complements a defect in step 2. Step 1 products that had accumulated during a 20 min reaction catalyzed by Δ18 extract were chased into mature mRNA upon addition of recombinant Prp18. An increase in step 2 products (mRNA and excised lariat intron) was evident 3 min after supplementation with Prp18 (Fig. 1, lanes 20–22). No step 2 products were formed during a parallel incubation without added Prp18 (Fig. 1, lanes 17–19).

Prp18 is dispensible for splicing of precursor RNAs in which the interval between the branch point and 3′ splice site is <12 nt

In the wild-type actin pre-mRNA (ACT_{WT}) the splice acceptor site is located 38 nt downstream of the branch point. We showed previously that the requirement for Slu7 in actin pre-mRNA splicing can be obviated by shortening the interval between the branch point and the 3′ splice site (12). This prompted us to ask whether the requirement for Prp18 might also depend on the branch point to 3′ splice site distance. To answer this question, we exploited a series of actin pre-mRNAs in which the interval between the branch point and 3′ splice site varied between 7 and 15 nt (12). Splicing of the ACT_{15} pre-mRNA, like that of ACT_{WT}, was arrested after step 1 in Prp18-depleted extracts and could be restored by adding back recombinant Prp18 (Fig. 2A, lanes 9 and 10). Splicing of ACT_{12} was also Prp18 dependent (Fig. 2A, lanes 7 and 8). In contrast, splicing of ACT_7 was clearly Prp18 independent. ACT_7 RNA was spliced very efficiently in the Prp18-depleted extract and step 1 intermediates did not accumulate (Fig. 2A, lane 3). Addition of recombinant Prp18 had no impact on the ACT_7 splicing reaction (lane 4). Splicing of ACT_9 was partially dependent on Prp18, i.e. although Δ18 extracts did form mature spliced ACT_9 product, they also accumulated lariat intermediate (lane 5). Adding back Prp18 increased the yield of mature ACT_9 mRNA and decreased lariat intermediate (lane 6). Thus, Prp18, like Slu7, is required to splice RNAs in which the branch point to splice site distance is ≥12 nt.

We considered the possibility that Prp18 and Slu7 are functionally redundant in the splicing of RNAs with short branch point–splice site intervals, but non-overlapping in the splicing of longer interval precursors. Were this so, we would expect that depletion of both proteins would block splicing of short interval RNAs. Yet, we observed that ACT_7 was spliced perfectly well by the doubly depleted Δ7Δ18 extract (Fig. 2B, lane 3). Splicing of ACT_{12}, ACT_{15} and ACT_{WT} by the Δ7Δ18 extract was blocked after step 1 and was complemented by adding back both purified proteins (Fig. 2B).

Excess Slu7 protein obviates the requirement for Prp18

Recombinant Prp18 alone could not complement the step 2 defect of the doubly depleted Δ7Δ18 extract (Fig. 3, lanes 1). Formation of mature actin mRNA in the presence of purified Prp18 (at 50 μg/ml in the splicing reaction mixture) required Slu7. The yield of spliced RNA increased as the concentration of recombinant Slu7 was titrated from 16 to 500 ng/ml; this occurred concomitant with a Slu7 concentration-dependent decline in the level of lariat intermediate (Fig. 3, lanes 2–5). A 100-fold increase in Slu7 concentration (to 50 μg/ml) neither enhanced nor hindered splicing (Fig. 3, lane 6).

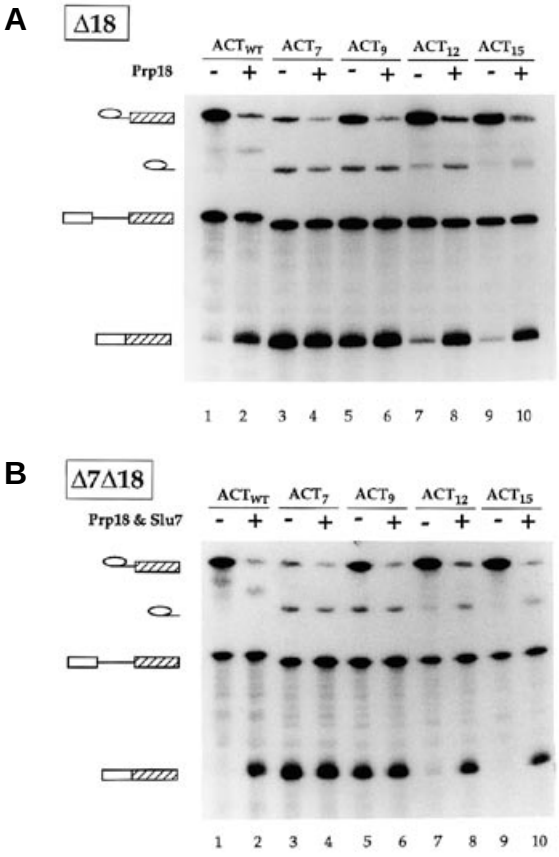

Figure 2. Prp18 is not essential for splicing of ACT_7 and ACT_9 precursor RNAs. (**A**) The indicated pre-mRNAs were incubated for 20 min in Δ18 extract (–) or Δ18 extract supplemented with 0.5 μg Prp18 (+). Wild-type actin precursor (ACT_{WT}) contains 38 nt between the branch point UACUA**A**CA and the 3′ splice site UAG (the branch site **A** and the intron/exon border are depicted in bold). Mutated versions of actin pre-mRNA (ACT_N) contain *N* nucleotides between the branch point and 3′ splice site sequences. The RNA between these two sequence elements are as follows: UCGAUUA in ACT_7; UCGAUUAUA in ACT_9; UCGAUUUG-UUUA in ACT_{12}; UCGAUUAUAUGUUUA in ACT_{15}. (**B**) The indicated pre-mRNAs were incubated for 20 min in Δ7Δ18 extract (–) or Δ7Δ18 extract supplemented with 0.25 μg Prp18 plus 0.25 μg Slu7 (+).

An unanticipated and mechanistically instructive finding was that recombinant Slu7 alone could relieve the step 2 defect of the Δ7Δ18 extract. However, the Slu7 concentration dependence of splicing in the absence of Prp18 was shifted significantly to the right, i.e. 10-fold higher concentrations of Slu7 were required to achieve the extents of splicing seen in Prp18-supplemented Δ7Δ18 extract (Fig. 3, lanes 8–12). We conclude that the requirement for Prp18 in the second step of splicing can be bypassed by excess Slu7. Prp18, while not strictly required for step 2, appears to facilitate the action of Slu7, which is strictly required.

Prp18 and Slu7 interact in the two-hybrid assay

We employed the two-hybrid assay (24) as a means to detect physical interactions between Prp18 and Slu7. The full-length Slu7 and Prp18 polypeptides were each fused to the Gal4 DNA binding domain (GBD) and to the Gal4 activation domain

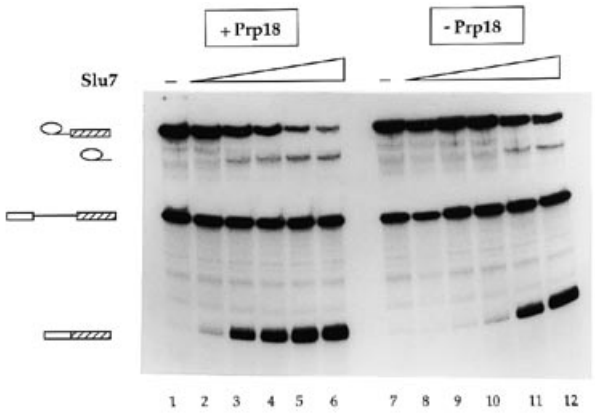

Figure 3. *In vitro* complementation of Δ7Δ18 extract by Slu7 alone and SLU7 plus Prp18. Actin pre-mRNA was incubated for 20 min in Δ7Δ18 extract (–Prp18, lanes 7–12) or Δ7Δ18 extract plus 0.5 µg Prp18 (+Prp18, lanes 1–6). The reaction mixtures were supplemented with increasing amounts of Slu7 protein as follows: 0.16 (lanes 2 and 8), 0.5 (lanes 3 and 9), 1.6 (lanes 4 and 10), 5 (lanes 5 and 11) and 500 ng (lanes 6 and 12). Control reactions contained no added Slu7 (lanes 1 and 7).

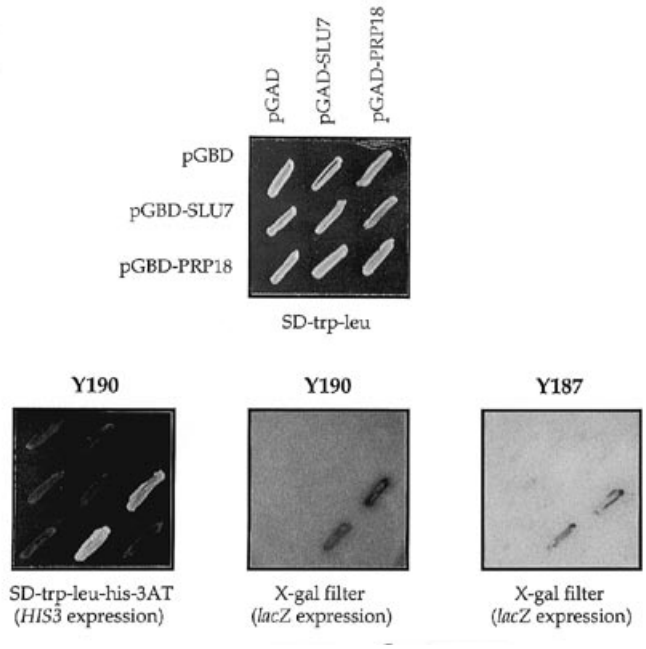

Figure 4. Slu7 and Prp18 interact in the two-hybrid assay. Yeast strains Y187 and Y190 were co-transformed with nine different combinations of plasmids as outlined. Leu^+ Trp^+ transformants were grown in patches on SD-trp-leu plates for 2 days at 30°C. Shown in the figure are Y190 transformants. Y190 cells were transferred to SD-trp-leu-3-AT medium to select for cells that expressed the *HIS3* reporter gene. The β-galactosidase activity of Y187 and Y190 transformants was tested using the X-gal filter assay.

(GAD). Four fusion plasmids were constructed: pGBD-PRP18, pGBD-SLU7, pGAD-PRP18 and pGAD-SLU7. The GBD–Slu7 fusion protein was functional *in vivo*, i.e. pGBD-SLU7 genetically complemented a *slu7* null mutant (not shown). Pairwise combinations of the GBD and GAD fusion plasmids were transformed into yeast strains Y187 and Y190, which contain a *lacZ* reporter gene with upstream Gal4 binding sites. Y190 also contains a *HIS3* reporter gene with upstream Gal4 binding sites (23). Expression of the *lacZ* and *HIS3* reporters is contingent on bridging interactions between the GBD and GAD fusion proteins. *lacZ* expression in yeast cells was evinced by the acquisition of blue color after incubation with X-gal. *HIS3* expression was manifested by growth in medium lacking histidine and containing 25 mM 3-AT. Control cells transformed with the pGAD and pGBD vectors did not express *HIS3* or *lacZ*. Strains transformed with pGAD-PRP18 plus pGBD-SLU7 or pGAD-SLU7 plus pGBD-PRP18 expressed both reporter genes (Fig. 4). All other combinations were negative in both assays. These results demonstrated that Prp18 and Slu7 can interact physically *in vivo*.

Slu7 deletion mutants define a minimum essential domain

A series of N- and C-terminal deletion mutants was designed to progressively truncate the 382 amino acid Slu7 protein. The *in vivo* function of the truncated genes was tested by using the plasmid shuffle procedure (22). Growth of the *slu7* null strain YXP3 depends on an extrachromosomal copy of *SLU7* on a *CEN*, *URA3* plasmid. YXP3 was transformed with a *CEN*, *TRP1* plasmid containing either full-length wild-type *SLU7* or a series of *SLU7* deletion mutants. Trp^+ transformants were plated on medium containing 5-FOA to select against retention of the *SLU7*, *URA3* plasmid. Selection was performed at 25 and 30°C. As expected, cells transformed with the *SLU7*, *TRP1* plasmid encoding full-length Slu7(1–382) grew readily on 5-FOA at both temperatures, whereas cells transformed with the *TRP1* vector were unable to form colonies (Fig. 5 and not shown). Deletion mutants Slu7(220–382), Slu7(1–335) and Slu7(1–307) were non-functional *in vivo*, i.e. they did not support growth on 5-FOA at either 25 or 30°C. All other less extensively truncated Slu7 variants supported growth at one or both temperatures. The 5-FOA survivors were subsequently tested for growth at different temperatures on YPD medium. The results are summarized in Figure 5 (and shown in Fig. 6). Deletion of up to 80 amino acids from the N-terminus of Slu7 had no effect on cell growth at any temperature from 17 to 37°C. However, additional deletions of 124, 162, 184 and 199 residues elicited a progressive temperature-sensitive growth phenotype (Figs 5 and 6). The break point for growth at 25°C versus lethality was between residues 200 and 222. A C-terminal deletion of 21 amino acids was well tolerated [Slu7(1–361)], whereas deletion of 47 residues was lethal [Slu7(1–335)].

Deletion analysis defines a domain of Slu7 required for interaction with Prp18

Deletion mutants of Slu7 were fused to the Gal4 DNA binding domain. The pGBD-SLU7Δ mutants were introduced into strain Y187 together with the pGAD-PRP18 fusion plasmid. Leu^+Trp^+ transformants were tested for expression of the *lacZ* reporter gene (Fig. 7). Elimination of 199 amino acids from the N-terminus of Slu7 did not affect interaction of the fusion protein with Prp18, i.e. GBD–Slu7(200–382) cells were blue in X-gal. However, cells transformed with GBD–Slu7(222–382) were white. Cells transformed with Slu7 C-terminal deletion mutants were $lacZ^+$ even after deletion of 158 residues [GBD–Slu7(1–224)]. More extensive

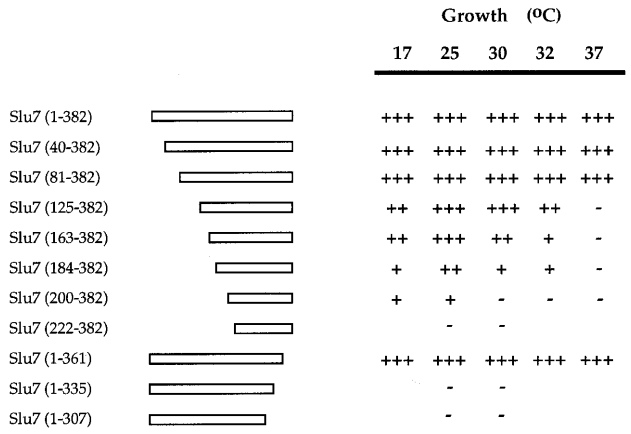

Figure 5. *SLU7* deletion mutants. Gene function was tested by plasmid shuffle. Wild-type and mutant alleles (on *TRP1*, *CEN*) were transformed into strain YXP3. A control transformation was performed with the vector pSE358. Trp^+ transformants were selected and streaked on medium containing 5-FOA at 25 and 30°C. –, no colonies were formed on 5-FOA medium. FOA-resistant colonies that had lost the *CEN*, *URA3*, *SLU7* plasmid were streaked to YPD medium and incubated for 4 days at different temperatures. Growth at 17°C was assessed after 6 days. +++, growth indistinguishable from that of wild-type cells; ++, cells grew more slowly than wild-type cells; +, strains formed only very small colonies.

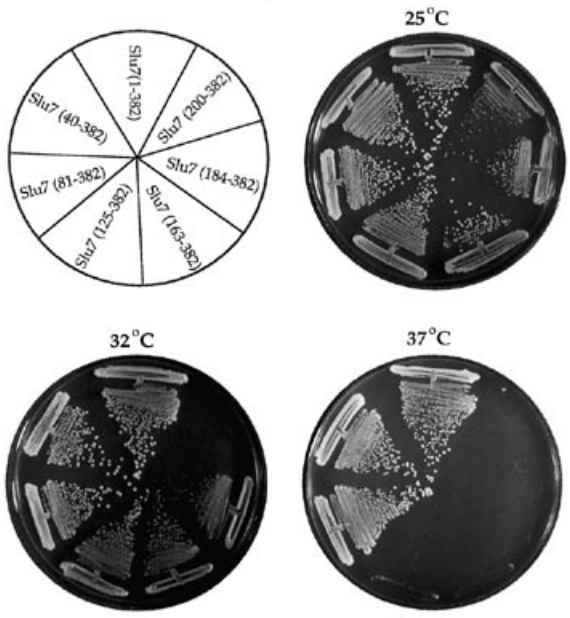

Figure 6. Temperature-sensitive growth of N-terminal SLU7 deletion mutants. Strains containing wild-type or mutant alleles as indicated were streaked to YPD medium. The plates were photographed after incubation for 3 days at the indicated temperatures.

deletions were inactive, e.g. GBD–Slu7(1–212) and GBD–Slu7(1–193). These results define a segment of Slu7 from residue 200 to 224 that is required for interaction of Slu7 with Prp18. Note that all Slu7 deletions that were viable in the plasmid shuffle assay were capable of interacting with Prp18 in the two-hybrid assay. None of the mutants that were defective in the two-hybrid assay

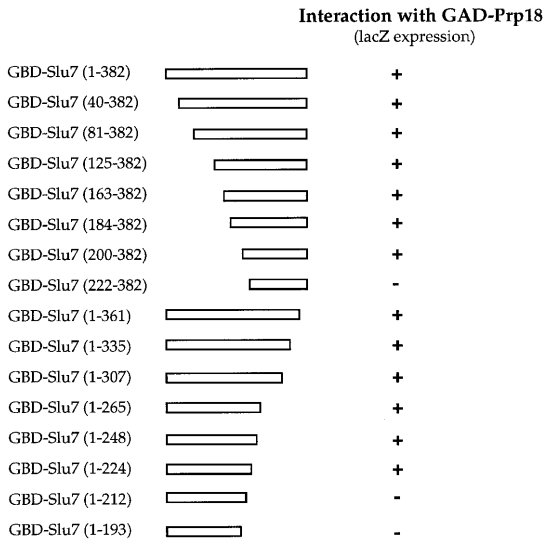

Figure 7. Identification of a domain of Slu7 required for two-hybrid interaction with Prp18. Strain Y187 was co-transformed with pGAD-Prp18 and the indicated deletion mutants of pGBD-Slu7. Positive interaction (+) was scored as blue color in the X-gal filter assay. White color was scored as negative (–).

were viable by plasmid shuffle. This suggests that the Prp18 interaction domain of Slu7 (and by inference the interaction between Slu7 and Prp18) contributes to Slu7 function *in vivo*.

DISCUSSION

Five major conclusions emerge from the work presented in this study: (i) Prp18 is dispensable for splicing of actin pre-mRNA in which the distance between the branch point and 3′ splice site is <12 nt; (ii) excess Slu7 protein obviates the need for Prp18 during the second step of splicing *in vitro*; (iii) Prp18 and Slu7 interact in the two-hybrid assay; (iv) the segment of Slu7 from residue 200 to 224 is necessary for this interaction; (v) the N-terminal 199 amino acids of Slu7 are not essential for Slu7 function *in vivo*. The mechanistic implications are considered below.

Slu7 and Prp18 act in concert

The catalytic center or 'active site' of the spliceosome will be located at the branch point upon completion of the first transesterification reaction (because the branched lariat intermediate is the product of that reaction). In order for the second transesterification reaction to occur, the 3′ splice site phosphodiester bond must be identified and positioned for attack by the 3′ hydroxyl of exon 1. It remains to be determined whether both transesterification reactions use the same active site, as suggested in a model for a two-metal ion mechanism for catalysis (25), or whether a new site is created for the second chemical step (26). In either case, structural rearrangements in the spliceosome need to occur to juxtapose the reactive moieties for the second catalytic step. Our studies show that the protein requirements for step 2 depend on the length of the RNA segment between the branch point and the 3′ splice site. Prp18 and Slu7 are required to splice pre-mRNAs in which this distance is ≥12 nt, but not when this interval is ≤9 nt. The data suggest that Slu7 and Prp18 act cooperatively, insofar as excess Slu7 can bypass the requirement

for Prp18. We found that 10-fold more Slu7 is needed to promote step 2 when Prp18 is lacking. This readily accounts for the observation that a *prp18* null mutant is viable (but temperature sensitive) (9) and that overexpression of *SLU7 in vivo* can suppress the temperature-sensitive growth defect of the *prp18-1* mutant strain (7).

We infer that Slu7 is involved in the structural changes that identify the 3′ splice site and position it for attack by the upstream exon. In principle, this could entail movement of the catalytic center (containing the 3′ hydroxyl of exon 1) away from the branch point to the 3′ splice site junction or recruitment of the 3′ splice site to the branch point region. This event is not rate limiting for transesterification chemistry when the branch point to 3′ splice site distance is short, but becomes rate limiting (and hence Slu7 dependent) when the distance is long. We suggest that Prp18 stabilizes the interaction of Slu7 with the spliceosome.

It is likely that Prp18 facilitates the Slu7-dependent step by interacting physically with Slu7. We have shown that Prp18 and Slu7 fusion proteins interact in the two-hybrid assay. Deletion mutations define a small region of Slu7, from amino acid 200 to 224, that is required for binding of Prp18. We assume that activation of the *HIS3* and *lacZ* reporter genes reflects direct contact between Slu7 and Prp18. However, the experiments do not exclude the participation of other macromolecules as molecular bridges between the Prp18 and Slu7 fusions. The finding that Slu7 and Prp18 can be removed individually from yeast extract by immunodepletion and that splicing activity is restored upon addition of the individual purified proteins argues against a tight association of the Prp18 and Slu7 proteins in yeast extracts.

It is conceivable that splicing factors engage in multiple transient interactions during the dynamic splicing process. For example, U5 snRNA is detected in immunoprecipitates when yeast extracts are reacted with anti-Prp18 antiserum (9). However, Prp18 cannot be considered an integral U5 snRNP component, because only a small fraction of the total U5 snRNA is precipitated by anti-Prp18. Moreover, anti-Prp18 does not functionally deplete yeast extract of U5 snRNP, because the depleted extract is perfectly capable of catalyzing step 1, a reaction that is U5 dependent. Note that human Prp18 (hPrp18) is not associated with U5 snRNA in HeLa cell extracts (27). Hence, we surmise that any association of Prp18 with U5 snRNP is likely to be transient or weak.

We speculate, nonetheless, that Prp18 and Slu7 interaction in the spliceosome involves U5 snRNP. This is suggested by several genetic interactions (5). For example, *slu7-1* was identified on the basis of synergistic lethality with a mutation in U5 snRNA and the same U5 snRNA lesion displays synergistic lethality with *prp18-1* (19). Also, the U5 snRNA and the U5 snRNP protein, Prp8, are implicated in 3′ splice site selection (28–30).

Mutational analysis of Slu7

Slu7 deletion mutations define a minimal essential domain of this splicing factor that extends from residue 200 to 361 of the 382 amino acid Slu7 polypeptide. Attention had been drawn earlier to a cysteine-rich motif of Slu7 (from residue 122 to 135) that resembles the so-called ‘zinc-knuckle’ found in retroviral nucleocapsid proteins (31). In the retroviral proteins, this motif is important for specific recognition of retroviral genomic RNA during encapsidation (32). It has previously been shown that replacement of two cysteine residues within the Slu7 zinc knuckle by two serines (*slu7-CCSS*) does not affect Slu7 function *in vivo* (13). We now show that an N-terminal Slu7 deletion mutant, Slu7(160–382), which lacks the cysteine-rich motif, supports cell growth at 25°C. Hence, the motif is not essential for Slu7 function. Progressive N-terminal deletions elicited a temperature-sensitive growth phenotype, but were not lethal until >199 amino acids were removed. The C-terminus was less tolerant of deletions; removal of >21 amino acids was lethal. The *slu7-ts* deletion mutants may prove useful for further genetic analysis of Slu7 interactions within the spliceosome, e.g. via the identification of extragenic suppressors of the temperature-sensitive growth defect. A more extensive mutational analysis of Slu7 in which amino acid substitutions are targeted to regions of interest (e.g. the Prp18 interaction domain), together with mutational analysis of Prp18, will provide additional insights into function of these proteins in pre-mRNA splicing.

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