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Characterization and functional ordering of Slu7p and Prp17p during the second step of pre-mRNA splicing in yeast

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ABSTRACT **Temperature-sensitive alleles in four genes (*slu7-1, prp16-2, prp17-1,* and *prp18-1*) are known to confer a specific block to the second chemical step of pre-mRNA splicing *in vivo* in the yeast *Saccharomyces cerevisiae*. Previous studies showed that Prp16p and Prp18p are required solely for the second step *in vitro*. The RNA-dependent ATPase, Prp16p, functions at a stage in splicing when ATP is required, whereas Prp18p functions at an ATP-independent stage. Here we use immunodepletion to show that the roles of Slu7p and Prp17p are also confined to the second step of splicing. We find that extracts depleted of Prp17p require both Prp17p and ATP for slicing complementation, whereas extracts depleted of Slu7p require only the addition of Slu7p. These different ATP requirements suggest that Prp16p and Prp17p function before Prp18p and Slu7p. Although *SLU7* encodes an essential gene product, we find that a null allele of *prp17* is temperature-sensitive for growth and has a partial splicing defect *in vitro*. Finally, high-copy suppression experiments indicate functional interactions between *PRP16* and *PRP17, PRP16* and *SLU7*, and *SLU7* and *PRP18*. Taken together, the results suggest that these four factors may function within a multicomponent complex that has both an ATP-dependent and an ATP-independent role in the second step of pre-mRNA splicing.**

The removal of introns from pre-mRNA, termed nuclear pre-mRNA splicing, is a highly conserved process that occurs in two catalytic steps (1, 2). Cleavage at the 5′ splice site in a trans-esterification reaction is followed by a second trans-esterification reaction that results in cleavage at the 3′ splice site, with concomitant ligation of the two exons. Splicing occurs in a large complex known as the spliceosome, which is composed of numerous proteins and five small RNAs [U1, U2, U4/U6, and U5 small nuclear RNAs (snRNAs)] in addition to the pre-mRNA. In yeast, most *non*-snRNA factors have been isolated in screens for temperature-sensitive mutations that affect splicing (called *prp* mutations for *p*re-m*R*NA *p*rocessing) (3).

Our interest has focused on studying factors involved in the second catalytic step. In yeast and mammals, U2, U5, and U6 snRNAs have been shown to play roles in both the first and the second step of splicing (for review, see ref. 4). In mammalian systems, one factor, PTB (pyrimine tract binding protein)-associated splicing factor, binds to the 3′ splice-site pyrimidine tract and is required *in vitro* for the second step (5). In yeast, temperature-sensitive mutations in four genes reveal a role for the encoded proteins in the second step of splicing *in vivo*: Prp16p, Prp17p, Prp18p, and Slu7p (refs. 6 and 7; for review, see ref. 3). In addition, an allele of the U5 snRNP protein encoded by *PRP8* has been shown to impair 3′ splice site recognition and inhibit the second step (8). Synthetic lethality is observed between alleles of all these genes in pairwise combination except between *PRP16* and *PRP18* (ref. 7; J. Umen and C.G., unpublished work). Furthermore, mutant alleles of both *PRP17* and *SLU7* are synthetically lethal in combination with certain U5 snRNA mutations (7).

Prp16p is an RNA-dependent ATPase and has been shown to effect an ATP hydrolysis-dependent conformational rearrangement at the 3′ splice site (9, 10). In contrast, the activity of Prp18p, a U5 snRNP protein, is ATP-independent, indicating that Prp18p acts subsequent to Prp16p (11, 12). Slu7p contains a "zinc knuckle" motif that in retroviral nucleocapsid proteins has been implicated in RNA binding; substrate competition experiments suggest that SLU7 plays a role in 3′ splice-site choice (13). Prp17p contains four WD-40 motifs, which are present in a variety of proteins and are thought to be involved in protein–protein interactions (14). In this study, we use antibodies raised against Slu7p and Prp17p to characterize their roles in splicing *in vitro* and perform genetic suppression experiments to study interactions between factors that act at the second step of splicing.

MATERIALS AND METHODS

Yeast Strains. The following strains were used for these studies: yPH274 (15) *Mata/*α*trp1-*Δ*his3-*Δ*200 ura3-52 ade2-101 lys2-801 leu2-*Δ*1*; BJ2168 (16) Mata *leu2 trp1 ura3-52 prb1-1122 pep4-3 prc1-407 gal2*; BJΔ17 (BJ2168 with *prp17::LEU2*, this study); yDFA7 (13) Mata *slu7-1 ura3 lys2 his3 ade2 trp1 leu2 snr7*Δ*::LEU2* pBW*URA-SNR7*; ts503 (6) *Mat*α *prp18-1 ade2-101 his3-*Δ*200 ura3-52 lys2-801*; ts365 (6) *Mat*α *prp17-1 ade2-101 his3-*Δ*200 ura3-52 lys2-801*; yS78 [all *prp16* alleles (17)] Mata *trp1 ura3 lys2 leu2 ade2 his3 prp16*Δ*::LYS2* pSB2*URA-PRP16*; HM121 (U5-A98, from H. Madhani, University of California at San Francisco) *Mat*α *cup1* Δ*lys2 leu2 ura3 trp1 his3 snr7*Δ*::LEU2* pSE360–*SNR7*.

Molecular Biology. All enzymes were purchased from New England Biolabs, and all protocols have been described (18) unless otherwise mentioned.

Yeast Genetic Techniques. Growth conditions and media, transformation, plasmid shuffle, tetrad dissection, and other genetic techniques were done as described (19).

Overexpression Plasmids. *SLU7, PRP17*, and *PRP16* genes were overexpressed in yeast from the GPD1 promoter of the multicopy plasmid pG-1 (20). The *PRP18* gene was expressed on a centromere-containing plasmid (low copy) and was from D. Horowitz (California Institute of Technology).

Construction of *PRP17* Gene-Disruption Strain. The *PRP17* clone was from U. Vijayraghavan, M. Company, and J. Abelson (California Institute of Technology). The disrupted *PRP17* gene contains LEU2 coding sequences, leaving three amino acids of *PRP17* at the amino terminus and none at the carboxyl terminus. After transformation, the gene disruption was marked by leucine prototrophy and confirmed by Southern

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Abbreviation: snRNA, small nuclear RNA.

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analysis (18). Ten tetrads of the diploid strain for each of three transformants were dissected and analyzed.

Prp17p and Slu7p Antibody Production. Polyclonal antibodies were raised against fusion proteins containing either Slu7p (described in ref. 21) or Prp17p (entire coding sequence) fused to the TrpE protein and expressed in *Escherichia coli* (22).

Heterologously Produced Slu7p and Prp17p. Expression of Slu7p in *E. coli* and preparation of extracts were accomplished as described (21). Prp17p was produced in a reticulocyte lysate using a coupled T7 transcription/translation system (Promega) programmed with pCITE1 (Novagen) or pCITE-*PRP17*.

Immunodepletion. Immunodepletion was done as described (9). Protein A-purified antibodies to Prp16p (9) were from S. Burgess and J. Umen (University of California at San Francisco).

***In Vitro* Splicing.** Yeast whole-cell extract was prepared, and splicing was done as described (23), except reaction temperature was 30°C. Splicing was quantitated using a Molecular Dynamics PhosphorImager. Inhibition of the second step and complementation of the block were assessed by comparing the ratios of lariat/lariat-intermediate, lariat-intermediate/mature mRNA, and exon 1/mature mRNA in a treated sample to those of a mock-depleted sample. The ratios gave similar numbers both relatively (i.e, α-16 vs. α-7 vs. α-17) and in terms of the degree of complementation.

RESULTS AND DISCUSSION

Detection and Slu7p and Prp17p with Polyclonal Antibodies. To directly assess the roles of Prp17p and Slu7p in the second step of pre-mRNA splicing, we raised polyclonal antibodies to TrpE fusions of each protein. When these antibodies were used to probe immunoblots containing wild-type yeast whole-cell extracts, polypeptides of the expected sizes of Slu7p (44 kDa) and Prp17p (50 kDa) were detected with the corresponding antibodies (Fig. 1*A* and *B*, respectively, lane 1), and not with preimmune serum; substantially greater amounts of these proteins were detected in extracts made from yeast carrying plasmids overexpressing either Slu7p (Fig. 1*A*, lane 2) or Prp17p (Fig. 1*B*, lane 2). For Slu7p, a correspondingly smaller protein was detected when the plasmid bore a truncated version of the *SLU7* gene (Fig. 1*A*, lane 3). Thus, the antisera specifically recognize Slu7p and Prp17p in yeast whole-cell extracts.

Slu7p and Prp17p Are Only Required for the Second Step of Splicing *in Vitro*. We first tested the effect on *in vitro* splicing of removing either the Slu7 or the Prp17 protein from a wild-type splicing extract by immunodepletion. Two control extracts were also prepared: one treated only with protein A bound to beads (mock) and one treated with antibodies to Prp16p (α-16), a protein known to act at the second step of splicing *in vitro*. The *in vitro* splicing (and complementation) protocol used in these experiments is outlined in Fig. 2*A*. Splicing reactions with extracts depleted of Prp16p (α-Prp16, lane 3), Slu7p (α-Slu7, lane 9), or Prp17p (α-Prp17, lane 14) resulted in the substantial accumulation of first-step splicing products (lariat–exon 2 and exon 1) and a decrease in the products of the second step (lariat and exon1–exon2) relative to mock treatment (lane 2). For both α-Slu7 and α-Prp17 extracts, the kinetics of splicing were assayed and revealed no block to the first step of splicing (data not shown). Although the second-step block for α-Slu7 extract was generally close to complete (i.e., no mRNA was produced), in α-Prp17 extracts a small amount of mRNA was always formed, despite >90% removal of the protein as assayed by immunoblot analysis (data not shown). This result suggested either that a small amount of residual protein is conferring the partial activity or that Prp17p is not absolutely required for the second step of splicing. A similar partial block has been observed in Prp18p immunodepleted extract (11).

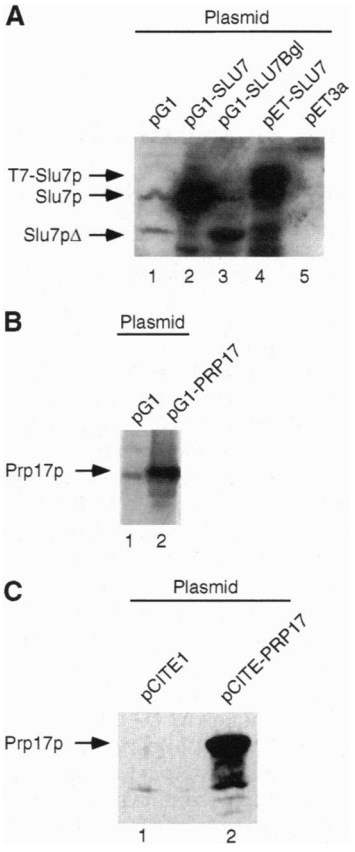

FIG. 1. Detection of Slu7p and Prp17p in yeast extracts and from heterologous sources. (*A*) Immunoblot analysis (enhanced chemiluminescence, Amersham) of Slu7p from either yeast strain BJ2168 (lanes 1–3) or from *E. coli* strain BL21 (lanes 4 and 5), transformed with either control (lanes 1 and 5) or SLU7-expressing plasmids (lanes 2–4). Arrows indicate the expected migration for the bacterial, yeast, and truncated yeast proteins, respectively. (*B*) Immunoblot analysis of Prp17p from yeast strain BJ2168 transformed with either control (lane 1) or *PRP17* (lane 2)-expressing multicopy plasmids. (*C*) Fluorography of SDS/PAGE gel containing ^{35}S-labeled reticulocyte lysate *in vitro* translation reactions programmed with either control (lane 1) or *PRP17* (lane 2)-expressing plasmids.

To test whether the observed splicing defects resulted from specific removal of Prp17p or Slu7p or removal of other coimmunoprecipitating proteins, we attempted complementation of the splicing defects with heterologously produced Slu7p or Prp17p. To this end, we synthesized Slu7p in *E. coli* (T7-Slu7p shown by immunoblot analysis in Fig. 1*A*, lane 4; note that the fusion added 14 amino acids) and show that it complements the second-step splicing block seen in α-Slu7 extracts (Fig. 2*B*, lane 11), whereas the control extract containing no T7-Slu7p (Fig. 1*A*, lane 5) does not (Fig. 2*A*, lane 10). Because Prp17p produced in *E. coli* is highly insoluble, we chose to synthesize Prp17p *in vitro* using a reticulocyte lysate transcription–translation system. Prp17 protein can be visualized by autoradiography when radioactive amino acids are included in the translation reaction (Fig. 1*C*, lane 2). This protein alleviates the second-step block to splicing (Fig. 2*B*, lane 16), whereas control lysate (Fig. 1*C*, lane 1) is inactive for complementation (Fig. 2*B*, lane 15). Thus, we conclude that both Slu7p and Prp17p are specifically required for the second step of splicing.

ATP Requirement for Complementation. Previous work has divided the second catalytic step of splicing into two stages: an ATP-dependent stage, defined at least in part by the Prp16p-

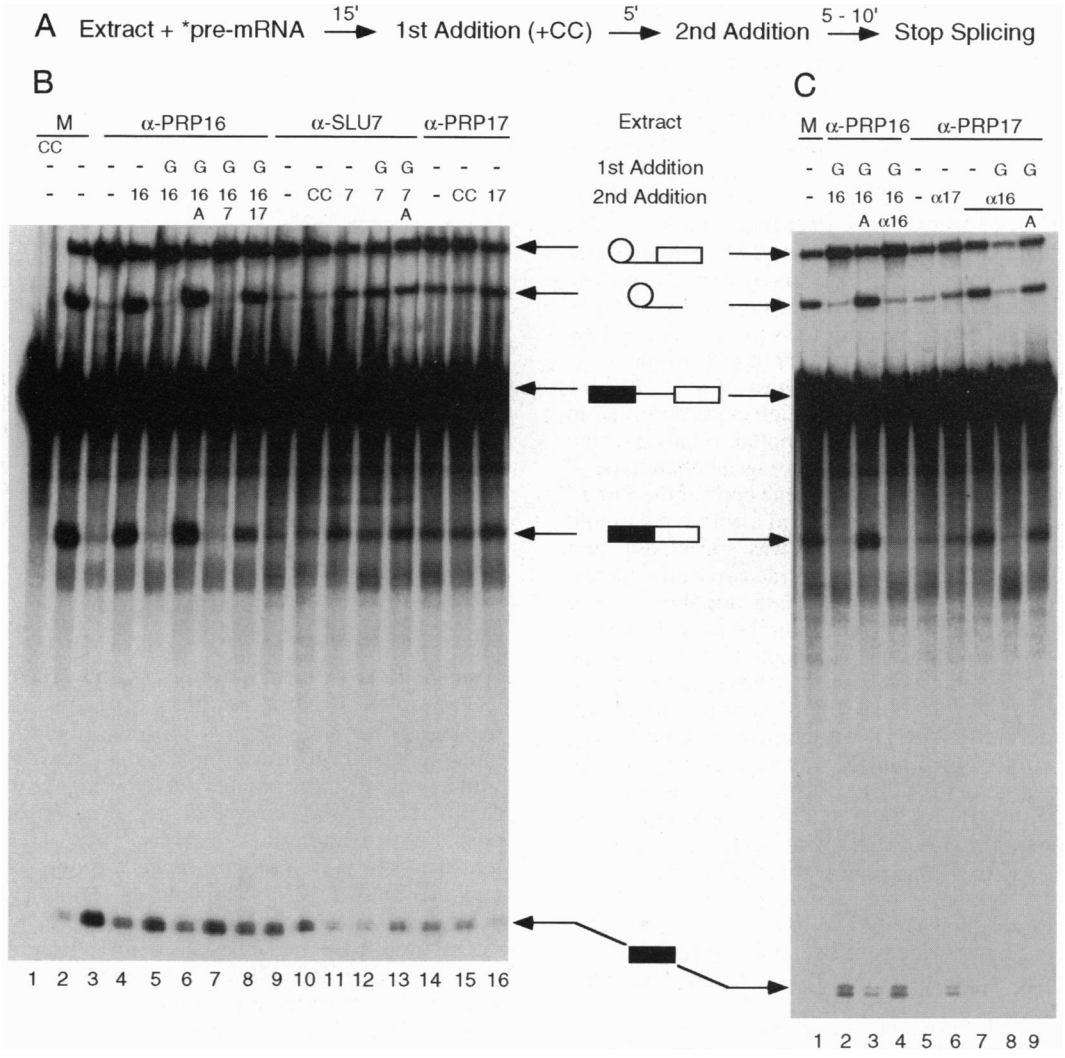

FIG. 2. *In vitro* splicing. (*A*) Diagram of general protocol with splicing started by addition of extract to other components of the splicing reaction. (*B* and *C*) Splicing extracts were either mock-depleted (protein A treatment alone) (M) or immunodepleted of Prp16p (α-PRP16), Slu7p (α-SLU7), Prp17p (α-PRP17). CC, excess cold competitor; G, glucose to 3 mM; C, control extract (bacterial or reticulocyte lysate); A, ATP; 16, purified Prp16p; 7, Slu7p-containing bacterial extract; 17, Prp17p-containing reticulocyte lysate. Arrows between *B* and *C* (top–bottom) indicate migration of lariat–exon 1, lariat, pre-mRNA, exon 1–exon 2, and exon 1, respectively. *, ^{32}P-radiolabeled.

requiring step (9), and an ATP-independent stage, during which Prp18p is known to function (12). The different ATP requirements for complementation by these two proteins formally places the Prp18p-dependent step "downstream" of the Prp16p-dependent step. We wanted to assess the ATP requirement for complementation of the splicing defect in Slu7p- and Prp17p-immunodepleted extracts to functionally order Slu7p and Prp17p relative to Prp16p and Prp18p. Because yeast splicing extract contains endogenous hexokinase, ATP can be rapidly depleted from splicing reactions by the addition of glucose (12). With immunodepleted extracts, pre-mRNA was incubated under splicing conditions to allow the first catalytic step to occur (outlined in Fig. 2*A*), after which an excess of cold competitor pre-mRNA (CC) was added to prevent further spliceosome assembly, and glucose (G) (1st addition) was added to deplete ATP. The complementing protein was then added (2nd addition) in the presence or absence of additional ATP (A). We used extract depleted of Prp16p as a control because this protein is known to require ATP for function. We also assayed our Slu7p and Prp17p fractions for ATP contamination by determining whether they could substitute for ATP in allowing complementation of α-16 extracts.

As expected, complementation with Prp16p (Y. Wang, University of California at San Francisco) requires the addition of ATP after glucose treatment (Fig. 2*B*, compare lanes 5 and 6). However, when α-Slu7 extract undergoes similar treatment to deplete ATP, complementation with Slu7p proceeds with only a slight reduction in the amount of exon1–exon2 and lariat produced (lane 12 vs. lane 11). The addition of ATP (lane 13) does not improve complementation and, in fact, seems to decrease it somewhat, probably by affecting the turnover of unstable intermediates. That the Slu7p fraction itself is free of ATP is shown in lane 7 by its inability to substitute for ATP in allowing Prp16p complementation. Thus, Slu7p, like Prp18p, appears to act during the second step of splicing after all ATP-dependent reactions (e.g., that catalyzed by Prp16p). The same conclusion was reached by Ansari and Schwer regarding Slu7p (B. Schwer, personal communication).

Two alternative approaches were taken to determine the ATP requirement for Prp17p function. These were necessary because our attempts to remove ATP from the reticulocyte lysate–Prp17p fraction, using dialysis and size-exclusion columns, were inadequate in that the Prp17p fraction was able to substitute partially for ATP in the Prp16p complementation assay (lane 8). First, we used an α-Prp16 extract as an

alternative source of Prp17p (Fig. 2*C*, complementation of α-17 shown in lane 7 vs. lane 5) and α-Prp17 extract as a negative control for complementation (lane 6). Using α-Prp16 extracts to complement the second-step block in α-17 extracts after glucose treatment requires the addition of ATP (Fig. 2*C*, lane 9 vs. lane 8). As a second approach, the reticulocyte lysate-Prp17p fraction itself was treated with glucose and exogenous hexokinase, followed by dialysis to remove glucose. Although this fraction is less active than that used in Fig. 2*B*, lane 16, the remaining activity depends on ATP addition (data not shown). Therefore, Prp17p appears to act during the second step of splicing before or concomitant with an ATP-dependent reaction. These results allow us to functionally position Prp16p and Prp17p before Slu7p and Prp18p.

Biological and Biochemical Phenotypes of *PRP17* Gene Disruption. The partial *in vitro* splicing defect we observed in α-17 extracts despite the apparently complete removal of the protein prompted us to determine the biological phenotype of a *PRP17* gene disruption. We disrupted one copy of the *PRP17* gene with the nutritional marked *LEU2* in a wild-type diploid strain (yPH274). The resulting strain was sporulated, and tetrads were dissected at 25°C; in each tetrad two wild-type and two slow-growing spores germinated (data not shown). The slow-growth phenotype segregated with the *LEU2* marked gene disruption. *PRP17* was also disrupted in a wild-type haploid strain BJ2168, with similar results. The resulting haploid strain (prp17Δ) was transformed with a plasmid-borne wild-type *PRP17* gene (*PRP17*-pG1) or a control plasmid (pG1), and growth at 25°C, 30°C, and 33°C was compared to the parental wild-type strain (Table 1). Although growth of the prp17Δ strain (with pG1) is similar to that of a wild-type strain at 25°C and somewhat slow at 30°C, the strain is inviable at 33°C or higher. These phenotypes are complemented in the prp17Δ strain that contains either a high-copy (*PRP17*-pG1) or a low-copy (data not shown) plasmid-borne wild-type *PRP17* gene. A similar temperature-sensitive phenotype has been shown for a gene disruption of *PRP18* (11). We conclude that,

Table 1. Biological phenotypes of splicing mutant strains

Splicing mutant allele*	Plasmid	Growth 25°C	Growth 30°C	Growth 33°C†
Wild type	Any	+++	+++	+++
prp17Δ	pG1	+++	+	–
	PRP17-pG1	+++	+++	+++
	SLU7-pG1	+++	+	–
	PRP16-pG1	+++	**++**	**+**
slu7-1	pG1	+++	+++	+
	PRP17-pG1	+++	+++	+
	SLU7-pG1	+++	+++	+++
	PRP16-pG1	+++	+++	**++**
prp18-1	pG1	+++	+	–
	PRP17-pG1	+++	+	–
	SLU7-pG1	+++	**+++**	**+**
	PRP16-pG1	+++	+	–
	PRP18-pDH	+++	+++	+++
prp16-2	pG1	+++	+++	++
	PRP17-pG1	+++	+++	++
	SLU7-pG1	+++	+++	++
	PRP16-pG1	+++	+++	+++
U5-A98	pG1	+++	+++	+
	PRP17-pG1	+++	+++	+
	SLU7-pG1	+++	+++	+
	U5-wt	+++	+++	+++

Boldface symbols represent supression by heterologous genes.
*Other mutant alleles tested that show similar suppression phenotypes to those shown are as follows: prp17-1, -2; prp16-1, -301 (two cold-sensitive strains); prp16-201, -203, -204, and -205.
†For the U5-A98 strain, growth was tested at 37°C.

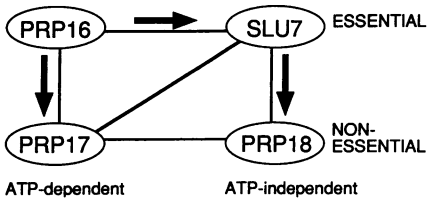

FIG. 3. Genetic interactions between second-step splicing factors. A line between the factors indicates a synthetic lethal interaction, whereas an arrow indicates that a mutant allele of the gene at the arrowhead can be suppressed by overexpression of the gene at the beginning of the arrow.

like Prp18p, Prp17p is not essential for splicing at low temperatures and, instead, may act by aiding in a process that occurs more inefficiently at higher temperatures. An example of such a process could be the stabilization of a functionally active conformation of U5, U2, or U6 snRNAs.

Assessing *in vivo* splicing of several mRNAs (*CYH2, RP51*) in the prp17Δ strain showed an identical defect to that which we reported for *prp17-2* [also called *slu4-1* (7)]: constitutive accumulation of lariat intermediate relative to mature mRNA at permissive temperature that is exacerbated after shift to nonpermissive temperature, as well as a decrease in the absolute level of mRNA (data not shown). *In vitro* splicing using whole-cell extract from the prp17Δ strain also shows a partial block of the second step similar to that observed using immunodepleted extract (data not shown). Neither extract shows a significantly greater defect at higher temperature. In summary, genetic removal of the Prp17 protein results in partial growth and splicing defects *in vivo* and *in vitro*, consistent with the partial *in vitro* defect we observe upon immunodepletion of Prp17p from splicing extract (Fig. 2).

Suppression of prp Mutant Phenotypes. Synthetic lethal interactions have been observed for alleles of all pairwise combinations of second-step factor genes (shown diagrammatically with connecting lines in Fig. 3) except between *PRP16* and *PRP18* [ref. 7; J. Umen and C.G., unpublished work]. In a complementary approach, we have examined whether the growth phenotypes of second-step mutants can be suppressed by overexpression of either *PRP16, PRP17*, or *SLU7* genes. As summarized in Table 1 (suppression in boldface type) and Fig. 3 (broad arrows), we observe partial suppression of the temperature-sensitive growth phenotypes of *slu7-1* and *prp17Δ* by overexpressing *PRP16*. Overexpressing *SLU7* strongly suppresses *prp18-1* at 30°C but does not bypass the need for Prp18p at temperatures at or >33°C. Finally, neither *PRP17* nor *SLU7* overexpression has any effect on the phenotype of the U5 snRNA mutation to which mutant alleles of these genes were recovered as synthetic lethal mutations (U5-A98) (7). Our genetic suppression experiments, like the previous synthetic lethal analysis, support the idea that second-step splicing factors interact functionally. They further reinforce the possibility of associations between Prp16p and Prp17p, Prp16p and Slu7p, and Slu7p and Prp18p.

SUMMARY

Our results indicate that the second step of splicing can be operationally divided into two substeps relative to the requirement for ATP hydrolysis: The functions of Prp16p and Prp17p functions are ATP-dependent, whereas those of Slu7p and Prp18p are ATP-independent. Interestingly, each substep is promoted by one essential (Prp16p and Slu7p) and one nonessential (Prp17p and Prp18p) factor. This pairwise relationship is further supported by the results of overexpression suppression. The temperature-sensitive phenotype of each null mutant can be complemented by the overexpression of the

essential member of the pair, consistent with an ancillary role for the dispensable gene product.

This picture is illuminated by recent crosslinking data (J. Umen and C.G., unpublished work), which show that after the first step of splicing, Prp16p can be efficiently cross-linked to the 3′ splice site. This initial binding step is Prp17p-independent. After ATP hydrolysis by Prp16p, Slu7p can be efficiently cross-linked in a manner that is dependent on Prp18p as well as Prp16p and Prp17p. Taken together with the functional ordering data presented here, these results place the Prp17p-dependent step after Prp16p binds the 3′ splice site but before or concomitant with ATP hydrolysis. In the simplest case, there may be a hand-off of the 3′ splice site from Prp16p to Slu7p. However, the fact that overexpression of Prp16p can partially suppress the temperature-sensitive phenotype of *slu7-1* suggest a more complex, interdependent relationship. Future experiments could be aimed at dissecting the specific functional contributions of each of these second-step factors.

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