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Evidence for a role of Sky1p-mediated phosphorylation in 3′ splice site recognition involving both Prp8 and Prp17/Slu4

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ABSTRACT

The SRPK family of kinases is specific for RS domain-containing splicing factors and known to play a critical role in protein–protein interaction and intracellular distribution of their substrates in both yeast and mammalian cells. However, the function of these kinases in pre-mRNA splicing remains unclear. Here we report that *SKY1*, a SRPK family member in *Saccharomyces cerevisiae*, genetically interacts with *PRP8* and *PRP17/SLU4*, both of which are involved in splice site selection during pre-mRNA splicing. Prp8 is essential for splicing and is known to interact with both 5′ and 3′ splice sites in the spliceosomal catalytic center, whereas Prp17/Slu4 is nonessential and is required only for efficient recognition of the 3′ splice site. Interestingly, deletion of *SKY1* was synthetically lethal with all *prp17* mutants tested, but only with specific *prp8* alleles in a domain implicated in governing fidelity of 3′AG recognition. Indeed, deletion of *SKY1* specifically suppressed 3′AG mutations in *ACT1-CUP1* splicing reporters. These results suggest for the first time that 3′AG recognition may be subject to phosphorylation regulation by Sky1p during pre-mRNA splicing.

Keywords: 3′ AG recognition; genetic interactions; phosphorylation control; second step factors; SR protein kinases

INTRODUCTION

Pre-mRNA splicing requires both small nuclear ribonucleoprotein particles (snRNPs) and non-snRNP protein factors. Genetic and biochemical studies have uncovered most of the essential components for catalyzing the splicing reaction within the spliceosome (reviewed by Kramer, 1996; Burge et al., 1999). Although the two transesterifications are likely to be catalyzed by the RNA core established in the spliceosome (reviewed by Nilsen, 1998), recognition of correct splice sites and formation of the catalytic core require multiple components assembled on pre-mRNA in an orderly fashion. Analysis of splicing complexes shows that spliceosome assembly is initiated by U1 snRNP base pairing with the 5′ splice site in an ATP-independent manner followed by ATP-dependent U2 snRNP recognition of the branchpoint sequence. The established prespliceosome is then converted to mature spliceosome by the joining of the U4/5/6 tri-snRNP (reviewed by Burge et al., 1999). Many initial contacts of critical splicing signals by snRNPs and non-snRNP factors likely take place much earlier, before the formation of stable complexes at distinct stages. For example, it was recently shown that U2 snRNP establishes a functional contact with the U1-pre-mRNA complex in the absence of ATP (Das et al., 2000) and that U4/5/6 tri-snRNP contacts the 5′ splice site in an ATP-dependent manner prior to the base pairing interaction between U2 and the branchpoint sequence (Maroney et al., 2000).

A central question regarding efficient and accurate intron removal is how the 5′ and 3′ splice sites are recognized during the splicing reaction. Selection of the 5′ splice site appears to be determined initially by base pairing between U1 snRNP and the consensus intronic sequence at the 5′ splice site. This base-pairing interaction is aided by the cap-binding protein CBP80 as well as a number of U1-associated proteins in budding yeast (Puig et al., 1999; Zhang & Rosbash, 1999). The consensus 5′ sequence is later proofread by base pairing with U6 (Kandels-Lewis & Séraphin, 1993; Lesser & Guthrie, 1993). The sequential recognition of the 5′ splice site by U1 and subsequently by U6 is mediated by the RNA helicase Prp28 (Staley &

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Guthrie, 1999; Chen et al., 2001). Selection of the 3′ splice site appears to involve a more complex strategy. In both yeast and humans, BBP/SF1 binds to the branchpoint sequence and Mud2/U2AF^{65} interacts with the polypyrimidine tract (Abovich & Rosbash, 1997; Berglund et al., 1997). These sequence-specific protein–RNA interactions set the stage for the RNA helicase Sub2/UAP56 to mediate U2 base pairing with the branchpoint sequence (Berglund et al., 1998; Kistler & Guthrie, 2001; Libri et al., 2001; Zhang & Green, 2001). These interactions, however, are not sufficient to define the 3′AG. Interestingly, a class of so-called AG-independent introns does not require the 3′AG for the first-step chemical reaction, indicating that 3′AG recognition can take place after the assembly of a catalytically competent spliceosome. Selection of the correct 3′AG, however, is critical for establishing the catalytic center for the second step in which U5 is positioned to contact both 5′ and 3′ exon sequences (Newman & Norman, 1992; Sontheimer & Steitz, 1993; O'Keefe & Newman, 1998), and the first and the last G in the intron form a non-Watson–Crick contact (Parker & Siliciano, 1993; Chanfreau et al., 1994; Deirdre et al., 1995).

The establishment of critical RNA–RNA interactions in the spliceosome likely depends on the function of a large number of protein factors. One such essential factor is the U5-associated protein Prp8, required for both transesterificiations. Most strikingly, Prp8 can be crosslinked to all important splicing signals within the pre-mRNA, that is, the 5′ and 3′ splice sites, the branchpoint sequence, and the polypyrimidine tract (Wyatt et al., 1992; MacMillan et al., 1994; Teigelkamp et al., 1995; Chiara et al., 1996; Reyes et al., 1996; Umen & Guthrie, 1996). Consistent with this biochemical property of Prp8, recent genetic experiments show that many *prp8* alleles can simultaneously suppress mutations in the 5′GU and 3′AG as well as a mutation in U6 (Collins & Guthrie, 1999; Siatecka et al., 1999). In addition, Prp8 has been shown to play a role in U4 release, which is essential for spliceosome activation (Kuhn et al., 1999). These observations suggest that Prp8 may directly contribute to the establishment of the catalytic center for the splicing reaction (reviewed by Collins & Guthrie, 2000).

Genetic and biochemical studies in budding yeast have also established that five proteins (Prp16, Prp17/Slu4p, Prp18, Slu7p, and Prp22) function exclusively in the second step by interacting with the 3′ splice site in a sequential fashion: Prp16 (an RNA-dependent ATPase) and Prp17/Slu4 interact with the 3′AG in an ATP-dependent manner (Jones et al., 1995). Following a conformational change triggered by Prp16-catalyzed ATP hydrolysis, Slu7 becomes strongly crosslinked to the 3′AG and the interaction requires the functions of both Prp17/Slu4 and Prp18 (Umen & Guthrie, 1995a). Finally, the RNA helicase Prp22 is not only required for the second-step chemical reaction but is also responsible for the release of spliced mRNA from the spliceosome (Schwer & Gross, 1998). Although this elegant picture of orderly recognition of the 3′AG is largely derived from studies in budding yeast, all of these second-step factors are conserved between yeast and humans (Ohno & Shimura, 1996; Horowitz & Krainer, 1997; Ben-Yehuda et al., 1998; Lindsey & Garcia-Blanco, 1998; Zhou & Reed, 1998; Chua & Reed, 1999a). In particular, human Slu7 has recently been shown to play a critical role in selecting the authentic 3′AG in HeLa nuclear extracts (Chua & Reed, 1999b).

Interestingly, among all known second step factors, only Prp8 and Prp16 are essential for splicing. Prp17/Slu4 only affects the splicing efficiency at the second step (Frank & Guthrie, 1992), whereas Prp18, Slu7p, and Prp22 are required only when the 3′AG is located beyond a certain distance from the branch site (Brys & Schwer, 1996; Zhang & Schwer, 1997; Schwer & Gross, 1998). Similarly, it was recently observed in higher eukaryotes that U2AF^{35} plays a critical role in 3′AG recognition, but is dispensable for splicing of introns with short distances between the branchpoint sequence and the 3′AG (Merendino et al., 1999; Wu et al., 1999; Zorio & Blumenthal, 1999). These observations indicate a tremendous plasticity in 3′ splice site recognition and raise the possibility for modulation of splicing efficiency and fidelity by as yet unrecognized mechanisms (i.e., involvement of additional factors and/or modification of existing factors). In this regard, it is interesting to note that a U2-associated protein, SAP155, becomes phosphorylated right before the second-step transesterification reaction in HeLa nuclear extracts, although the functional significance of this phosphorylation event remains unclear (Wang et al., 1998a).

Our current study was prompted by the finding that a family of SR protein-specific kinases (SRPKs) is conserved from yeast to higher eukaryotes (Tang et al., 1998, 2000; Siebel et al., 1999). The single SRPK family member in budding yeast is called Sky1p and displays similar activity and substrate specificity to its mammalian counterparts (Siebel et al., 1999; Yeakley et al., 1999; Nolen et al., 2001). SRPKs can affect many biochemical properties of their substrates, including protein–protein interaction, nuclear distribution, and more recently, nuclear import (Wang et al., 1998b; Siebel et al., 1999; Yeakley et al., 1999; Yun & Fu, 2000; Gilbert et al., 2001). However, no direct evidence is available to document a role of SRPKs in splicing, although phosphorylation has long been thought to be important for pre-mRNA splicing in mammalian cells (Mermoud et al., 1994; Cao et al., 1997). Here we took advantage of budding yeast as a genetically tractable system to address the function of Sky1p and found that the kinase genetically interacts with two conserved splicing factors, Prp8 and Prp17/Slu4, involved in the second step of the splicing reaction. Our findings have not

only provided a novel insight into the function of Sky1p, but also revealed an unanticipated connection between phosphorylation and 3′AG recognition.

RESULTS

SKY1 genetically interacts with the splicing factor *PRP8*

Because *SKY1* is not essential for growth (Siebel et al., 1999), a synthetic lethal screen was conducted to identify genes that genetically interact with the kinase using the sectoring strategy described by Holm and coworkers (Kranz & Holm, 1990). This screen exploits the accumulation of red pigment in yeast containing defective *ade2* and wild-type *ADE3*. As diagramed in Figure 1A, *SKY1* was deleted from a yeast strain (CH1305) carrying defective chromosomal alleles of both *ade2* and *ade3*. The resulting SL-sky1Δ strain was covered with wild-type *SKY1* in a *URA3* and *ADE3*-marked plasmid (pCH1675-*SKY1*). Because *SKY1* is dispensable for growth, the plasmid is easily lost and cells without *ADE3* become white. A colony may appear white or sector red and white, depending on how early the plasmid is lost during growth. Red (nonsectoring) colonies will appear if a second site mutation makes *SKY1* essential and therefore prevents the growth of cells that lose the plasmid bearing the wild-type *ADE3* and *SKY1* genes.

We mutagenized the SL-sky1Δ strain with UV light and isolated red colonies from approximately 100,000 surviving colonies (see Materials and Methods). Individual nonsectoring colonies were then subjected to three tests: (1) growth on 5-FOA plates to select for the loss of the *SKY1* plasmid (true synthetic lethals cannot grow); (2) the ability to regain sectoring and growth on 5-FOA plates when the cells were transformed with a *LEU2*-marked plasmid bearing wild-type *SKY1* (YCpLac111-*SKY1*) (this test eliminates false positives; e.g., a colony may appear red due to integration

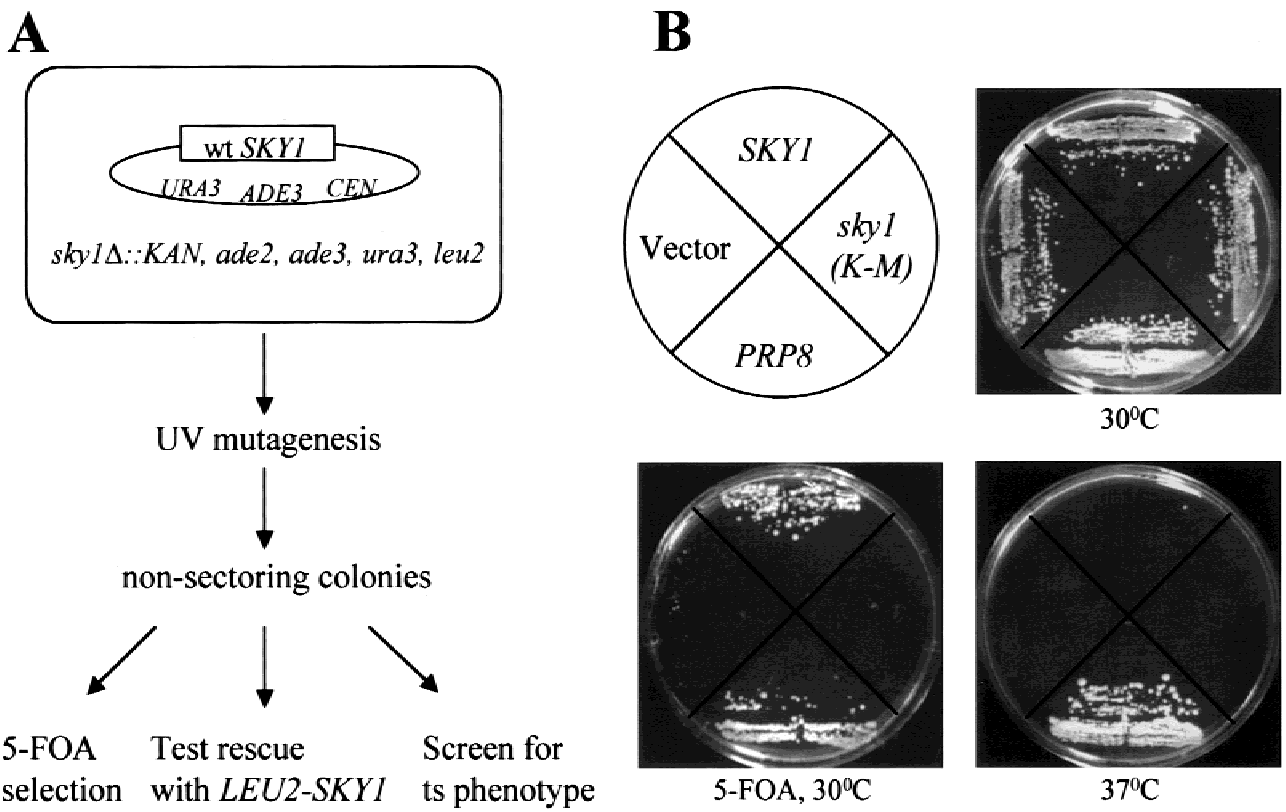


FIGURE 1. Genetic interaction between *SKY1* and *PRP8*. **A:** Diagramed is the strategy used for the synthetic lethal screen. *SKY1* was deleted from the yeast strain CH1305 to create SL-sky1Δ (the genotype shown in the box). The strain carries wild-type *SKY1* in the plasmid pCH1675-*SKY1* marked with *URA3* and *ADE3*. **B:** Diagram of location of the *SL39* strain transformed with vector (YCpLac111), *SKY1* (YCpLac111-*SKY1*), *sky1 (K-M)* (YCpLac111-*sky1 K-M*), and *PRP8* (YCpLac111-*PRP8*). Top right: Growth phenotype in the YP galactose minus Leu and Ura at 30 °C; Bottom left: Growth phenotype in the YP galactose media minus Leu and containing 5-FOA at 30 °C. The presence of *LEU2-SKY1* or *LEU2-PRP8* plasmids allowed the loss of the *URA3-SKY1* plasmid. *LEU2-sky (K-M)* could not replace *LEU2-SKY1* in this assay, indicating that kinase activity is required for the genetic interaction between *SKY1* and *PRP8*. Bottom right: Growth phenotype of the *SL39* strain in the YP galactose media minus Leu at 37 °C, showing the ts phenotype of the *SL39* strain and complementation by wild-type *PRP8*. All plates shown were photographed after 3 days of culturing.

of *ADE3* into the yeast genome or conversion of the chromosomal *ade3* allele to wild type by homologous recombination); and (3) potential temperature sensitive (ts) phenotypes associated with isolated mutants. We noted that many isolated red colonies had the tendency to acquire variable ability to grow on 5-FOA plates containing glucose during extended culturing. However, this reversion/adaptation could be largely suppressed if glucose was replaced with galactose as the carbon source in the media. We therefore conducted our screen and initial characterization of candidates in galactose media and then confirmed the synthetic lethal phenotype in glucose media. For consistency, all data shown in this report are derived from analysis in galactose media.

Among several candidates, we chose to pursue the synthetic lethal *SL39* because it displayed a temperature sensitive defect at 37 °C (Fig. 1B). To clone the gene responsible for the ts phenotype, we transformed the *SL39* strain with a *LEU2*-marked yeast genomic library (Heiter library; Rose & Broach, 1991). A plasmid was recovered from a temperature-resistant transformant, and partial sequencing revealed that it contained a single full-length ORF encoding the splicing factor Prp8. The plasmid (now referred to as p366-*PRP8*) also relieved the requirement of episomal *SKY1* for *SL39* cells to grow on 5-FOA plates (Fig. 1B). These results along with mutation mapping below demonstrate the synthetic lethality between *prp8* and *sky1*Δ. The synthetic lethal mutant was thus renamed *prp8-39*.

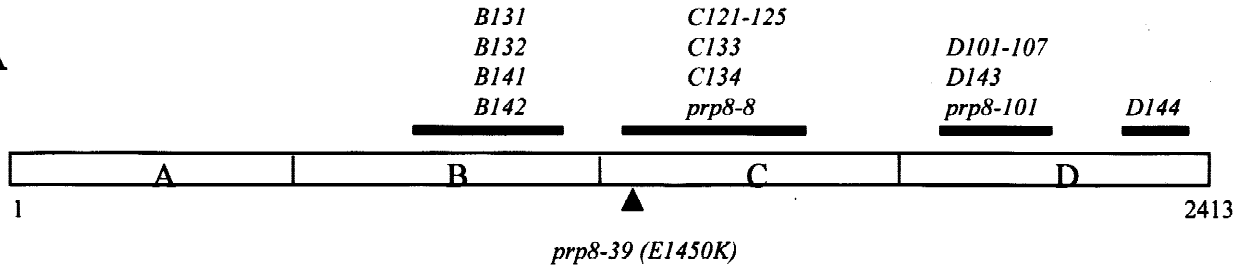
The mutation associated with the ts phenotype in *prp8-39* was mapped by gap repair (see Materials and Methods). Sequencing of the repaired plasmid carrying the ts mutation revealed that *prp8-39* contains a single missense mutation changing glutamic acid (E) to lysine (K) at position 1450. This mutation does not correspond to any previously published mutation in Prp8. To confirm that this E-to-K mutation was responsible for the ts and synthetic lethal phenotypes in the *prp8-39* strain, the point mutation was engineered into a *LEU2*-marked *PRP8* expression plasmid (pSE363-*PRP8*), which was then introduced by plasmid shuffling into a *prp8* null strain covered with an *URA3*-marked wild-type *PRP8* (yJU71) as well as a double mutant strain (yJU71-sky1Δ). Reintroduction of the E-to-K mutation into *PRP8* indeed reestablished the ts phenotype and synthetic lethality with *SKY1* deletion (data not shown). To determine whether the kinase activity of Sky1p is required for the genetic interaction, we transformed the *prp8-39* strain with wild-type *SKY1* or *sky1 (K-M)* bearing a lysine-to-methionine mutation in the ATP binding site (Siebel et al., 1999). Temperature sensitivity and growth on 5-FOA were not rescued when *sky1 (K-M)* was combined with *prp8-39* (Fig. 1B). Therefore, the kinase activity rather than a structural motif in the protein is required for its genetic interaction with *PRP8*. This result provides the first functional link between Sky1p and a splicing factor known to play a crucial role in organizing the catalytic core in the spliceosome.

Allele-specific genetic interactions between *SKY1* and *PRP8*

Prp8 is highly conserved between yeast and mammals and is known to form multiple contacts with conserved splicing signals in both pre-mRNA and snRNAs in the spliceosome (reviewed by Collins & Guthrie, 2000). However, this largest known splicing factor has no recognizable protein motifs. To aid in deciphering its function, the protein has been arbitrarily divided into four domains, A to D, for PCR mutagenesis, and a large number of recessive alleles were mapped into clusters within the B, C, and D domains by the Guthrie and Konarska groups (listed in Fig. 2A; Umen & Guthrie, 1996; Collins & Guthrie, 1999; Siatecka et al., 1999). For example, alleles *D101–107* and *C121–125* were previously characterized as suppressors of mutations in the polypyrimidine tract and the 3′YAG, respectively (Umen & Guthrie, 1995b, 1996). More recently, many *prp8* alleles were found to simultaneously suppress mutations in both the 5′GU and 3′YAG (Collins & Guthrie, 1999; Siatecka et al., 1999). Characterization of these *prp8* alleles has led to functional assignment of Prp8 in spliceosome assembly and splice site recognition (Brown & Beggs, 1992; Collins & Guthrie, 1999; Siatecka et al., 1999). More recently, characterization of a novel *prp8* mutant (known as *prp8-201*, a U4-cs1 suppressor) indicates that Prp8 is also critical for RNA rearrangement (Kuhn et al., 1999; Kuhn & Brow, 2000). We therefore asked whether Sky1p function could be related to a specific pathway involving Prp8 by surveying previously characterized *prp8* alleles with regard to their genetic interaction with the kinase.

A large collection of *prp8* alleles was surveyed for possible genetic interactions with *sky1*Δ (Fig. 2B). Two alleles located in the C domain (C133 and C134) were found to be synthetically lethal with deletion of *SKY1*. Interestingly, the newly identified *prp8-39* mutation was also mapped in the C domain near the mutations in C133 and C134 (Fig. 2A). None of the B and D domain alleles including the recently described *prp8-201* displayed synthetic lethality with *sky1*Δ (Fig. 2B; A. Kuhn, unpubl. observation). Importantly, the analysis also revealed that a ts phenotype is not a prerequisite for the genetic interaction between *PRP8* and *SKY1*: C134 was synthetically lethal with *sky1*Δ, but did not display a ts phenotype. On the other hand, a number of ts alleles in the C and D domains were not synthetically lethal with *sky1*Δ. Together, these results establish an allele-specific genetic interaction between *SKY1* and *PRP8* and suggest that Sky1p may play a role in pre-mRNA splicing in a specific pathway involving a small region in the C domain of Prp8.

A



B

PRP8 allele	Domain (mutations*)	ts	SL with *sky1*Δ
pJU225 (PRP8)	Wild Type	no	-
B131	B (Y923C, E942G)	no	-
B142	B (I857T, S894G, Y923C, E942G, S1018P)	no	-
C122	C (W1575R)	no	-
C133	C (I1444V, T1565A, V1621A)	yes (mild)	+++
C134	C (Q1455P, D1485G, H1592R)	no	++
prp8-39	C (E1450K)	yes	+++
prp8-8	C (G1563R)	yes	-
D103	D (F1834L, V1946A)	no	-
D143	D (K1864E)	no	-
D144	D (F2176S, Q2313R, T2364A)	no	-
prp8-101	D (E1960K)	yes	-
prp8-201	D (T1861P)	no	-

* Mutation information is from Umen and Guthrie, 1996; Collins and Guthrie, 1999.

FIGURE 2. *SKY1* functionally interacts with *PRP8* in an allele-specific manner. **A:** Diagram of the *PRP8* gene. Shown above are *prp8* alleles in B, C, and D domains. Thick lines indicate the regions containing the mutations. The *prp8-39* mutation was mapped in the C domain as shown (arrow). **B:** Summary of the genetic interaction between *SKY1* and the *PRP8* alleles tested. The *prp8* mutations were previously described (Umen & Guthrie, 1996; Collins & Guthrie, 1999). yJU71-sky1Δ was transformed with *prp8* alleles and the transformants were screened for viability on 5-FOA. The degree of synthetic lethal (SL) with *sky1*Δ: −: wild-type growth; ++: appearance of some small colonies after 1 to 2 weeks of culturing; +++: no growth.

SAD1 overexpression suppresses the splicing defect in *prp8-39* but not the synthetic lethality with *sky1*Δ

Prp8 is known to play many roles in pre-mRNA splicing. We reasoned that the synthetic lethal phenotype may reflect a common pathway for both Prp8 and Sky1p-mediated phosphorylation, and thus, characterization of the *prp8-39* mutation may provide clues to the function of the kinase. We therefore tested whether *prp8-39* produced a splicing defect by primer extension after shifting cultures from the permissive (30 °C) to the non-permissive temperature (37 °C; Fig. 3A). As indicated by the accumulation of unspliced U3 pre-snoRNAs, a splicing defect was detectable in *prp8-39* before the temperature shift and the defect was exacerbated after the shift (Fig. 3A, lanes 7–9). These results were comparable to those associated with a known *prp2* splicing mutant (Fig. 3A, lanes 4–6). The splicing defect was completely suppressed by wild-type *PRP8* (Fig. 3A, lanes 10–12), indicating that *prp8-39* has a recessive splicing phenotype.

We next asked whether it was possible to suppress both the ts phenotype of *prp8-39* and synthetic lethality with *sky1*Δ by overexpressing a third gene. For instance, overexpression of a Sky1p substrate may partially compensate for its reduced activity in the absence of the kinase. We therefore transformed the *prp8-39* strain with a high-copy yeast expression library (see Materials and Methods) and isolated genes capable of suppressing the ts phenotype. This approach led to the isolation of *SAD1*, a recently described factor involved in the assembly of newly synthesized U4 into the U4/6 particle (Lygerou et al., 1999). Overexpression of *SAD1* was able to suppress both the ts phenotype (Fig. 3B) and the splicing defect (Fig. 3A, lanes 12–15) in *prp8-39*, although the suppression was less effective than that by wild-type *PRP8* (Fig. 3A, compare lanes 10–12 with lanes 12–15). These results suggest a novel functional link between Sad1p and the U5-associated Prp8,

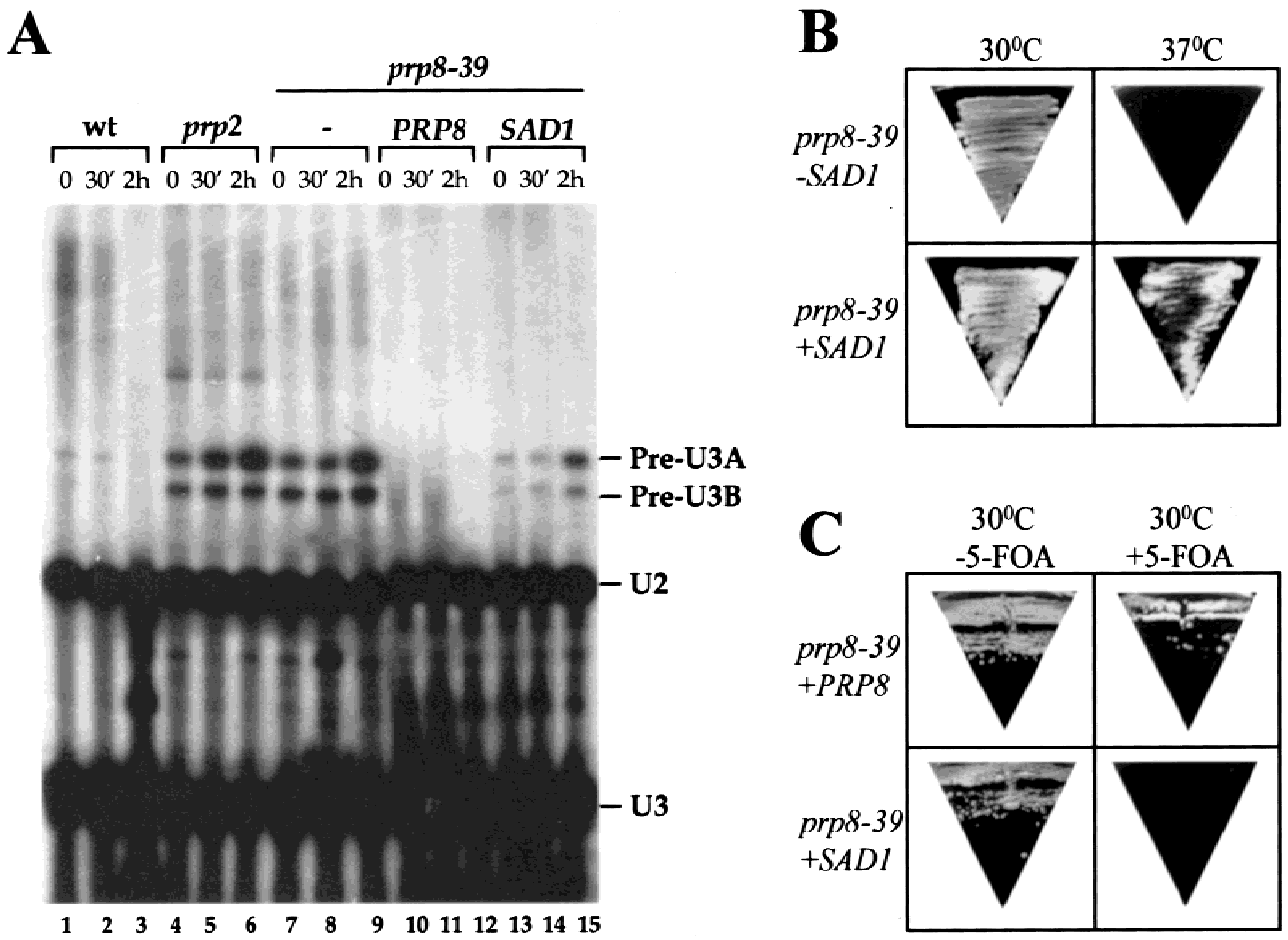


FIGURE 3. High-copy *SAD1* suppresses the splicing defect in *prp8-39*, but not the synthetic lethality with *sky1*Δ. **A:** Characterization of the splicing defect in *prp8-39* cells by primer extension. *SAD1* was isolated by a high copy suppression screen in *prp8-39*. Strains containing wild-type *PRP8* (lanes 1–3), *prp2* (lanes 4–6), *prp8-39* (lanes 7–9), *prp8-39* complemented by wild-type *PRP8* (lanes 10–12), or high-copy *SAD1* (lanes 13–15) were grown at 30 °C to mid-log and then shifted to 37 °C for 0.5 and 2 h. RNAs were extracted and analyzed by primer extension using an oligonucleotide complementary to *SNR17A* (pre-U3A) and *SNR17B* (pre-U3B) genes. U2 snRNA was extended in the same reactions as a control. Unspliced U3 transcripts (pre-U3A and pre-U3B) and spliced U3 are indicated. The splicing defect is indicated by accumulation of pre-U3 transcripts. **B:** Overexpression of *SAD1* suppresses the ts phenotype of *prp8-39*. The *prp8-39* strain (*SL39*) was mock transformed or transformed with the *LEU2*-marked 2μ *SAD1* plasmid followed by incubation at 30 or 37 °C. **C:** Overexpression of *SAD1* does not rescue the synthetic lethality between *prp8-39* and *sky1*Δ. The *prp8-39* strain bearing the *URA3*-marked *SKY1* (pCH1675-*SKY1*) was transformed with either *LEU2*-marked *PRP8* (pJU225, Fig. 2) or *LEU2*-marked 2μ *SAD1* followed by selection on 5-FOA plates at 30 °C. Overexpression of *SAD1* was not sufficient to rescue the synthetic lethality between *prp8-39* and *sky1*Δ.

either in the assembly of functional U4/5/6 tri-snRNP before splicing or during RNA rearrangement within the spliceosome. However, *prp8-39* remained synthetically lethal with *sky1*Δ regardless of *SAD1* overexpression (Fig. 3C). Thus, the ts phenotype associated with *prp8-39* may be distinct from its genetic interaction with *SKY1*. More importantly, this result in conjunction with the lack of genetic interaction between *prp8-201* and *sky1*Δ implies that the synergy between Sky1p-mediated phosphorylation and Prp8 may not occur at the level of snRNP assembly and rearrangement during splicing.

A role of Sky1p-mediated phosphorylation in 3′ AG recognition

To explore the biochemical basis for the genetic interaction between *SKY1* and *PRP8*, we next examined the potential role of Sky1p in splice site recognition, as Prp8 is a well-known component in the process. For example, Prp8 has been shown to play a role in polypyrimidine tract recognition, which can be measured by scoring the utilization of two competing 3′ splice sites, one of which is uridine rich and the other is uridine poor (Umen & Guthrie, 1995b). The normal uridine-rich tract

is preferred in wild-type yeast, but the preference is lost in a class of *prp8* mutants mapped in the D domain (Umen & Guthrie, 1995b). Because *sky1*Δ is not synthetically lethal with D domain mutants, it appears unlikely that Sky1p functions in polypyrimidine tract recognition. This was confirmed by showing that the preference for uridine-rich tract is not impaired in both *prp8-39* and *sky1*Δ cells (data not shown).

Prp8 is also known to play a critical role in splicing fidelity as many recessive *PRP8* alleles were able to suppress mutations in both 5′GT and 3′AG dinucleotides (Collins & Guthrie, 1999; Siatecka et al., 1999). Importantly, many of those mutations were mapped to the Prp8 C region, which we have now linked to the allele-specific genetic interaction with *sky1*Δ (Fig. 2). It was proposed that the suppression phenotype may result from relaxation of a constraint imposed by Prp8 at the catalytic core (reviewed by Collins & Guthrie, 2000). We reasoned that a Sky1p function could also contribute to the normal constraint so that, with a combined effect of *prp8-39* and *sky1*Δ, the loss of this constraint could be lethal. To test this hypothesis, we determined whether *SKY1* contributes to splice site recognition by utilizing an *ACT1-CUP1* splicing reporter system, which allows rapid evaluation of *cis* and *trans* mutations based on cell growth in the presence of copper sulfate (Lesser & Guthrie, 1993).

A panel of *ACT1-CUP1* reporters containing point mutations at the 5′ splice site, the branchpoint, and the 3′ splice site were transformed into both wild-type and *sky1*Δ cells and the transformants were inoculated onto plates containing increasing concentrations of copper sulfate (see Materials and Methods). Indeed, two mutations at the 3′AG were modestly but reproducibly suppressed in *sky1*Δ cells (Fig. 4A). To confirm the suppression phenotype, we analyzed the reporter RNA by primer extension. We detected a higher level of the *ACT1-CUP1* mRNA from reporter G303C in *sky1*Δ than in wild-type cells (Fig. 4B, compare lanes 7 and 8), but the effect of *SKY1* deletion on splicing of reporter A302U was not as obvious (Fig. 4B, compare lanes 4 and 5). The latter is likely due to increased sensitivity of the copper-resistance test compared to primer extension as noted previously (Ben-Yehuda et al., 2000). *prp8-39* displayed a similar suppression phenotype by the copper assay (data not shown) as well as by primer extension, especially at position A302 (Fig. 4B, lanes 6 and 9). In contrast, mutations at the 5′GT or the branchpoint were not suppressed in *sky1*Δ or *prp8-39* (Fig. 4A; data not shown). These results indicate that the recognition of 3′AG may be the converging point for the concerted action of Prp8 and Sky1p.

Functional interaction between *SKY1* and the second-step splicing factor *PRP17/SLU4*

The recognition of 3′AG is a complex process involving not only *PRP8* but also a number of second-step splicing factors, including *PRP16*, *SLU7*, *PRP18*, *PRP17/SLU4*, and *PRP22*. If Sky1p plays a role in 3′AG recognition, we might expect that the kinase function is also synergistic with one or multiple second-step factors. To test this hypothesis, yeast strains harboring ts or deletion mutations in individual second-step genes were transformed with *URA*-marked plasmids bearing prospective wild-type genes (Table 1). *SKY1* was then deleted from the strains followed by selection on 5-FOA plates (Fig. 5A). A synthetic lethal phenotype resulted in *sky1*Δ cells when combined with mutations in *PRP17/SLU4* (Fig. 5B). To determine whether the kinase activity of Sky1p is also required for the genetic interaction, we carried out the plasmid shuffling experiment with *LEU2*-marked *SKY1* or *sky1 (K-M)* in a *prp17*Δ*/sky1*Δ double knockout strain bearing an *URA3*-marked *RPP17/SLU4* plasmid (pSJ3). We found that only *SKY1* was able to support cell growth on 5-FOA (Fig. 5C). Thus, as in the case with *PRP8*, the kinase activity is also essential for the genetic interaction between *SKY1* and *PRP17/SLU4*.

Interestingly, synthetic lethality has been observed between alleles of all second-step genes except between *PRP16* and *PRP18*, implying a physical and functional association of these proteins during the formation of the catalytic core for the second step (Frank et al., 1992; Jones et al., 1995). More recently, extensive genetic interactions between *PRP8* and *PRP17/SLU4* were reported (Ben-Yehuda et al., 2000). In this study, two classes of *prp8* mutants were isolated: one class suppressed the ts phenotype of *prp17*Δ whereas the other class displayed synthetic lethality with *prp17*Δ. Of particular interest is that all mutations were mapped to the C-domain in *PRP8*. Because *prp8-39* is a C-domain mutant and *sky1*Δ is synthetically lethal with both *prp8-39* and *prp17* mutations, we predicted that *prp8-39* would also be synergistic with *prp17* mutations. However, it remained to be determined whether a combination of both mutations would suppress the ts phenotype or generate synthetic lethality. We deleted *PRP17* in the *prp8-39* strain and observed a clear synthetic lethal phenotype with the double *prp17*Δ*/prp8-39* mutant (Fig. 5D). Together, the pairwise synthetic lethality between *prp8-39*, *prp17*Δ, and *sky1*Δ provides strong evidence for their functions in a common pathway in 3′AG recognition during the second step of the splicing reaction.

DISCUSSION

In the current study, we took a genetic approach to address the function of an SR protein-specific kinase (Sky1p) in budding yeast (Siebel et al., 1999). Interestingly, although the kinase is conserved from yeast to humans, the existence of an SR system in budding yeast remains controversial. On the one hand, the yeast genome does not appear to encode proteins with ex-

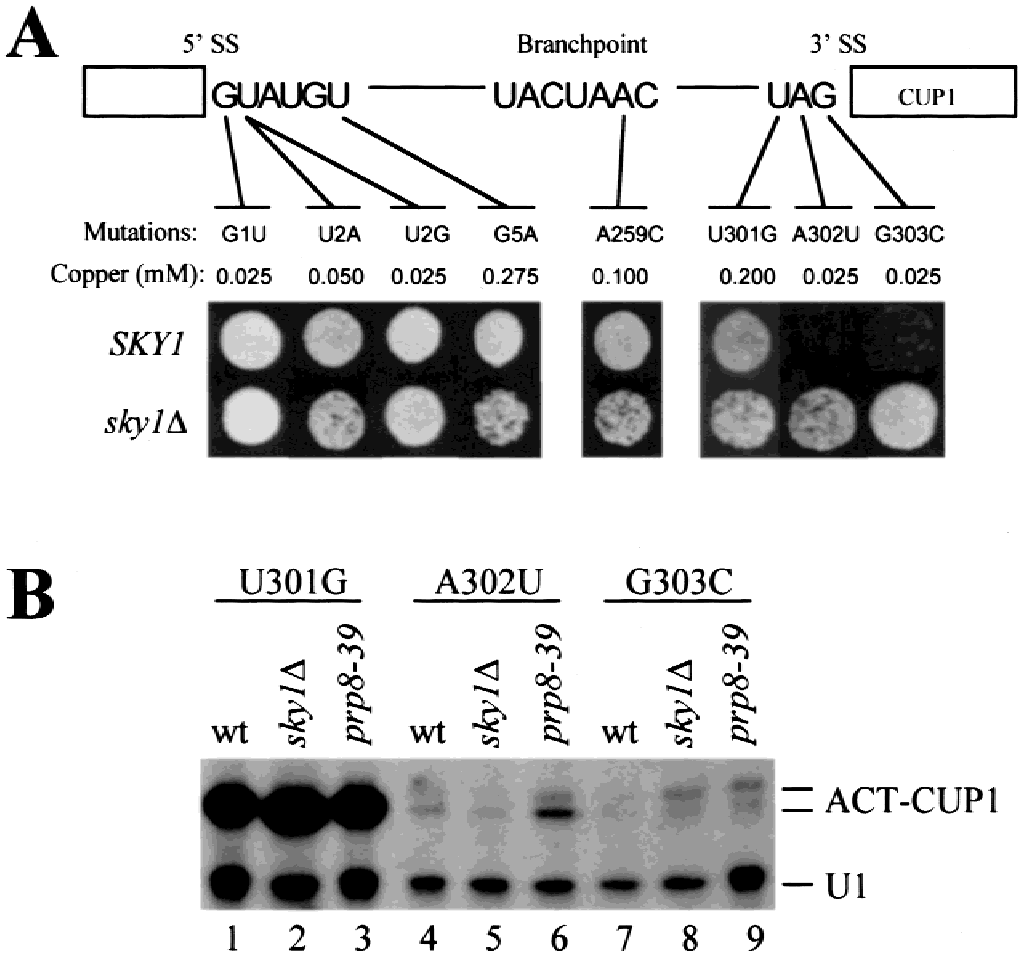


FIGURE 4. Suppression of 3′AG mutations by deletion of *SKY1*. **A:** Splicing suppression analysis using *ACT1-CUP1* splicing reporters. The *ACT1-CUP1* reporters carrying mutations at the 5′ splice site (G1U, U2A, U2G, and G5A), the branchpoint (A259C), and the 3′ splice site (U301G, A302U, and G303C) were transformed into wild-type (yJU75) and *sky1*Δ (yJU75-sky1Δ) strains. The transformants were inoculated onto plates containing increasing concentrations of copper sulfate. Growth at the maximal copper sulfate concentration was shown with each reporter. **B:** Analysis of splicing suppression by primer extension. Three *ACT1-CUP1* reporters carrying mutations (U301G, A302U, and G303C) at the 3′ splice site were transformed into yJU75 (wt *PRP8*), yJU75-sky1Δ (*sky1*Δ), or yJU75-*prp8-39* (*prp8-39*). The transformants were grown to OD_{600} of 2, and RNAs were extracted and analyzed by primer extension. The upper *ACT1-CUP1* band represents splicing to a cryptic 3′ splice site and the lower *ACT1-CUP1* band corresponds to the use of the wild-type 3′ splice site as previously described (Umen & Guthrie, 1996). Intronless U1 was extended as a control.

plicit characteristics of mammalian SR proteins bearing multiple RS or SR repeats. In addition, only a small fraction of yeast genes contain a single intron and the splicing signals are nearly invariant. Thus, although the SR protein system is critical for enhancing the selection of weak splice sites in mammalian cells; such a system might be dispensable in yeast. On the other hand, it is striking that the SRPK family of kinases is conserved during evolution and Sky1p from budding yeast displays the same sequence features, enzymatic activity, and substrate specificity as its mammalian counterparts (Wang et al., 1998b; Siebel et al., 1999; Yeakley et al., 1999; Nolen et al., 2001). Here, we present genetic evidence that Sky1p contributes to 3′AG recognition during the second splicing step and the effect is likely mediated by a kinase substrate(s). This unexpected finding not only suggests a novel phosphorylation control pathway but also echoes recent findings in higher eukaryotic cells that U2AF[35], an RS domain-containing protein, functions in 3′AG recognition (Merendino et al., 1999; Wu et al., 1999; Zorio & Blumenthal, 1999) and that a splicing enhancer presumably functioning through an SR protein(s) is required for the second step of the splicing reaction (Chew et al., 1999). These observations raise the possibility that some features of SR proteins are conserved in yeast at the functional level, but are less apparent at the sequence level.

Our findings presented in this report suggest the model illustrated in Figure 6. The fact that all introns in budding yeast appear to be AG-independent makes it possible to clearly relate experimental evidence to the

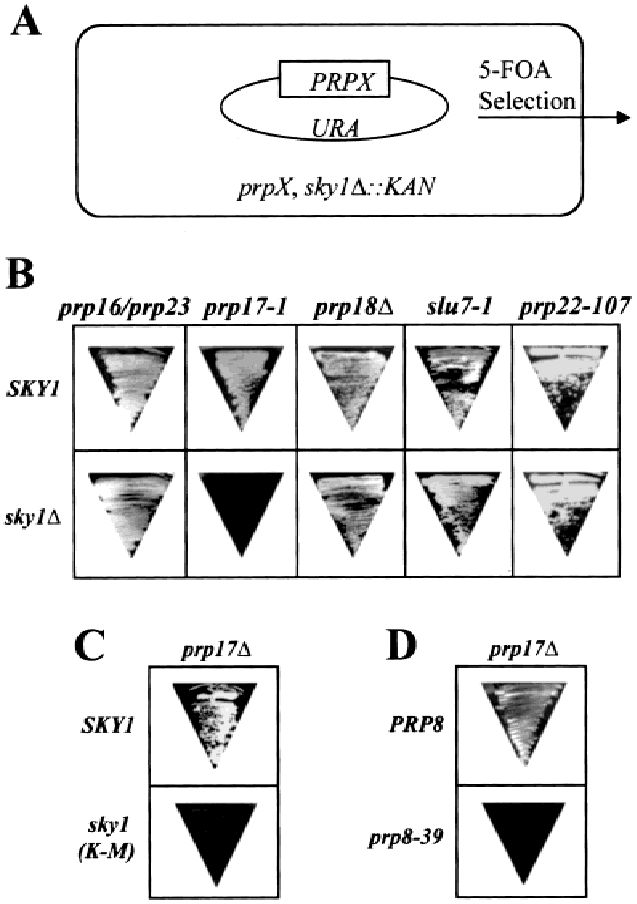


FIGURE 5. Functional interaction between *SKY1* and *PRP17/SLU4*. **A:** The strategy to examine potential genetic interactions between *SKY1* and genes encoding splicing factors required for the second step. Yeast strains carrying mutations of individual second step factor genes were first transformed with *URA3*-marked plasmids carrying corresponding wild-type genes (see Table 1). *SKY1* was deleted from individual yeast followed by 5-FOA selection to determine synthetic lethality when deletion of *SKY1* was combined with mutations in a second-step factor gene. **B:** Specific genetic interaction between *SKY1* and *PRP17/SLU4*. **C:** Requirement of the kinase activity for the genetic interaction between *SKY1* and *PRP17/SLU4*. **D:** Synthetic lethality resulted from combining *prp8-39* with *prp17*Δ.

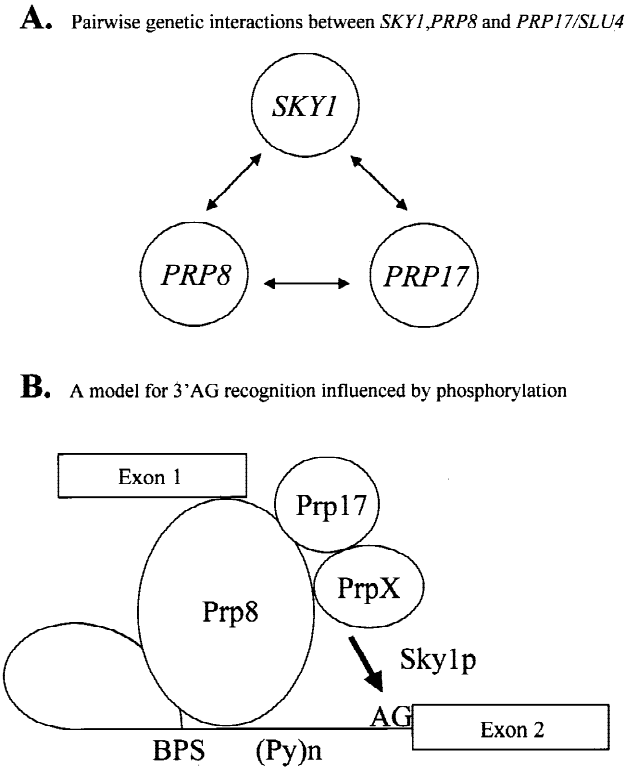


FIGURE 6. A: Pairwise genetic interactions between *SKY1*, *PRP8*, and *PRP17/SLU4* established in the current study. **B:** A model for a role of Sky1p-mediated phosphorylation in 3′AG recognition involving both Prp8 and Prp17/Slu4. Large arrowhead indicates the proposed constrained recognition of the 3′AG by a protein complex including Prp8 and Prp17/Slu4. Sky1p-mediated phosphorylation may either enhance the joining of a presumed PrpX to the 3′AG recognition complex or modulate the configuration of the complex at the 3′ splice site.

first or the second splicing step. In this report, we show that the removal of the constraint imposed by Sky1p-mediated phosphorylation selectively suppresses mutations at the 3′ splice site, suggesting that Sky1p plays a role in the second step. In budding yeast, the conserved 3′AG dinucleotide appears to be recognized multiple times by a number of second-step factors, which may represent an important mechanism for ensuring splicing fidelity (reviewed by Reed, 2000). UV crosslinking experiments indicate that sequential 3′AG recognition takes place at two distinct stages: an ATP-dependent stage involving Prp17/Slu4 and the RNA helicase Prp16 and a subsequent ATP-independent stage involving Slu7, Prp18, and Prp22 (Jones et al., 1995; Umen & Guthrie, 1995a; Schwer & Gross, 1998). We place Sky1p at the ATP-dependent step because *SKY1* genetically interacts with both *PRP8* and *PRP17/SLU4*, but not with *SLU7*, *PRP18*, and *PRP22*. Furthermore, Sky1p-mediated phosphorylation may be synergistic with a function of Prp8 prior to the action of Prp16 because depletion of Prp16 had no effect on Prp8 crosslinking to the 3′AG (McPheeters et al., 2000).

We show that kinase activity is required for *SKY1* to functionally interact with both *PRP8* and *PRP17/SLU4*, suggesting that the constraint on 3′ splice site recognition may be mediated by a Sky1p substrate(s). We tested whether Prp8 and Prp17/Slu4 are themselves substrates for the kinase by in vitro phosphorylation of bacterially expressed proteins using purified Sky1p and found no evidence for such a simple scenario (data not shown). Thus, the observed genetic interactions likely involve a Sky1p substrate(s) yet to be identified. This possibility is consistent with a recent crosslinking experiment showing that the 3′ splice site is contacted in an early ATP-dependent step by additional unidentified RNA-binding proteins, indicating that the recognition of 3′AG may be more complex than previously thought (McPheeters et al., 2000). In fact, despite intensive biochemical and genetic studies, the precise mechanisms

TABLE 1. Strains used for the current study.

Strain	Genotype	Reference
CH1305	*MATa ura3 leu2 his3 ade2 ade3*	Kranz and Holm (1990)
SL-sky1Δ	*MATa ura3 leu2 his3 ade2 ade3 sky1Δ::KAN* pCH1675-*SKY1* (*URA SKY1 CEN ARS ADE3*)	This study
SL39 (prp8-39)	*MATa prp8-39 ura3 leu2 his3 ade2 ade3 sky1Δ::KAN* pCH1675-*SKY1* (*URA SKY1 CEN ARS ADE3*)	This study
yJU71	*MATα prp8Δ:LEU leu2 tyr1 his3 ura3 ade2 trp1* pY8000 (*URA PRP8 CEN ARS*)	Brown and Beggs (1992)
yJU71-sky1Δ	*MATα prp8Δ:LEU leu2 tyr1 his3Δ ura3 ade2 trp1 sky1Δ::KAN* pY8000 (*URA PRP8 CEN ARS*)	This study
yJU75	*MATa ade2 his3 leu2 lys2 cup1::ura3 prp8Δ::LYS2* pJU169 (*URA PRP8 CEN ARS*)	Umen and Guthrie (1996)
yJU75-sky1Δ	*MATa ade2 his3 leu2 lys2 cup1::ura3 prp8Δ::LYS2 sky1Δ::KAN* pJU169 (*URA PRP8 CEN ARS*)	This study
yDFA7C	*MATa slu7-1 ura3 trp1 leu2 his3 lys2 ade2* pDF64 (*URA SLU7 CEN ARS*)	Frank and Guthrie (1992)
yDFA7C-sky1Δ	*MATa slu7-1 ura3 trp1 leu2 his3 lys2 ade2 sky1Δ::KAN* pDF64 (*URA SLU7 CEN ARS*)	This study
ySJ134	*MATa prp17-1 ura3 trp1 his3*	Gift from Guthrie lab UCSF
ySJ134-pSJ3	*MATa prp17-1 ura3 trp1 his3* pSJ3 (*URA SLU4 CEN ARS*)	Gift from Guthrie lab UCSF
ySJ134-sky1Δ	*MATa prp17-1 ura3 trp1 his3 sky1Δ::KAN* pSJ3 (*URA SLU4 CEN ARS*)	This study
ySJ136	*MATa prp17Δ::LEU ura3 trp1 his3*	Gift from Guthrie lab UCSF
ySJ136-pSJ3	*MATa prp17Δ::LEU ura3 trp1 his3* pSJ3 (*URA SLU4 CEN ARS*)	Gift from Guthrie lab UCSF
ySJ136-sky1Δ	*MATa prp17Δ::LEU ura3 trp1 his3 sky1Δ::KAN* pSJ3 (*URA SLU4 CEN ARS*)	This study
DH120R	*MATa prp18Δ::HIS3 ade2 ade3 his3 ura3*	Horowitz and Abelson (1993)
DH120R-pDH101	*MATa prp18Δ::HIS3 ade2 ade3 his3 ura3* pDH101 (*URA3 PRP18 CEN ARS*)	Horowitz and Abelson (1993)
DH120R-sky1Δ	*MATa prp18Δ::HIS3 ade2 ade3 his3 ura3 sky1Δ::KAN* pDH101 (*URA3 PRP18 CEN ARS*)	This study
prp23-ts514	*MATα prp23-ts514 ade2 his3 ura3 tyr1*	Vijayraghavan et al. (1989)
prp16-pS32	*MATα prp23-ts514 ade2 his3 ura3 tyr1* pS32 (*URA PRP16 CEN ARS*)	Vijayraghavan et al. (1989)
prp16-sky1Δ	*MATα prp23-ts514 ade2 his3 ura3 tyr1 sky1Δ::KAN* pS32 (*URA PRP16 CEN ARS*)	This study
prp22-ts107	*MATα prp22-ts107 ade2 his3 ura3 lys2*	Vijayraghavan et al. (1989)
prp22-p360	*MATα prp22-ts107 ade2 his3 ura3 lys2* p360 (*URA PRP22 CEN ARS*)	Vijayraghavan et al. (1989)
prp22-sky1Δ	*MATα prp22-ts107 ade2 his3 ura3 lys2 sky1Δ::KAN* p360 (*URA PRP22 CEN ARS*)	This study

for the function of Prp8 and Prp17/Slu4 in 3′AG recognition remain obscure. Prp8 can be crosslinked to both splice sites and the polypyrimidine tract, indicating that this largest spliceosome component plays a critical role in organizing the catalytic center for splicing (reviewed by Collins & Guthrie, 2000). Although deletion of *PRP17/SLU4* generates a ts phenotype and impairs the second step of the splicing reaction (Ben-Yehuda et al., 1998; Vijayraghavan et al., 1989), the gene is not essential for growth and there is no evidence that it directly contacts the 3′ splice site. However, given its genetic interactions with U2, U5, and all second-step genes (Frank et al., 1992; Xu et al., 1998; Ben-Yehuda et al., 2000), Prp17/Slu4 must play a crucial role in the definition of the 3′ splice site, and the data presented in this report indicate that the process may also be subject to phosphorylation control by Sky1p.

As illustrated in Figure 6, pairwise genetic interactions between *PRP8*, *PRP17/SLU4*, and *SKY1* indicate that these genes may function in the same pathway. We propose that an unidentified Sky1p substrate (*PrpX*) interacts with Prp8 and/or Prp17/Slu4 in a phosphorylation-dependent manner, thereby contributing to the faithful recognition of the 3′AG. This presumed factor may be important for the efficacy in 3′AG recognition as well as for the establishment of a tight constraint (large arrowhead in Fig. 6B) at the 3′ splice site in wild-type yeast. Given the observed suppression phenotype, this constraint may be relaxed to some extent by conformational changes of a hypothetical 3′AG recognition complex, which can be triggered either by mutations in Prp8 or by removing Sky1p-mediated phosphorylation. Support to this theory awaits future experiments to identify the presumed factor, map the Sky1p phosphorylation site(s), and determine the impact of mutations at the phosphorylation site(s) on 3′AG recognition as well as potential synergy with specific mutations in *PRP8* and *PRP17/SLU4*. Finally, it is unclear whether a potential phosphatase is also involved in the transition from 3′AG recognition to the second-step catalytic reaction. This question may be addressed in the future by determining whether the second step of the splicing reaction requires dephosphorylation of a SRPK substrate(s).

A kinase action at the 3′ splice site may also take place in mammalian systems. As mentioned in the Introduction, U2AF^{35} can directly contact the 3′AG when complexed with the polypyrimidine tract binding protein

U2AF^{65} (Merendino et al., 1999; Wu et al., 1999; Zorio & Blumenthal, 1999). In addition, Krainer and colleagues provide evidence that an exonic enhancer is important for the second splicing step (Chew et al., 1999). Because all SR proteins and both subunits of U2AF can be phosphorylated by SRPKs (Colwill et al., 1996; Wang et al., 1998b), it is possible that these RS domain-mediated interactions can be influenced by SRPK-mediated phosphorylation in mammalian cells. It is also interesting to note a similarity in 3′AG recognition between yeast and mammalian systems. In yeast, Prp18 and Slu7 are dispensable for splicing when the distance between the branchpoint and the 3′AG is less than 12 nt (Brys & Schwer, 1996; Zhang & Schwer, 1997), whereas Prp22 becomes nonessential when the branchpoint-to-3′AG distance is less than 21 nt (Schwer & Gross, 1998). Although it is unclear whether these rules apply to all introns in yeast, these observations strongly suggest that 3′AG recognition may involve distinct complexes for introns with different configurations. In this case, it is interesting that the recognition of the 3′AG by U2AF^{35} in higher eukaryotic cells is also dependent on the distance between the branchpoint and the 3′AG (Merendino et al., 1999). Because it is unclear whether budding yeast express a functional homolog of U2AF^{35}, it remains a mystery how many other factors could also influence 3′AG selection in an intron-configuration-dependent manner in both yeast and mammalian cells. With more variations in intron configurations and more factors involved in splice site selection processes, it is conceivable that many different strategies may be used for 3′AG recognition in higher eukaryotic cells.

Our results provide genetic evidence that the SRPK family of kinases may play an important part in modulating 3′AG recognition. Furthermore, other kinases including the Clk/Sty family members and the Prp4 kinase described in fission yeast are also documented to play roles in constitutive and regulated splicing (Prasad et al., 1999; Schwelnus et al., 2001). Our current study illustrates the power of yeast genetics in elucidating fundamental functions without prior assumptions for the action of Sky1p in splicing or at a specific step in the splicing reaction. The genetic evidence now sets the stage for future investigation of Sky1p-mediated phosphorylation in 3′AG recognition using biochemical approaches.

MATERIALS AND METHODS

Yeast strains and plasmids

Yeast strains used in the current study are listed in Table 1. The strain used for the synthetic lethal screen (CH1305) was a gift from the Holm laboratory (Kranz & Holm, 1990). *SKY1* was deleted from all strains by using a kanamycin-resistance expression unit flanked by *SKY1* genomic sequence as previously described (Yeakley et al., 1999). The plasmid pCH1675-*SKY1* was constructed by inserting the *Bam*HI-*Sal*I fragment containing *SKY1* from pRS316-*SKY1* (Siebel et al., 1999) into the *Bam*HI-*Sma*I sites of pCH1675 (Kranz & Holm, 1990). Wild-type *SKY1* was similarly subcloned into the *LEU2*-marked plasmid YCpLac111. The mutant YCpLac111-*sky1 K-M* was constructed by exchanging the *Psh*AI-*Pfl*MI fragment from YCpLac111-*SKY1* with the corresponding fragment from pYES2-*sky1 K-M* (Siebel et al., 1999). The *prp8* null strains yJU71 and yJU75 used for allele specificity and splice site suppression analyses were gifts from the C. Guthrie laboratory. The *slu4* [ySJ134 (*prp17-1*), ySJ136 (*prp17*Δ)] and *slu7* [yDFA7C (*slu7-1*)] mutant strains, and plasmids pSJ3 (wild-type *SLU4* in YCp50) and pDF64 (wild-type *SLU7* in YCp50) were also gifts from the C. Guthrie laboratory. The *prp18* null strain DH120R and the plasmid pDH101 bearing wild-type *PRP18* were gifts from the D. Horowitz laboratory. The *prp16* (prp23-ts514) and *prp22* (prp22-ts107) mutant strains were gifts from the J. Abelson laboratory (Vijayraghavan et al., 1989). The *URA/CEN* plasmids carrying *PRP16* (pS32) and *PRP22* (p360) were gifts from the B. Schwer laboratory. All wild-type and mutant *PRP8* alleles listed in Figure 2 and all *ACT-CUP1* reporters used in Figure 4 were kindly provided by the C. Guthrie laboratory (Lesser & Guthrie, 1993; Umen & Guthrie, 1996; Collins & Guthrie, 1999).

Synthetic lethal screen

SKY1 was first disrupted in CH1305 and the resulting SL-sky1Δ strain was covered with pCH1675-*SKY1* (*CEN, ARS, URA3, ADE3*). UV mutagenesis conditions were chosen to produce 25–30% viability on YP galactose plates. Nonsectoring cells were isolated from approximately 100,000 surviving colonies and then subjected to 5-FOA selection. Three synthetic lethals (*SL34*, *SL39*, and *SL44*) were obtained and all showed ts growth at 37 °C. *SL34* and *SL44* had high reversion rates on 5-FOA plates following long incubation. In contrast, *SL39* was stable and therefore chosen for further functional studies. The *SL39* gene was cloned by complementing the ts phenotype with the Heiter yeast genomic DNA library, and identified as *PRP8* by partial sequencing. The *prp8-39* mutation was determined by gap repair (Guthrie & Fink, 1991). Briefly, a series of restriction fragments was prepared from pSE363-*PRP8* (*HIS3, CEN, ARS*) (a gift from the C. Guthrie laboratory) and transformed into the *SL39* strain. All but the *Psh*AI-*Spe*I fragment were able to correct the ts phenotype, indicating the *SL39* mutation(s) resides in this region. The *Psh*AI-*Spe*I gapped pSE363-*PRP8* was then transformed into the *SL39* strain and the repaired plasmid was isolated. Sequencing of the *Psh*AI-*Spe*I region in the plasmid revealed a missense glutamic acid (E)-to-lysine (K) mutation at position 1450.

High copy suppressor screen

A high copy CV13 library (Rose & Broach, 1991) was transformed into the *prp8-39* strain. One temperature resistant clone was isolated. Sequencing of the recovered plasmid showed that it encodes a number of genes, including the

newly described *SAD1* (Lygerou et al., 1999). Deletion analysis revealed the *SAD1* gene was responsible for the observed ts suppression.

Splicing suppression assay using *ACT-CUP1* reporters

Growth assays in the presence of copper sulfate were as previously described (Lesser & Guthrie, 1993). To determine the impact of Sky1p on splice site selection, *SKY1* was deleted from the parental yJU75 strain to generate the yJU75-sky1Δ strain for subsequent copper assays. To determine the effect of *prp8-39* on splice site recognition, we shuffled wild-type *PRP8* in the *URA3* plasmid (pY8000) with a *HIS3*-marked plasmid (pSE362, Elledge & Davis, 1988) bearing either wild-type or mutant *PRP8* (pSE362-*PRP8* and pSE362-*prp8-39*).

Primer extension

Cells were grown at 30 °C or 37 °C in YP galactose media to OD_{600} of 2.0–3.0. RNA was extracted from harvested yeast as previously described (Guthrie & Fink, 1991) except the guanidinium buffer was replaced by 50 mM Tris-pH 7.4, 100 mM NaCl, 10 mM EDTA, and 1% SDS. The following primers were used: U3: 5′-CCAAGTTGGATTCAGTGGCTC-3′; U2-23T: 5′-GTCTCTTCCCGTCCATTTTATTA-3′; U1: 5′-CAATGACTTCAATGAACAATTAT-3′; Cup/exon 2: 5′-CTTCATTTTGGAAGTTAATTAATT-3′. Primer extensions were performed as previously described (Frank & Guthrie, 1992). Briefly, 13 μg RNA was mixed with 1 ng ^{32}P-labeled oligos (primers) in 6.4 μL and added to 1.6 μL annealing buffer (250 mM Tris-pH 8.3, 300 mM NaCl, 50 mM DTT). The mixture was heated for 3 min at 68 °C, frozen in dry ice/ethanol, and then allowed to thaw on ice. To this mixture was added 13 μL of the premixed RT solution (20 mM Tris-pH 8.3, 21 mM NaCl, 3.6 mM DTT, 11 mM $Mg(OAc)_2$, 0.72 mM each dNTP, and 14 U of AMV reverse transcriptase). The reaction mixtures were incubated at 37 °C for 5 min and then at 42 °C for 20 min. The reactions were stopped by adding 20 μL sequencing stop buffer, heated at 92 °C for 3 min, and analyzed by electrophoresis in 6% polyacrylamide/7 M urea gels followed by autoradiography.

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