A novel role for a U5 snRNP protein in 3' splice site selection

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The choice of a 3' splice site in *Saccharomyces cerevisiae* introns involves recognition of a uridine-rich tract upstream of the AG dinucleotide splice junction. By isolating mutants that eliminate the normal preference for uridine-containing 3' splice sites in a *cis*-competition, we identified a mutation that is an allele of *PRP8*, *prp8-101*. This was unexpected because previous analysis has demonstrated that the U5 snRNP protein encoded by *PRP8* is required for spliceosome assembly prior to the first catalytic step of splicing. In contrast, the uridine recognition defect caused by the *prp8-101* mutation selectively inhibits the second catalytic step of splicing. This defect is seen not only in 3' splice site *cis*-competitions but also in the splicing of an unusual intron in the *TUB3* gene and in the *ACT1* intron when utilization of its 3' splice site is rate limiting for splicing. Consistent with a direct role in 3' splice site selection, Prp8 can be cross-linked to the 3' splice site during the splicing reaction. These data demonstrate a novel function for Prp8 in 3' splice site recognition and utilization.

[Key Words: Yeast; pre-mRNA splicing; PRP8; U5 snRNP; 3' splice site]

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Nuclear pre-mRNA introns are identified by the presence of conserved sequences at their 5' splice site, internal branch site, and 3' splice site. These sequences are recognized by a set of small ribonucleoprotein particles (U1, U2, U4, U5, and U6 snRNA-containing snRNPs) that, along with other protein factors, bind to pre-mRNAs in an ordered set of events that lead to spliceosome assembly and catalysis. The two-step chemical reaction of splicing proceeds via successive transesterifications. First, the 2'-hydroxyl of the branch site adenosine attacks the 5' splice site phosphodiester bond to generate the lariat intermediate and the free 5' exon. Then, the 3'-hydroxyl of the 5' exon attacks the 3' splice site phosphodiester bond forming ligated exons and an excised lariat intron (for review, see Green 1991; Guthrie 1991; Rymond and Rosbash 1992; Moore et al. 1993).

Prior to the catalytic steps of splicing, the reaction partners must be accurately selected and juxtaposed. Multiple snRNA-pre-mRNA and snRNA-snRNA interactions are involved in recognizing and aligning the 5' splice site and branch site before the first catalytic step. In contrast, much less is known about the events that lead to 3' splice site selection and the second catalytic step of splicing. An AG dinucleotide preceded by a pyrimidine-rich tract comprises the 3' splice site of both yeast and mammalian introns. Moreover, an interaction between the first and last nucleotides of yeast introns plays a functional role in 3' splice site utilization (Parker and Siliciano 1993). However, *trans*-acting factors that

contribute to 3' splice site recognition have been difficult to identify. Two conserved loop nucleotides of U5 snRNA interact with residues in the second exon, although the lack of sequence conservation in exons makes this interaction unlikely to play a major instructive role in 3' splice site selection (Newman and Norman 1992; Sontheimer and Steitz 1993). In mammals, several proteins have been shown to bind the 3' splice site [IBP, 70K, U2AF, heterogeneous-nuclear RNP (hnRNP) C, hnRNP D, PTB, hnRNP A1, and PSF] (Gerke and Steitz 1986; Tazi et al. 1986; Ruskin et al. 1988; Swanson and Dreyfuss 1988; Garcia-Blanco et al. 1989; Buvoili et al. 1990; Patton et al. 1993)]. U2AF and PSF are required for the first and second steps of splicing, respectively (Zamore and Green 1991; Gozani et al. 1994), but the specific roles that these proteins play in splice site selection have not been determined. Multiple interactions with the 3' splice site probably reflect that this sequence is often required for early steps of spliceosome assembly in mammals and is also required for later steps in the splicing reaction (Reed and Maniatis 1985; Ruskin and Green 1985; Lamond et al. 1987; Reed 1989; Zhuang and Weiner 1990).

In the yeast *Saccharomyces cerevisiae*, the uridine-rich tract and AG dinucleotide splice acceptor appear to be required only during the second catalytic step (Rymond and Rosbash 1985; Rymond et al. 1987). Although the uridine residues in yeast 3' splice sites are generally less conserved than the pyrimidines in mammalian introns (Parker and Patterson 1987), they play an important role in efficient 3' splice site utilization. Patterson

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and Guthrie (1991) demonstrated that a uridine-rich tract placed upstream of a 3' splice site greatly enhances that splice site's ability to compete in *cis* with a non-uridine-containing 3' splice site. This, in turn, implies the existence of *trans*-acting factors that recognize this sequence and preferentially recruit uridine-containing 3' splice sites into the spliceosome.

U5 snRNP has been linked genetically and biochemically to the 3' splice site and the second catalytic step of splicing (Chabot et al. 1985; Gerke and Steitz 1986; Tazi et al. 1986; Patterson and Guthrie 1987; Winkelmann et al. 1989; Frank et al. 1992; Newman and Norman 1992; Sontheimer and Steitz 1993). Therefore, Frank and Guthrie (1992) tested the hypothesis that *slu* mutants, which were identified by their synthetic lethality with mutant U5 snRNAs, are defective in uridine-specific 3' splice site selection. This work uncovered a role for the Slu7 protein in selection of 3' splice sites that are located distally from the branch site; however, this factor is unlikely to interact directly with the uridine-rich tract (Frank and Guthrie 1992).

Here, we have modified the 3' splice site *cis*-competition reporters developed by Patterson and Guthrie (1991) for use in a genetic selection for factors that mediate uridine-rich tract recognition in yeast 3' splice sites. We have identified *prp8-101*, a novel mutation in the integral U5 snRNP protein encoded by *PRP8* (Lossky et al. 1987; Jackson et al. 1988); *prp8-101* specifically impairs recognition of the uridine-rich tract in a 3' splice site *cis*-competition. This finding was unexpected, because all previous alleles of *PRP8* are defective at or prior to the first catalytic step of splicing (Jackson et al. 1988; Brown and Beggs 1992). The *prp8-101* mutation severely inhibits the second step of splicing of the *TUB3* intron, which depends critically on its uridine tract for efficient 3'

splice site recognition. Other introns also require the uridine recognition function of Prp8 when utilization of the 3' splice site is made rate limiting for splicing. Consistent with a direct role in 3' splice site recognition, we have used site-specific UV cross-linking to demonstrate that Prp8 contacts the 3' splice site during the splicing reaction. In conjunction with data implicating Prp8 and the U5 snRNP in the first step of splicing (Brown and Beggs 1992; Wyatt et al. 1992), these results suggest a model in which Prp8 can function to juxtapose the two reaction partners for the second catalytic step.

Results

Isolation of mutants defective in 3' splice site uridine recognition

To identify factors involved in uridine tract recognition, we modified two 3' splice site *cis*-competition constructs developed by Patterson and Guthrie (1991). These constructs each contain a single 5' splice site and branch site and two potential 3' splice acceptors. In +T PyDOWN (Fig. 1A), use of the uridine-rich branch site-proximal 3' splice site is favored over the branch site-distal, adenosine-rich 3' splice site (Fig. 2A; Table 1). We reasoned that if the factors that recognize the uridine-rich tract polyuridine splicing factor (PSF) were mutated to abrogate uridine recognition, then the splicing pattern of +T PyDOWN would be altered to give a more balanced usage of the two competing splice sites (Fig. 1B). The net result would be a decrease in splicing to the proximal, uridine-rich 3' splice site and an increase in splicing to the distal, adenosine-rich 3' splice site.

To allow a genetic selection for *psf* mutants, we fused the +T PyDOWN competition to the selectable marker

Figure 2. Primer extension of splice site competition RNAs in wild-type and *psf1-1* strains. (A) Primer extension +T PyDOWN RNAs from a wild-type strain, *PSF1*, or mutant strain, *psf1-1*. Primer extension products corresponding to use of the branch site–proximal (MP), branch site–distal (MD) 3' splice site, or U1 snRNA internal control (U1) are indicated. (B–D) Primer extension of +A WT, +TA, and +AT RNAs from a wild-type and *psf1-1* strain. Primer extension products are labeled as in A.

CUP1, which encodes the yeast metallothionein homolog and confers dosage-dependent copper resistance to cells (Lesser and Guthrie 1993). In this fusion, use of the branch site–distal 3' splice site results in a message with the initiator AUG in-frame with the *CUP1*-coding sequence, and use of the branch site–proximal 3' splice site results in a message with the *CUP1*-coding sequence out-of-frame. *psf* mutants were selected by their predicted phenotype of increased use of the distal splice site and, therefore, increased copper resistance (Fig. 1B; see Materials and methods).

We isolated two independent and allelic mutants, *psf1-1* and *psf1-2*, that displayed similar phenotypes. Primer extension analysis of the +T PyDOWN RNA products from *psf1-1* and its wild-type parent strain (Fig. 2A; Table 1) revealed a dramatic increase in use of the distal (uridine-poor) 3' splice site and a decrease in use of

the proximal (uridine-rich) 3' splice site in the *psf1-1* mutant (proximal/distal = 1.6/1) versus wild type (proximal/distal = 22/1). The change in ratio of splice site usage in the *psf1-1* mutant is accompanied by a greater than threefold increase in copper resistance (0.18 mM) versus wild type (0.05 mM).

In principle, the change in splice site choice in the *psf1-1* strain could result from either a loss of uridine recognition or a change in preference from branch site–proximal to branch site–distal 3' splice sites ("polarity"). To distinguish these hypotheses, we used the construct +A WT (Fig. 1A), which is the opposite of the +T PyDOWN construct in that the branch site–proximal 3' splice site is uridine-poor and the distal 3' splice site is uridine-rich. Because branch site proximity is preferred in a wild-type strain (Patterson and Guthrie 1991), the proximal 3' splice site of +A WT is utilized almost as efficiently as the distal 3' splice site. We introduced this construct into the *psf1-1* strain and its wild-type parent strain and assayed splicing by primer extension and copper growth (Fig. 2B; Table 1). If the *psf1-1* mutation only affects polarity, then splice site usage in the +A WT construct would have shifted toward the distal 3' splice site as it does in the +T PyDOWN construct. Instead, we found that 3' splice site usage was shifted away from the uridine-rich, distal 3' splice site and toward the uridine-poor, proximal splice site with +A WT (wild-type proximal/distal = 0.86/1; *psf1-1* proximal/distal = 3.1/1). The *psf1-1* mutant is therefore impaired in uridine recognition at the 3' splice site and not in polarity of splice site choice.

To confirm these results and rule out context-specific effects, we tested an isogenic set of constructs, +TA and +AT (Fig. 1A). These constructs are similar to +T PyDOWN and +A WT except that the adenosine-rich and uridine-rich tracts preceding the competing 3' splice sites are uniform and identical. Primer extension analysis of +TA and +AT introduced into wild-type and *psf1-1* strains yields the same qualitative results as seen with +T PyDOWN and +A WT (Fig. 2C,D; Table 1); that is, the uridine-rich 3' splice site is less favored in the *psf1-1* mutant than in the wild-type strain. The total amount of mature message produced with these constructs is markedly lower in the *psf1-1* mutant versus wild type. Importantly, however, the influence that the *psf1-1* mutation exerts on utilization of uridine-rich 3' splice sites is very similar for both sets of constructs (Table 1, loss of uridine preference for +T PyDOWN and +TA; +A WT and +AT). Thus, the *psf1-1* mutant is specifically impaired in recognizing the uridine-rich tract at the 3' splice site.

Splicing of the TUB3 intron requires *Psf1*

The altered pattern of splice site choice in the *psf1-1* strain prompted us to determine whether splicing of endogenous yeast genes is affected by this mutation. Whereas most yeast 3' splice sites are enriched for uridines (Parker and Patterson 1987; Rymond and Rosbash 1992), mutations in the uridine tract often have no

Table 1. Splice site competitions in *psf1-1* strains: quantitation of splice site usage by primer extension and copper growth

Strain	RNA	MP/MD ^a	Loss of uridine preference ^b (×)	Copper growth ^c (mM)
WT	+ T PyDOWN	22 ± 0.3		0.05 ±
<i>psf1-1</i>	+ T PyDOWN	1.6 ± 0.10	14	0.18
<i>psf1-1</i> /WT	+ T PyDOWN	2.9 ± 0.3	7.6	0.1
WT	+ A WT	0.86 ± 0.04		1
<i>psf1-1</i>	+ A WT	3.1 ± 0.03	3.6	0.1
WT	+ TA	11 ± 2		0.075
<i>psf1-1</i>	+ TA	0.63 ± 0.03	17	0.1
WT	+ AT	0.037 ± 0.003		1
<i>psf1-1</i>	+ AT	0.17 ± 0.01	4.6	0.075

^aRatio of branchpoint–proximal (MP) to branchpoint–distal (MD) mature message quantitated by a Molecular Dynamics PhosphorImager. This ratio reflects the relative rates of splicing to each competing splice site (see Frank and Guthrie 1992). Data are expressed as the mean of at least three independent measurements ± s.d.

^bRelative shift in the MP/MD ratio away from use of the uridine-rich 3' splice site in the mutant strain compared with wild type: (MP/MD)_{WT}/(MP/MD)_{*psf1-1*} for + T PyDOWN and + TA; (MD/MP)_{WT}/(MD/MP)_{*psf1-1*} for + A WT and + AT.

^cMaximum copper resistance of strain measured with distal splice site (MD) in-frame and proximal splice site (MP) out-of-frame. (±) marginal growth at that concentration of copper. Note that copper resistance reflects only the absolute amount of (MD) mature message and not the relative ratios of splice site usage.

apparent phenotype, suggesting that recognition of this sequence is not usually rate limiting for 3' splice site selection (Patterson 1989). Primer extension analysis of several endogenous spliced yeast genes (*RP51*, *CYH2*, and *UBI1*) revealed no apparent splicing defects in the *psf1-1* strain (data not shown). We then examined the splicing of an unusual intron in the α -tubulin homolog, *TUB3*. The distance between the branch site and 3' splice site of this intron is atypically long (139 nucleotides) for yeast, and the 3' splice site is highly enriched for uridines (Fig. 3A) (Schatz et al. 1986). A non-uridine-rich spacer sequence of similar length, when introduced into the 3' splice site of the yeast actin intron, strongly inhibits its splicing (Cellini et al. 1986). We reasoned that the *TUB3* intron, because of its unusual architecture, might be highly dependent on its uridine tract and, thus, the *PSF1* gene product, for efficient splicing. Because *TUB3* is expressed at relatively low levels, we chose to move the intron into the well-expressed *ACT1*–*CUP1* gene fusion construct to facilitate analysis. As shown in Figure 3A, this construct (*TUB3* WT) contains the actin 5' exon and 5' splice site, most of the *TUB3* intron (except for the 5' splice site region), and the 3' exon encoding *CUP1*. For comparison, splicing of the wild-type *ACT1*–*CUP1* gene fusion is shown in lanes 1 and 2 of Figure 3B. The *TUB3* intron is spliced as efficiently as the wild-type *ACT1*–*CUP1* fusion in a wild-type strain, whereas it is spliced poorly in the *psf1-1* mutant, as evidenced by primer extension (mature message/lariat intermediate diminished 20-fold) and by copper growth (copper resistance diminished 8-fold in *psf1-1* mutant) (Fig. 3B, lanes 3,4; Table 2). The block to the second step of splicing indicated by the decreased ratio of (mature message/lariat intermediate) is expected for a mutant impaired in 3' splice site recognition. We ob-

serve no change in *TUB3* WT pre-mRNA levels, indicating that the splicing defect in the *psf1-1* strain occurs exclusively at the second catalytic step (data not shown). The splicing of the actin intron, which has a more typical spacing of 43 nucleotides between its branch site and 3' splice site, is only mildly affected (mature message/lariat intermediate diminished threefold) in the *psf1-1* strain (Fig. 3B, lanes 1,2; Table 2).

To establish that impaired recognition of the uridine-rich tract, and not another feature of the *TUB3* intron, is the basis for the *psf1-1* splicing defect, we mutated the 3' splice-site uridine tract in our *TUB3* fusion construct to adenosines [*TUB3* poly(A)] (Fig. 3A). We predicted that without a uridine-rich 3' splice site, the splicing of the *TUB3* poly(A) intron would be impaired and would be insensitive to the presence of the *psf1-1* mutation. This construct was introduced into wild-type or *psf1-1* mutant strains and analyzed by primer extension and copper growth. As predicted, splicing of the *TUB3* poly(A) intron is almost completely insensitive to the presence of the *psf1-1* mutation (Fig. 3B, lanes 5,6; Table 2). Furthermore, in a wild-type strain, the *TUB3* poly(A) intron is spliced more poorly than is the *TUB3* WT intron (Fig. 3B, lane 3 vs. lane 5; Table 2). Surprisingly, however, the *TUB3* poly(A) intron is spliced better in a *psf1-1* strain than is the *TUB3* WT intron (Fig. 3B, lane 4 vs. lane 6). Therefore, it appears that adenosines, though not preferred, can be recognized by the splicing machinery when uridines are not present at the 3' splice site. Moreover, the ability to utilize adenosine-rich 3' splice sites must be independent of the uridine recognition mediated by *Psf1*, because the *TUB3* poly(A) intron is spliced at similar efficiencies in both wild-type and *psf1-1* strains. Thus, despite our finding that adenosines at the 3' splice site can be recognized better than expected, this experi-

Figure 3. Splicing of the *TUB3* intron in wild-type and *psf1-1* strains. (A) Structure of the *ACT1-TUB3-CUP1* (*TUB3* WT) fusion construct and *TUB3* poly(A) fusion construct. Actin sequences are indicated by the left-hatched region, *TUB3* sequences by the lines, and nucleotides and *CUP1* sequences by the right-hatched region. The sequence of the *TUB3* 3' splice site is shown. (B) Primer extension of the *ACT1-CUP1*, *TUB3* WT, and *TUB3* poly(A) RNAs in wild-type, *PSF1*, and mutant, *psf1-1*, strains. (Left) Markers are for products of *ACT1-CUP1* splicing (lanes 1,2) and indicate mature message (M) and lariat intermediate (LI). A strong primer extension stop (U1*) from the *ACT1-CUP1* RNA obscures the U1 internal control in lanes 1 and 2. (Right) Markers are for products of *TUB3* WT and *TUB3* poly(A) splicing (lanes 3–6) and indicate mature message (M), lariat intermediate (LI), and U1 snRNA internal control (U1). Lane marker (m) indicates size markers that are underexposed. The *ACT1-CUP1* lariat intermediate is underexposed and not visible here. The size difference between the two mature messages is the result of a ~15-nucleotide shortening of the *ACT1-CUP1* 5'-untranslated region during cloning of *ACT1-TUB3-CUP1* and other constructs used in this work.

ment demonstrates that loss of uridine tract recognition is the basis of the *TUB3* splicing defect in the *psf1-1* strain.

psf1-1 exacerbates the defects of 3' splice site PyAG mutations

Although uridine recognition by Psf1 clearly plays a role in splice site selection and in the efficient splicing of

introns such as that in the *TUB3* gene, it might also make a contribution to the splicing of other introns. This contribution would normally be masked if 3' splice site recognition were not the rate-limiting step in splicing these introns. Therefore, to test for a more general role of uridine recognition by Psf1 in 3' splice site selection, we constructed point mutations in the PyAG motif of the actin 3' splice site. Point mutations in this sequence partially or completely block the second catalytic step of splicing and make 3' splice site utilization rate limiting for production of mature message (Vijayraghavan et al. 1986; Fouser and Friesen 1987). In this situation, one could imagine that recognition of the uridine-rich tract might be critical, even for an apparently "uridine-independent" intron. Thus, a defect in the *psf1-1* strain would be apparent in the splicing of these 3' splice site mutant RNAs.

We tested a number of actin 3' splice site mutations in the *ACT1-CUP1* reporter. Splicing of three representative mutants (TAG to GAG, TTG, or TGG) and the wild-type intron was analyzed by primer extension and copper growth (Fig. 4, Table 3). As shown in lanes 1 and 2 of Figure 4, the splicing of the wild-type actin intron is only mildly affected by the *psf1-1* mutation. As expected, in a wild-type strain, all three intron mutations result in increased amounts of lariat intermediate and reduced amounts of mature message, indicating their partial defect in the second catalytic step of splicing. Moreover, in a *psf1-1* strain, the mutant introns show an even greater

Table 2. Quantitation of splicing of wild-type *ACT1-CUP1* and *ACT1-TUB3-CUP1* derivatives by primer extension and copper growth

Strain	RNA	M/L ^a	Copper resistance ^b (mM)
WT	<i>ACT1-CUP1</i>	338 ± 31	2
<i>psf1-1</i>	<i>ACT1-CUP1</i>	110 ± 3	1.5
WT	<i>TUB3</i> WT	203 ± 2.0	2
<i>psf1-1</i>	<i>TUB3</i> WT	10.2 ± 10.30	0.25
WT	<i>TUB3</i> poly(A)	62 ± 5.8	1
<i>psf1-1</i>	<i>TUB3</i> poly(A)	41 ± 0.75	1
WT	none ^c	N.A.	0.013
<i>psf1-1</i>	none	N.A.	0.013

^aRatio of mature message (M) to lariat intermediate (L) quantitated by a Molecular Dynamics PhosphorImager. M/L provides a measure of the overall efficiency of the second catalytic step of splicing (see Frank and Guthrie 1992). Data are expressed as the mean of at least three independent measurements ± S.D. (N.A.) Not applicable.

^bMaximum copper resistance of a strain measures absolute amounts of mature message produced. Note that copper growth values (M only) will not always correspond to those for M/L. For discussion of this, see Pikeilny and Rosbash (1985). Also, copper resistance is not entirely linear with respect to the amount of message (Lesser and Guthrie 1993).

^cCopper resistance values below 0.025 mM are indistinguishable from a *CUP1* null mutant that grows at 0.013 mM copper.

Figure 4. Effect of the *psf1-1* mutation on splicing of actin 3' splice site derivatives. Primer extension of wild-type actin and actin 3' splice site mutants in wild-type *PSF1* or mutant *psf1-1* strains. The 3' splice site mutations all reside in the final 3 nucleotides of the actin intron in the *ACT1-CUP1* construct and are indicated above each lane. Primer extension products are marked as in Fig. 3, except L indicates lariat intermediate.

decrease in splicing efficiency (5- to 10-fold reduction in mature message/lariat intermediate). In addition, we observe a similar effect when substitutions in the uridine tract are made in *cis* with PyAG mutations (data not shown). Similar to *TUB3* pre-mRNA splicing, we see no change in pre-mRNA levels for these constructs in the *psf1-1* strain, indicating that the impairment of splicing is at the second catalytic step. In summary, *Psf1*-dependent uridine tract recognition can make a significant contribution to splicing efficiency, even in the actin intron that does not normally depend on its uridine tract for efficient splicing.

PSF1 is identical to PRP8

To determine the molecular basis for the *psf1-1* phenotype, we isolated genomic clones of *PSF1* and *psf1-1*. Wild-type *PSF1* clones were obtained from a standard yeast plasmid library that was transformed into a *psf1-1* strain harboring the *TUB3* WT construct. A wild-type copy of the *PSF1* gene was expected to improve the splicing efficiency of the *TUB3* WT RNA in a *psf1-1* strain and thus confer increased copper resistance (see Materials and methods).

Table 3. Quantitation of splicing of wild-type *ACT1-CUP1* and *ACT1-TUB3-CUP1* 3' splice site derivatives by primer extension and copper growth

Strain	RNA	M/L ^a	Copper resistance ^b (mM)
WT	<i>ACT1-CUP1</i> WT (3' TAG)	338 ± 31	2
<i>psf1-1</i>	<i>ACT1-CUP1</i> WT (3' TAG)	110 ± 13	1.5
WT	<i>ACT1-CUP1</i> 3' GAG	0.65 ± 0.04	0.1
<i>psf1-1</i>	<i>ACT1-CUP1</i> 3' GAG	0.056 ± 0.002	0.013
WT	<i>ACT1-CUP1</i> 3' TTG	0.0054 ± 0.0019	0.013
<i>psf1-1</i>	<i>ACT1-CUP1</i> 3' TTG	0.00085 ± 0.0005	0.013
WT	<i>ACT1-CUP1</i> 3' TGG	0.18 ± 0.00	0.025
<i>psf1-1</i>	<i>ACT1-CUP1</i> 3' TGG	0.014 ± 0.002	0.013

See Table 2 footnote for details.

Because the *psf1-1* mutant causes a partial splicing phenotype in the presence of a wild-type *PSF1* allele (Table 1; data not shown), to isolate the *psf1-1* allele, we constructed a plasmid library from the mutant strain and used it to transform a wild-type strain harboring the +T PyDOWN reporter construct (see Materials and methods). A *psf1-1* clone introduced into this strain is predicted to activate use of the distal splice site and, therefore, confer increased copper resistance.

Both strategies yielded clones with overlapping restriction maps. Partial sequence analysis revealed that the cloned inserts encoded the U5 snRNP protein Prp8 (Lossky et al. 1987; Jackson et al. 1988). Both clones

Figure 5. Allele specificity of the *psf1-1* phenotype. Primer extensions of +T PyDOWN RNAs from various *prp8* strains grown at the permissive temperature of 25°C. Products of primer extension are marked as in Fig. 2A. The *prp8* strains are described in Materials and methods.

Figure 6. UV cross-linking of Prp8 to the 3' splice site. (A) Diagram of splicing substrate XL7. The 3' splice site region is shown in detail with the A → G point mutation at the 3' splice site indicated by an arrow and the single radiolabeled phosphate by an asterisk (*). The 15-nucleotide product of RNase T1 digestion is underlined. (B) Splicing of XL7 in extracts derived from PRP8 epitope-tagged and untagged extracts. Presence or absence of triple HA epitope on Prp8 is indicated by + or - next to (HA)₃ above each lane. Presence or absence of ATP in the splicing reaction is similarly indicated. Bands corresponding to unspliced precursor, lariat intermediate, and excised lariat intron are indicated by cartooned symbols (right), with the radiolabeled phosphate molecule represented by an asterisk (*). (C) UV cross-linking of samples from B. (Lanes 1–3) Total cross-linked proteins from B; (lanes 4–6) cross-linked proteins that were immunoprecipitated with an anti-HA epitope antibody. Markers above each lane are as in B. (D) Analysis of cross-linked and immunoprecipitated RNase T1 fragment. (Lane 1) Proteinase-treated and extracted immunoprecipitate fractionated on a 20% denaturing polyacrylamide gel. (Lane m) Sizes of 5' end-labeled DNA markers (*Hpa*II-digested PBR325). (E) Splicing of XL7 and its derivatives. Products of splicing are cartooned next to each band. Derivatives are described in the text. (F) Cross-linking of Prp8 to XL7 and its derivatives. SDS-PAGE analysis of samples from E that were cross-linked and immunoprecipitated as in C. Only the region of the gel where Prp8 migrates is shown.

complemented a chromosomal deletion of the *PRP8* gene; however, only the *psf1-1* clone could activate the distal splice site in the +T PyDOWN construct, as expected (data not shown). The *psf1-1* mutation was mapped using gap repair (Guthrie and Fink 1991) and shown to reside in the carboxyl-terminal portion of the gene. Sequence analysis of this region revealed a single G → A transition in the *psf1-1* clone that changes a GAG glutamic acid codon to an AAG lysine codon at amino acid position 1960. Subsequent analysis of *psf1-2* revealed the identical nucleotide change.

To demonstrate that this G → A mutation causes the *psf1-1* phenotype, a small, sequenced restriction fragment containing the mutation was used to replace the corresponding region of the wild-type gene (see Materials

and methods). The swap produced a single point mutation in an otherwise wild-type *PRP8* clone and was both necessary and sufficient to generate the *psf1-1* phenotypes. The formal designation of *psf1-1* will be *prp8-101*, but for clarity we will continue to refer to it as *psf1-1* in this report.

Allele specificity of the *psf1-1* phenotype

The identity of *PSF1* with *PRP8* raised the question of whether the *psf1-1* phenotype is unique to this allele or commonly associated with *prp8* mutants. The low frequency of *psf* mutants in our genetic selection and the fact that both independently isolated *psf1* alleles contain identical mutations suggest that *psf1-1* confers a novel

phenotype. To test this directly, we introduced the +T PyDOWN reporter construct (Fig. 1) into *prp8* strains carrying either one of two temperature-sensitive alleles, *prp8-1* or *prp8-7*, or a cold-sensitive allele, *prp8^{cs}* (see Materials and methods for strain references). RNA was prepared from these strains grown at a permissive temperature (25°C), and the splicing products were analyzed by primer extension (Fig. 5). Use of the alternative 3' splice sites in the +T PyDOWN construct was essentially the same as in the wild-type strain for these alleles. Only the *psf1-1* allele of *PRP8* causes loss of uridine recognition and consequent activation of the distal splice site with this construct. Thus, the *psf1-1* mutation defines a novel function for the Prp8 protein.

Site-specific UV cross-linking of Prp8 to the 3' splice site

The simplest model to explain the role of Prp8 in 3' splice site selection is that Prp8 directly binds and recognizes the 3' splice site. To test this model, we used the technique of Moore and Sharp (1992) to introduce a single ³²P label at the 3' splice site of an in vitro splicing substrate (XL7) for the purpose of site-specific UV cross-linking (Fig. 6A). XL7 is a derivative of the *ACT1-CUP1* gene fusion with the 5' exon and intron containing 50% uniformly incorporated 5-bromouridine as a photoaffinity reagent (Hanna 1989). We also introduced an A → G point mutation (TAG → TGG) in the penultimate residue of the intron (Fig. 6A). The effect of this mutation, in vivo as well as in vitro, is to slow the normally rapid kinetics of the second catalytic step of splicing without changing 3' cleavage site choice.

For in vitro splicing, we employed a novel method for preparing splicing extracts. The procedure, which involves grinding cells in liquid nitrogen, allows rapid and reproducible preparation of highly active whole cell splicing extracts (see Materials and methods). Extracts were prepared from two isogenic *prp8* chromosomal deletion strains. One harbors a wild-type *PRP8*-containing plasmid, and the other harbors a plasmid with an epitope-tagged version of *PRP8*, containing three copies of a hemagglutinin (HA) epitope at its carboxyl terminus (Guthrie and Fink 1991; Materials and methods). The epitope-tagged version of *PRP8*, *PRP8*-HA3, complements a chromosomal deletion of *PRP8* and supports wild-type growth rates at all temperatures tested (16°C–37°C; data not shown).

XL7 undergoes the first catalytic step of splicing efficiently, accumulates lariat intermediate, and shows a reduced amount of mature message, indicated by the excised lariat intron (Fig. 6B, lanes 1,3). The placement of the labeled phosphate in the intron (Fig. 6A) precludes visualization of mature spliced RNA and the free first exon. As expected, XL7 was not spliced in the absence of ATP (Fig. 6B, lane 2).

A portion of each sample shown in Figure 6B was subjected to UV cross-linking followed by digestion with RNase T1. The samples were then analyzed by autoradiography following SDS-PAGE, before or after immuno-

precipitation using a monoclonal antibody specific for the HA epitope. Numerous polypeptides became cross-linked to the XL7 3' splice site in splicing extract, most of them in an ATP-independent manner (Fig. 6C, lanes 1 and 3 vs. lane 2). The cross-links were all dependent on irradiation with UV light (data not shown). Immunoprecipitation of Prp8-HA3 revealed a single labeled band that migrated at the molecular mass of Prp8 (280 kD) (Fig. 6C, lane 4). Furthermore, appearance of this band was dependent on ATP and the presence of an epitope tag on Prp8. These results demonstrate that the cross-linked species corresponds to Prp8 (Fig. 6C, lane 4 vs. lane 6) and that Prp8 associates with the 3' splice site in a splicing-dependent manner (Fig. 6C, lane 4 vs. lane 5).

Because Prp8 may make multiple contacts with the splicing substrate, incomplete RNase T1 digestion combined with cross-linking at a more distant site in the substrate would give the appearance of specific cross-links in our experiments. To confirm that the RNase T1 digestion we performed was complete, the immunoprecipitated material from a sample identical to that in Figure 6C (lane 4) was digested with protease and extracted (see Materials and methods). The RNA was precipitated from this sample and size fractionated (Fig. 6D). The labeled RNA in Figure 6D appears slightly larger and more heterogeneous than the 15-nucleotide fragment from digestion of pure XL7 RNA with RNase T1 (data not shown). This size distribution, slightly above the 15-nucleotide size marker, is probably the result of peptide adducts that remain covalently attached to the RNA after digestion and extraction. Importantly, no RNAs larger than these are present in the immunoprecipitate, indicating that the 3' splice site is the site of cross-linking detected in our experiments.

To demonstrate further that the interaction between Prp8 and the 3' splice site is splicing specific, we tested cross-linking using derivatives of XL7 that contain additional mutations. XL7-A5 contains a G → A mutation in the fifth position of the intron. This mutation strongly blocks the first step of splicing (Fig. 6E, lane 2) and results in no detectable Prp8–3' splice site cross-linking (Fig. 6F, lane 2). XL7-C259 contains an A → C mutation at the branch site A residue. This substrate is spliced 5- to 10-fold more poorly than XL7 (Fig. 6E, lanes 1,3) and shows a similar reduction in Prp8–3' splice site cross-linking (Fig. 6F, lanes 1,3). The strong correlation between the levels of splicing and cross-linking establishes that Prp8 interacts with the 3' splice site in active spliceosomes.

To address the possibility that Prp8 interacts nonspecifically with all regions of the substrate during splicing, we examined cross-linking in a derivative of XL7, XL7-E2, that contains a uniformly labeled second exon (the first labeled RNase T1 fragment begins 9 nucleotides downstream of the 3' splice site; see Materials and methods). With this substrate we see no detectable cross-links to Prp8 (Fig. 6E,F, lanes 4,5). When the specific activities of individual RNase T1 fragments are taken into account, the assay could detect cross-linking to any fragment in exon 2, even with a 10- to 50-fold reduction in

efficiency compared with cross-linking to XL7. Therefore, Prp8 does not interact promiscuously with all regions of the substrate during splicing. It interacts specifically with the 3' splice site (and perhaps other conserved sequences; see Discussion).

Discussion

Prp8 promotes use of the uridine-rich tract at the 3' splice site

The conserved uridine-rich tract upstream of the 3' splice site in yeast is important for efficient utilization of that splice site (Patterson and Guthrie 1991). Here, we have presented evidence for a functional interaction between Prp8 and the uridine-rich tract. Using a sensitive *cis*-competition assay, we have identified the *psf1-1* mutant allele of *PRP8* based on its loss of preference for a uridine-rich 3' splice site and consequent activation of a nonpreferred, competing, adenosine-rich 3' splice site. This phenotype contrasts that of the *slu7-1* mutant that is impaired in utilization of branch site-distal 3' splice sites independent of sequence content (Frank and Guthrie 1992). *slu7-1*, therefore, appears to affect spacing constraints or polarity. The relationship between Prp8 and Slu7 is discussed below.

Because yeast introns do not normally contain competing 3' splice sites, we searched for endogenous yeast introns that might require the uridine-recognition function of Prp8 and thus be affected by the *psf1-1* mutation. The *TUB3* intron is a good candidate for such an intron because of its unusual architecture. It has an atypically long branch site to 3' splice site distance (139 nucleotides) and a high enrichment for uridines preceding its 3' splice site (10 out of 15 residues preceding the PyAG are uridines). The *TUB3* intron is spliced very poorly in the *psf1-1* strain. Furthermore, the effect of *psf1-1* on the splicing of the *TUB3* intron can be obviated when the uridine tract is mutated to adenosines. Thus, the splicing defect of the *TUB3* intron in a *psf1-1* strain can be attributed to loss of uridine recognition.

To determine whether the uridine recognition function of Prp8 contributes to the splicing of other types of introns, albeit to a lesser extent, we made 3' splice site selection rate limiting in the actin intron by introducing point mutations in the PyAG motif. In this situation the *psf1-1* mutation exerts a large deleterious effect on splicing, demonstrating the contribution of uridine-tract recognition by Prp8, even in the actin intron, whose architecture is typical of yeast introns. Thus, uridine-tract recognition by Prp8 probably contributes to the splicing efficiency of many yeast introns.

The psf1-1 mutation defines a novel function for Prp8

Previous studies of Prp8 have implicated this protein in early steps of spliceosome assembly. Depletion of Prp8 or inactivation of a temperature-sensitive mutant, *prp8-1*, leads to an early block in spliceosome formation, at a step prior to U4, U5, and U6 triple snRNP addition.

However, the functions of Prp8 in the spliceosome, subsequent to the spliceosome assembly block, could not be assessed by these methods (Brown and Beggs 1992).

Our genetic approach has allowed us to identify a novel function for Prp8 by demanding a precise functional defect, loss of preference for a uridine-rich 3' splice site. The *psf1-1* phenotype most likely results from a specific inactivation of the domains in Prp8 responsible for uridine-rich 3' splice site recognition rather than a general inactivation of the protein. Supporting this is our finding that the uridine-recognition phenotype is specific for the *psf1-1* allele of *PRP8*. Most notably, unlike other alleles of *PRP8* that are blocked prior to the first catalytic step of splicing, the biochemical block caused by the *psf1-1* mutation is at the second catalytic step of splicing, which is when 3' splice site selection takes place. Moreover, other *prp* mutants that are specifically defective for the second step of splicing do not display the uridine-recognition defect of *psf1-1* (Frank and Guthrie 1992). Finally, the novel phenotype of the *psf1-1* allele provides an important tool for the dissection of the 280-kD Prp8 protein into structure/function modules. As a first step, we are isolating additional alleles of *PRP8* that confer a *psf1*-like phenotype.

Prp8 interacts directly with the 3' splice site

The above observations of the *psf1-1* mutant phenotype are consistent with a mechanism whereby Prp8 binds directly to the uridine tract of a 3' splice site, activating its use. To test this model, we used site-specific UV cross-linking to determine whether the Prp8 protein contacts the 3' splice site during the splicing reaction. Because the kinetics of the second catalytic step of splicing are rapid (Schwer and Guthrie 1991), we wished to slow down this reaction in order to accumulate possible intermediates in 3' splice site recognition. The substrate we used contains an A → G point mutation in the dinucleotide splice acceptor (AG → GG) and undergoes the first catalytic step of splicing efficiently, accumulating lariat intermediates. The second step of splicing occurs accurately but at a reduced rate. With this substrate, we were able to identify a UV-induced cross-link between Prp8 and a 15-nucleotide region of the intron that contains the uridine-rich tract and the 3' splice site. This cross-link is splicing specific as it is absent when ATP is omitted and correlates with the reduced levels of splicing activity when mutant substrates are utilized. The absence of cross-linking between Prp8 and the uniformly labeled second exon indicates that the interaction between Prp8 and the 3' splice site is specific. The demonstration of a direct physical contact between Prp8 and the 3' splice site, combined with the genetic data above, provides strong support for a model in which Prp8 binds and activates uridine containing 3' splice sites (see below; Fig. 7).

Our results are interesting in light of previous work that demonstrated cross-linking between Prp8, or its mammalian homolog, p220, and the splicing substrate (Garcia-Blanco et al. 1990; Whittaker and Beggs 1991).

Figure 7. Model for the role of Prp8 and U5 snRNA in 3' splice site selection and the second step of splicing. A splicing substrate that has formed lariat intermediate and is about to undergo the second step of splicing is cartooned. (Dark lines) Lariat structure; (nucleotides) 3' splice site sequences; (shaded rectangles) exons 1 and 2. Interactions that might serve to position exon 1 and the 3' splice site together are depicted. Prp8 protein is shown interacting with the uridine tract at the 3' splice site (this paper) and with exon 1 (Wyatt et al. 1992). Prp8 is also a constituent of U5 snRNP (U5) (Lossky et al. 1987) that is depicted only in RNA form (thick dark lines) with its conserved loop nucleotides interacting with exons 1 and 2 (Newman and Norman 1991; Wasserman and Steitz 1992; Wyatt et al. 1992; Cortes et al. 1993; Sontheimer and Steitz 1993).

Although these cross-links were not mapped, subsequent work identified the -2 position of the first exon (relative to the 5' splice site) as one p220 cross-linking site (Wyatt et al. 1992). The functions of these Prp8/p220-pre-mRNA interactions have not yet been determined but could reflect an earlier event involving the U5 snRNP (see below). Given the conservation of the polypyrimidine tract and, in particular, the sequence and large size of the *PRP8* gene (Anderson et al. 1989; Pinto and Steitz 1989; Garcia-Blanco et al. 1990; J.G. Umen and C. Guthrie, unpubl.), it is likely that the 3' splice site recognition function for Prp8 that we have identified in yeast is similar in mammals and other organisms. Consistent with this, experiments in mammalian extracts suggest a role for the polypyrimidine tract late in the splicing pathway, similar to one that has been found in

yeast (Reed 1989). As in yeast, the polypyrimidine tract may not be critical for the second step of splicing in all mammalian introns (Smith et al. 1989, 1993).

The role of U5 snRNP in 3' splice site selection and the second catalytic step of splicing

In yeast, three *trans*-acting factors have now been shown to affect 3' splice site selection. They are Prp8, which interacts with the conserved uridine-rich tract at the 3' splice site (this paper); the conserved loop in U5 snRNA, which can interact with nonconserved sequences in the second exon adjacent to the 3' splice site (Newman and Norman 1992; Sontheimer and Steitz 1993); and Slu7, which promotes use of branch site-distal 3' splice sites in a sequence nonspecific manner (Frank and Guthrie 1992). The former two are components of U5 snRNP, whereas Slu7 interacts genetically with U5 snRNA.

How might these factors function to promote 3' splice site recognition and the second catalytic step of splicing? The conserved loop of U5 snRNA and Prp8 are both in the same snRNP particle and are, therefore, very likely to interact with the 3' splice site region simultaneously. Although neither of these interactions would normally provide the necessary specificity for 3' splice site selection, together, they might serve to enhance recognition of a properly situated AG dinucleotide that is bound by an, as yet, unidentified factor. Our observation of a synergistic inhibition of splicing of 3' splice site PyAG mutants in a *psf1-1* strain is consistent with the idea that Prp8 and this factor bind the 3' splice site cooperatively. How Slu7 functions in this process is less clear. Based on the phenotype of *slu7-1*, Frank and Guthrie (1992) have proposed that Slu7 interacts with the splicing factor that directly recognizes the uridine-rich tract at the 3' splice site. If so, then Slu7 might serve to monitor or promote the binding of Prp8 to the 3' splice site.

As described above, the mammalian Prp8 homolog p220 and the conserved loop of U5 snRNA have also been shown to interact with the first exon during splicing (Newman and Norman 1991, 1992; Wyatt et al. 1992; Cortes et al. 1993; Sontheimer and Steitz 1993). These findings have implicated Prp8 and U5 snRNP as playing an important role during the first catalytic step. Taken together with our results, Prp8 thus appears to promote both catalytic steps of the splicing reaction. Furthermore, if the interactions between U5 snRNP and exon 1 are maintained after the first catalytic step, then Prp8 and U5 snRNA would each bind the 5' exon and 3' splice site simultaneously; in this case, Prp8 could serve to juxtapose the reaction partners for the second catalytic step or, conceivably, participate directly in catalysis (Fig. 7). The precise timing of the Prp8-3' splice site interaction is currently under investigation.

Materials and methods

Materials

The following materials were purchased from the indicated sources: restriction enzymes (New England Biolabs); T4 poly-

nucleotide kinase (Pharmacia); T4 DNA ligase and Sequenase (U.S. Biochemical); MLV reverse transcriptase (BRL); 5-FOA (PCR Incorporated); ethylmethane sulfonate (EMS) and 5-bromo-UTP (Sigma); [γ - 32 P]ATP, [α - 32 P]ATP and Hot Tub DNA polymerase (Amersham); 12CA5 antibodies (BAbCo); RNase T1 (Boehringer Mannheim). Oligonucleotides were synthesized by Hiten Madhani, Suzanne Noble, Yan Wang, and Chris Siebel on a MilliGen Plus Cyclone DNA Synthesizer. Benomyl was a generous gift from DuPont Chemicals.

Genetic methods

All genetic manipulations including EMS mutagenesis were carried out as described (Guthrie and Fink 1991). For EMS mutagenesis, $\sim 10^9$ haploid cells from strain L5 containing *ACT1-CUP1* + T PyDOWN were mutagenized to 50% lethality and plated directly on media containing 0.18 or 0.25 mM CuSO₄. After 4 days of growth at 30°C, colonies were picked and mated to strain I4. Dominant mutants were discarded, and the remaining strains were cured of the reporter plasmid and retested for copper growth. Those that did not behave as *cup1* null mutants (0.013 mM maximum copper resistance) without the reporter plasmid were also discarded. Two mutant strains fulfilled these criteria. A mating test and subsequent analysis (see Results) showed that the two mutants, *psf1-1* and *psf1-2*, were allelic. Copper growth assays were performed as described previously (Lesser and Guthrie 1993).

Strains

The following *S. cerevisiae* strains were used in this study: L5 [*MAT α cup1 Δ ::ura3 leu2 trp1 ura3-52 lys2 his3 ade*, derivative of K3 (Lesser and Guthrie 1993) constructed by J. Lefstin]; I4 [*MAT α cup1 Δ ::URA3 leu2 trp1 ura3-52 lys2 his3 ade* (Lesser and Guthrie 1993)]; YJU69, identical to L5 except it contains a chromosomal *psf1-1* (*prp8-101*) mutation; N13/L5, diploid formed from mating of YJU69 and the α mating-type version of L5; JDY8.02 [*prp8 Δ ::LEU2 MAT α leu2-3 leu2-112 tyr1 his3 Δ 1 ura3-52 ade2-101 trp1-289 pY8000, URA3 marked PRP8 plasmid* (Brown and Beggs 1992)]; YJU74 [*prp8 Δ ::LEU2 MAT α leu2-3 leu2-112 tyr1 his3 Δ 1 ura3-52 ade2-101 trp1-289 pep4 Δ ::TRP1 pY8000*]; YJU76, identical to YJU74 except it contains *PRP8* plasmid pJU186; YJU77, identical to YJU74 except it contains *PRP8-HA3* plasmid pJU204; *rna8-1* [*prp8-1 MAT α ade arg trp1 ura3* (gift from Anita Hopper, Pennsylvania State University School of Medicine, University Park)]; DJY76 [*prp8-7 MAT α leu2-3 leu2-112 tyr1 his3 ura3-52* (gift from Jean Beggs, University of Edinburgh, Scotland)]; and YSN157 [*prp8cs MAT α ura3 leu2 his3 lys2 ade 2* (gift from Suzanne Noble, University of California, San Francisco)]. *Escherichia coli* strain DH5 α was used as a recipient for all cloning procedures.

Plasmids

Standard cloning procedures were used for all plasmid constructions (Maniatis et al. 1982). pGAC14 (*ACT1-CUP1*) (Lesser and Guthrie 1993) was the starting plasmid for all reporter constructs used here. Derivatives were made by PCR mutagenesis. TUB3 WT (*ACT1-TUB3-CUP1*) was made by replacing the pGAC14 *XhoI-KpnI* fragment with *TUB3* intron nucleotides 67–298 amplified from plasmid pRB300 (Schatz et al. 1986). Other *ACT1-TUB3-CUP1* and *ACT1-CUP1* derivatives were constructed by replacing the *BamHI-KpnI* fragment from one of these constructs with mutant PCR fragments.

PRP8 plasmids were constructed from the YCP50 *PRP8* or *psf1-1* clones (see below) by cutting with *XhoI* and *NheI* and

ligating the gene into the *XbaI* and *SaII* sites of the HIS3–CEN–ARS vector pSE362 (Elledge and Davis 1988) to generate pJU186 and pJU187. PCR mutagenesis was used to introduce a unique *NotI* restriction site into the carboxyl terminus of pJU186 just before the termination codon. A small restriction fragment containing three tandem copies of the HA epitope (Tyers et al. 1992) was inserted into this site to generate pJU204 (*PRP8-HA3*).

Cloning *psf1-1* and PRP8

The wild-type *PSF1* (*PRP8*) gene was cloned by complementing the copper growth defect of a *psf1-1* strain containing the TUB3 WT reporter using a YCP50-based genomic library (gift from Mark Rose, Princeton University, New Jersey). Transformants were selected for growth at 1 mM copper. The mutant *psf1-1* (*prp8-101*) allele was cloned by constructing a library of Sau3a partially digested *psf1-1* genomic DNA that was inserted into the *BamHI* site of YCP50. This library was transformed into strain L5 that contained the +T PyDOWN reporter. Transformants were screened for growth at 0.1 mM copper. Three wild-type and 12 *psf1-1* mutant clones containing overlapping inserts were recovered (Dower et al. 1988; Strathern and Higgins 1991). A mutant clone was gap-repaired to map the *psf1-1* mutation and a 1.9-kb *ClaI* fragment containing the mutation was sequenced to find the change. The single point mutation was reintroduced into the wild-type clone pJU186 by replacing its 1.9-kb *ClaI* fragment with the mutant version.

RNA preparation and primer extension analysis

Cells were grown to mid-logarithmic phase to harvest RNA. Total RNA was prepared according to Lesser and Guthrie (1993). The exon 2 and U1 primers used were Cup 24-mer, 5'-CTTCATTTTGGGAAGTTAATTAATT-3', and 19K, 5'-CAATGACTTCAATGAACAATTAT-3'. Primer extensions were performed by annealing 1 ng of 5' end-labeled primer to 15 μ g of total RNA in TE with 0.25 M KCl in 10 μ l. Annealing was performed by slow cooling the mixture from 70°C to 42°C, at which point 25 μ l of RT mix (15 mM Tris-HCl at pH 8.3, 7.5 mM MgCl₂, 75 μ g/ml of actinomycin D, 0.625 mM dNTPs, 1 mM DTT, 4 U/ μ l of MLV reverse transcriptase) was added. Samples were incubated at 42°C for 30 min and ethanol-precipitated. cDNAs were fractionated on 6% acrylamide/7 M urea/TBE sequencing gels.

RNA substrates

Templates for in vitro transcription were generated by PCR using the PG14 *ACT1-CUP1* plasmid as a target for amplification. Oligonucleotide primers for the 5' half molecule were T7-5'II, 5'-GCGCTAATACGACTCACTATAGGGAGATTTTTCACGCTTAC-3' and XL7, 5'-GCGGTACCAAGGAAAAATATAGCAAAAAAGAATGAAG-3'. Oligonucleotides for the 3' half-molecule were XL1, 5'-GCGCTAATACGACTCACTATAGGGTTGCTGGTACCGGT-3' and CUPE2HindIII, 5'-GGGGAAGCTTACATTGGCATTGGCACTC-3'. The 5' half-template was cut with *StyI*, and the 3' half-template was cut with *HindIII* prior to transcription. RNAs were synthesized using an Ambion T7 Megascript kit according to instructions. For the 5' half-RNA, 50% of the UTP was substituted with 5-bromo-UTP. RNAs were gel-purified, end-labeled, and ligated as described previously (Wyatt et al. 1992), with each RNA at 2 μ M in a 10- or 20- μ l ligation reaction. The bridging deoxyoligonucleotide, 5'-ACCCGGTACCAACCCCAAGGAAAAATATAGCAA-3', was also at 2 μ M. Efficiency of ligation reactions was 10–20%. XL7-A5 and XL7-C259 were constructed as above

Umen and Guthrie

except that the appropriate mutant PCR templates were utilized. XL7-E2 was constructed by labeling the 3' half RNA uniformly with [$\alpha^{32}\text{P}$]ATP and incorporating 50% 5-bromo-UTP. The first labeled RNase T1 fragment begins 9 nucleotides downstream of the 3' splice site, and the fragments range in size between 2 and 21 nucleotides. The 21-nucleotide fragment contains half of the ^{32}P label.

Splicing extract preparation

Whole cell extracts were prepared using an adaptation of a method described previously (Sorger et al 1989). One to three liters of cells from strains YJU76 and YJU77 were grown to late logarithmic phase in YEPD. Cells were harvested by centrifugation and washed twice with 50 and 20 ml of buffer AGK (10 mM HEPES at pH 7.9, 1.5 mM MgCl_2 , 200 mM KCl, 10% vol/vol of glycerol). Cell pellets were resuspended in 0.4 cell pellet volumes of AGK with protease inhibitors (final concentrations: 1 mM PMSF, 1 $\mu\text{g}/\text{ml}$ of leupeptin, 1 $\mu\text{g}/\text{ml}$ of aprotinin, 1 mM benzamidin). The suspension was added to a 5'' porcelain mortar (Fisher) containing liquid nitrogen and ground to a fine powder with occasional addition of liquid nitrogen. The frozen powder was transferred to a centrifuge tube, thawed rapidly at 25°C or 30°C, and centrifuged in a SS34 rotor at 17K, 4°C for 30 min. From this point, the samples were treated as described previously for yeast splicing extract preparation except that the dialysis buffer volume was 1.5 or 2 liters and was changed once during the procedure (Lin et al. 1985).

In vitro splicing and UV cross-linking

Standard in vitro splicing reactions (Lin et al. 1985) were in 40 μl with 3×10^5 cpm substrate. After 30 min incubation at 25°C, a small aliquot was removed for analysis of the RNA. The remainder was placed in a microtiter dish on ice and irradiated at 312 nm with a hand held UV lamp (Fisher) for 5 min at a distance of 0.5–1 cm. Samples were removed and protease inhibitors (see above) and RNase T1 (10 U/ μl (final) were added and the samples were incubated at 37°C for 30 min. An aliquot of 5 μl was removed and added to SDS-PAGE sample buffer for total protein analysis. KCl and NP-40 were added to the remaining sample to final concentrations of 300 mM and 0.05%, respectively, and 1 μg of protein A purified 12CA5 antibody was added to each sample. After 1 hr on ice, protein A–Sepharose beads (Pharmacia) were added in 500 μl of KTN buffer (0.3 M KCl, 20 mM Tris at pH 7.4, 0.05% NP-40) and the samples gently rocked at 4°C for an additional hour. The protein A–Sepharose beads were washed three times with KTN buffer, and sample buffer was added to the pellet. After boiling for 2 min, the samples were fractionated on a 7.5% SDS–polyacrylamide gel and autoradiographed. For analysis of the cross-linked RNA fragment, the pellet from the immunoprecipitate was resuspended in 40 μl of 1% SDS, 0.5 mg/ml of proteinase K (Sigma), and 10 mM EDTA and incubated at 50°C for 30 min. The sample was then extracted twice with phenol/chloroform (1:1), ethanol-precipitated, fractionated on a 20% acrylamide/TBE/7 M urea gel, and autoradiographed.

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J G Umen and C Guthrie

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