Molecular Cell, Vol. 6, 329–338, August, 2000, Copyright ©2000 by Cell Press

A Yeast Intronic Splicing Enhancer and Nam8p Are Required for Mer1p-Activated Splicing

Marc Spingola and Manuel Ares, Jr.*
Center for the Molecular Biology of RNA
Sinsheimer Laboratories
University of California, Santa Cruz
Santa Cruz, California 95064

Summary

Three introns whose splicing is activated during meiosis in *S. cerevisiae* contain a Mer1p-dependent splicing enhancer. The enhancer can impose Mer1p-activated splicing upon the constitutively spliced actin intron provided the basal splicing efficiency of actin is first reduced. Of several nonessential splicing factors tested, only the U1 snRNP protein Nam8p is indispensable for Mer1p-activated splicing. We show that Mer1p associates with the U1 snRNP even in the absence of Nam8p or pre-mRNA. This work defines a yeast splicing enhancer and shows that constitutively expressed and cell type-specific factors combine to regulate splicing of a specific subset of pre-mRNAs including *SPO70*, *MER2*, and *MER3*.

Introduction

Eukaryotic pre-mRNAs contain many introns that are removed in the cell nucleus by a dynamic protein-RNA complex called the spliceosome (reviewed in Staley and Guthrie, 1998). Some metazoan pre-mRNAs can be alternatively spliced to produce mature mRNAs with different combinations of exons to encode distinct proteins (Smith et al., 1989; Lopez, 1998). In metazoa and in yeast, splicing can be switched between production of a functional and a nonfunctional mRNA, in effect turning genes on or off at a posttranscriptional level (Smith et al., 1989). Thus, alternative splicing provides a genetic mechanism to increase both the protein coding capacity and the regulatory flexibility of the eukaryotic genome.

In metazoan cells, a number of different *cis*-acting sequence elements and *trans*-acting factors regulate alternative splicing. One class of *trans*-acting factors is produced only in specific tissues or at specific stages of development. This class includes the well-characterized *Drosophila* proteins Tra, Sxl (produced in female cells) (reviewed in Burtis and Baker, 1989; Chabot, 1996; Hodgkin, 1989; Valcarcel et al., 1993), and PSI (produced in somatic cells) (Siebel et al., 1992, 1994, 1995). These cell type-specific proteins bind to pre-mRNAs at discrete sites and activate (Tra) or repress (PSI and Sxl) splice sites by either recruiting splicing factors to the proper splice site (Lynch and Maniatis, 1996) or blocking basal splicing factors from selecting the default splice site (Siebel et al., 1992; Valcarcel et al., 1993). Another class of *trans*-acting factors is more widely expressed and in some cases is considered part of the basal splicing machinery. This class includes the SR proteins, which bind pre-mRNA and activate nearby splice sites (Valcarcel and Green, 1996; Tacke and Manley, 1999). Similarly, the hnRNPs A and B appear to bind pre-mRNA and repress nearby splice sites (Mayeda and Krainer, 1992; Mayeda et al., 1993), while others like hnRNP H and F might activate (or derepress) nearby splice sites (Min et al., 1995; Chou et al., 1999). SR proteins and the hnRNP A/B family have been hypothesized to act antagonistically to each other because changes in the relative amount or activity of these two classes of factors influence splice site selection in vivo and in vitro (Mayeda and Krainer, 1992; Mayeda et al., 1993; Caceres et al., 1994; Hanamura et al., 1998). Some proteins of this class, like polypyrimidine tract binding protein, have been shown to repress splicing in one system (Ashiya and Grabowski, 1997; Chan and Black, 1997) and activate in another (Lou et al., 1999). The *cis*-acting sequences that bind the cell type-specific or general splicing factors can be found in exons or in introns and have been called splicing enhancers or silencers. The precise mechanisms by which these proteins influence splice site usage remain a topic of intensive investigation.

In budding yeast, few examples of regulated splicing or alternative splicing have been observed (Engebrecht et al., 1991; Vilardell and Warner, 1994; Barta and Iggo, 1995; Nakagawa and Ogawa, 1999; Davis et al., 2000). Positive regulation of splicing occurs during meiosis. The *MER1* gene is expressed only during meiosis, and Mer1p is necessary to activate splicing of *MER2* (Engebrecht et al., 1991), *MER3* (Nakagawa and Ogawa, 1999), and *SPO70* (Davis et al., 2000) pre-mRNAs. Mer1p contains a KH domain RNA binding motif (Siomi et al., 1993) and has been shown to bind *MER2* pre-mRNA and intron in vitro (Nandabalan and Roeder, 1995). Despite these examples, the features of *MER2*, *MER3,* and *SPO70* pre-mRNAs that make them responsive to Mer1p have not been determined. Both the *MER2* and *MER3* introns have 5′ splice sites that match the consensus poorly and may be inefficiently recognized (Nandabalan et al., 1993; Nandabalan and Roeder, 1995; Nakagawa and Ogawa, 1999). Consistent with this, altering the *MER2* 5′ splice site to a consensus 5′ splice site or expressing mutant U1 snRNA with improved base pairing to the *MER2* 5′ splice site bypasses the need for Mer1p (Nandabalan et al., 1993; Nandabalan and Roeder, 1995). Weak 5′ splice sites cannot be the sole determinant of Mer1p target specificity however, since other introns have weak 5′ splice sites, such as *HOP2* (Leu and Roeder, 1999), but do not require Mer1p for efficient splicing. In addition, *SPO70* has a canonical 5′ splice site. If the large 5′ exon of *MER2* is shortened, Mer1p is no longer needed for efficient splicing (Nandabalan and Roeder, 1995). However, other genes with large 5′ exons, like *REC114* (Malone et al., 1997), are insensitive to Mer1p. Thus, no pre-mRNA feature has been identified that specifically mediates splicing activation by Mer1p. While it has been suggested that Mer1p activates splicing through the U1 snRNP (Nandabalan et al., 1993), an interaction between the two has not yet been demonstrated.

In this work, we compare the three Mer1p-responsive pre-mRNAs and identify a short, shared sequence element that acts as a Mer1p-dependent intronic splicing

*To whom correspondence should be addressed (e-mail: ares@darwin.ucsc.edu).

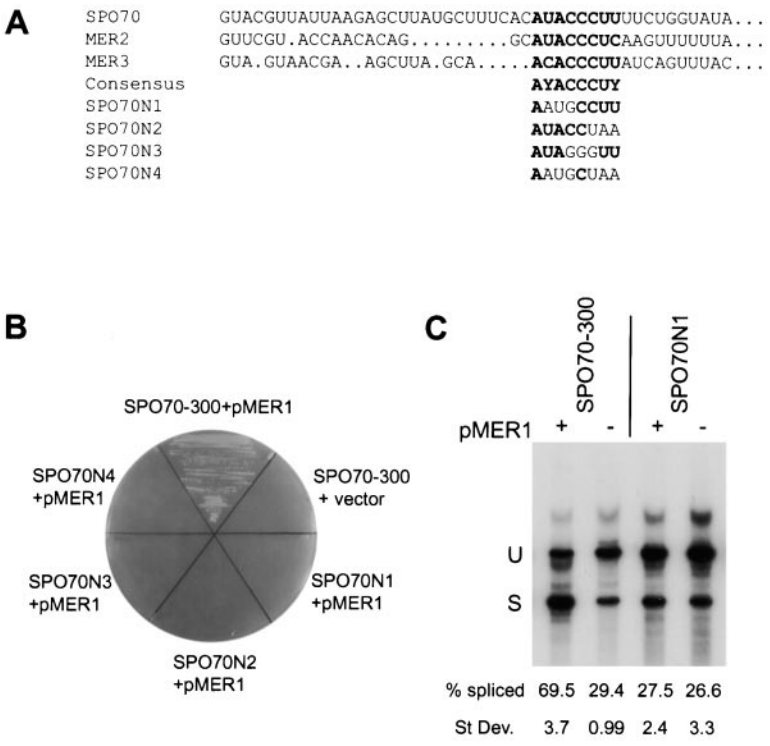

Figure 1. *SPO70* Contains a *MER1*-Dependent Splicing Enhancer

(A) The sequences of three Mer1p-activated introns starting with the 5′ splice site and ending after the enhancer sequence (boldfaced). The SPO70N1 through N4 mutations are listed below.

(B) Growth of *cup1*Δ yeast on plates containing 0.25 mM copper. Yeast contain either a *MER1* constitutive expression plasmid (pMER1) or the parental vector lacking the *MER1* open reading frame (vector) and one of five *SPO70-CUP1* fusion plasmids described in (A). The -300 suffix refers to the size of the 5′ exon in nt.

(C) Primer extension analysis of SPO70-300 and N1 splicing with pMER1 (+) or with control vector (-). Phosphorimager quantitation of the average splicing efficiencies (percent spliced, using the formula S/(S+U)*100) from at least two independent experiments are reported at the bottom of each lane with standard deviations. Bands corresponding to spliced (S) and unspliced RNA (U) are indicated in the left margin.

enhancer. We find that splicing of *SPO70* pre-mRNA is inefficient even though it contains standard splice sites and branchpoint sequences. This inefficiency is due primarily to a nonconserved inhibitory element adjacent to the 5′ splice site and splicing enhancer. We have used our findings to build Mer1p-activated splicing into the actin intron. Despite the presence of a standard 5′ splice site in *SPO70*, a nonessential U1 snRNP-associated protein involved in selecting weak 5′ splice sites, Nam8p (Puig et al., 1999), increases the basal level of *SPO70* splicing and is required for splicing activation by Mer1p. An additional test of nonessential splicing factors reveals that thus far only Nam8p is specifically required for Mer1p-activated splicing. Recombinant Mer1p binds more tightly to pre-mRNA containing the enhancer sequence than to pre-mRNA containing a mutant enhancer. Tagged Mer1p produced in yeast specifically coimmunoprecipitates U1 snRNA, demonstrating an association between Mer1p and the U1 snRNP. This interaction is independent of Nam8p or base pairing between U1 snRNA and the 5′ splice site. These results explain the specificity of Mer1p-activated splicing, link Mer1p action to U1 snRNP function, and provide a foundation for understanding how general splicing factors and cell type-specific splicing factors can combine to produce positive regulation of splicing.

Results

The *SPO70*, *MER2*, and *MER3* Introns Contain a Splicing Enhancer Necessary for Mer1p-Activated Splicing

Inspection of the three Mer1p-activated introns reveals a common sequence between the 5′ splice sites and branchpoints with the consensus AYACCCUY (Figure 1A). In each intron, this element is found within 25 nt of the 5′ splice site. We have developed a system for monitoring Mer1p-activated splicing in vegetative cells using a constitutively expressed *MER1* gene on a 2 μ plasmid (pMER1) (Engebrecht et al., 1991) and a constitutively expressed *SPO70-CUP1* fusion splicing reporter plasmid that allows for splicing-dependent growth of yeast on media containing copper (Lesser and Guthrie, 1993). Yeast containing the *SPO70-CUP1* fusion plasmid grow on plates containing copper only if they also contain the *MER1* expression plasmid (Figure 1B). Primer extension analysis of *SPO70* RNA from yeast containing the *CUP1* fusion plasmid and either pMER1 or a control vector lacking the *MER1* open reading frame indicates that *MER1* expression activates splicing of *SPO70* (Figure 1C; Davis et al., 2000). To test if the 8 nt conserved intronic sequence is important for Mer1p-activated splicing, we made mutations to it in the *SPO70-CUP1* fusion plasmid (Figure 1A). In contrast to the wild-type *SPO70* fusion plasmid, none of the mutant plasmids support growth on plates containing copper even when the yeast contain pMER1 (Figure 1B). RNA analyzed by primer extension shows that the copper-sensitive growth phenotypes reflect the absence of activation of splicing by Mer1p (Figure 1C; Table 1). The enhancer mutations affect only activated splicing since their splicing efficiencies are similar to the wild-type intron in the absence of pMER1 (Figure 1C; Table 1). To test if this sequence functions as an enhancer in the other two Mer1p-activated introns, the A*YAC*CCUY to A*AUG*C CUY mutation was created in *MER2*- and *MER3*-containing *CUP1* fusion plasmids. Primer extension analysis shows that Mer1p-activated splicing does not occur when the conserved element is altered but that the basal level of splicing is unaffected (Table 1). We were concerned that the low level of splicing of *SPO70* pre-mRNA observed in the absence of pMER1 (Figure 1C) might

Table 1. Splicing Efficiencies for Various Constructs in Cells Containing Mer1p or Lacking Mer1p

RNA	Percent Spliced +pMER1	Percent Spliced + Control
SPO70-300	69.5 ± 3.7	29.4 ± 0.98
SPO70-N2	27.5 ± 1.3	26.6 ± 1.7
SPO70-N3	26.8 ± 3.0	27.2 ± 0.78
SPO70-N4	26.0 ± 0.71	26.3 ± 2.1
MER2	45.1 ± 1.6	17.8 ± 1.1
MER2-N1	16.2 ± 0.63	18.0 ± 0.28
MER3	31.5 ± 1.5	9.5 ± 1.3
MER3-N1	8.2 ± 2.9	9.4 ± 0.63
SPO70-150	69.1 ± 1.9	58.9 ± 0.22
SPO70-50	78.4 ± 0.78	79.4 ± 1.2
SPO70-300 nam8Δ	11.8 ± 1.9	11.2 ± 1.9
SPO70-150 nam8Δ	32.0 ± 1.7	33.5 ± 0.92
SPO70-50 nam8Δ	59.8 ± 0.57	59.9 ± 0.99
SPO70-N1 nam8Δ	12.8 ± 1.2	11.5 ± 1.3
MXACT2	56.6 ± 1.4	55.0 ± 1.7
MXACT3	86.2 ± 2.4	60.2 ± 3.4

The -300, -150, -150 suffixes represent the size of the *SPO70* 5′ exon in nt. RNA isolated from *nam8*Δ cells is indicated. MXACT2 is the actin-CUP1 plasmid with the 8 nt enhancer insertion (+9 nt flanking sequences from *SPO70*) at 50 nt from the mutant 5′ splice site described in the text. MXACT3 contains the 8 nt enhancer (only 8 nt) inserted 18 nt from the mutated 5′ splice site. All other RNAs are described in the text.

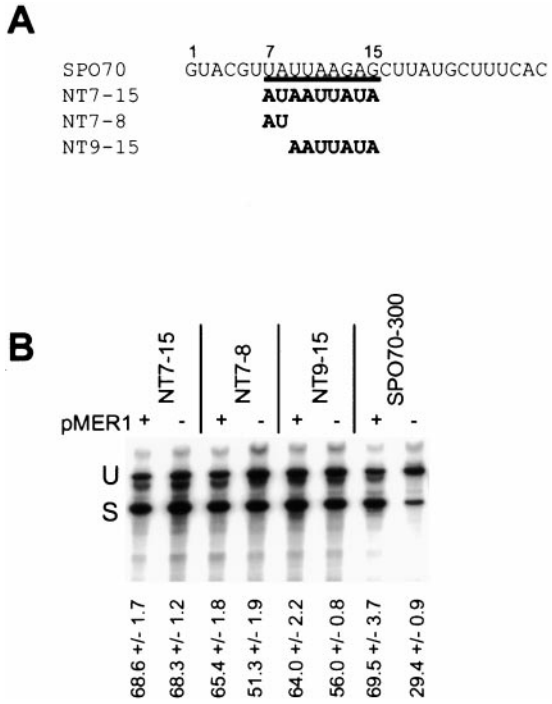

Figure 2. The *SPO70* Intron Contains a Splicing Silencer

(A) The sequence of the *SPO70* intron starting with the first nucleotide of the intron. The silencer region is underlined and the splicing activation mutations are boldfaced. The intron sequence stops at the nucleotide upstream of the splicing enhancer.

(B) Primer extension analysis of *SPO70-CUP1* fusion RNA isolated from yeast containing pMER1 (+) or control plasmid (-). Wild-type *SPO70* intron (SPO70-300) is compared to introns with mutations at intron nucleotides 7–15 (NT7–15), 9–15 (NT9–15), and 7–8 (NT7–8). Bands representing spliced and unspliced RNA are indicated on the left margin. Splicing efficiency for each sample is indicated at the bottom of each lane.

be due to leaky *MER1* expression in vegetative cells. However, we observe the same basal level of splicing in a *MER1* deletion strain (data not shown). These results indicate that the conserved intronic element of all three introns acts as a splicing enhancer that is necessary for Mer1p-activated splicing but not for basal splicing.

The *SPO70* Intron Contains a Silencer Sequence between the 5′ Splice Site and Enhancer

For reasons that are not obvious, *SPO70* splicing is inefficient without Mer1p. The basis of this may provide insight into the mechanism of Mer1p action. Unlike *MER2* and *MER3*, the *SPO70* 5′ splice site, GUACGU, is the same as that found in efficiently spliced introns but is not the most common 5′ splice found in yeast introns, GUAUGU (Spingola et al., 1999). To test if this slight difference contributes to poor splicing efficiency of *SPO70*, we altered the 5′ splice site to GUAUGU. This mutation did not increase basal splicing efficiency nor did it affect Mer1p-activated splicing (data not shown). Like *MER2*, *SPO70* has a large 5′ exon. Serial truncations of the *SPO70* 5′ exon improve splicing efficiency in the absence of Mer1p, but even a short (50 nt) 5′ exon construct is spliced only to 80% efficiency (Table 1). Mer1p-activated splicing is also affected by the *SPO70* 5′ exon size (Table 1). As the 5′ exon is truncated and basal splicing efficiency increases, Mer1p-activated splicing becomes less apparent. These studies indicate that (1) the *SPO70* 5′ splice site sequence does not contribute to its inefficient splicing, (2) the large 5′ exon contributes to but is not the sole cause of inefficient splicing of *SPO70* pre-mRNA, and (3) Mer1p-activated splicing is not apparent for introns that are efficiently spliced.

Since neither the 5′ splice site nor 5′ exon size can account for all of the inhibition of *SPO70* splicing, the possibility that some other feature of the *SPO70* intron contributes to its poor basal splicing was addressed. 8–10 nt blocks of nonconserved sequences of the intron were mutated, and yeast containing these mutant plasmids were screened for efficient splicing by copper-resistant growth in the absence of pMER1. A mutation that alters the nucleotides immediately downstream of the 5′ splice site, starting at nt 7 of the intron (nt 7–15, Figure 2A), allows growth on plates containing copper in the absence of pMER1. The first 2 nt of this substitution are AU and could extend base pairing of the 5′ splice site region to both U1 and U6 snRNAs. Increased base pairing of the U1 snRNA to the *MER2* 5′ splice site activates its splicing without Mer1p (Nandabalan et al., 1993). To determine if the nt 7–15 mutation activates splicing by extending base pairing of the *SPO70* 5′ splice site region to snRNAs or if it activates splicing by some other means, we constructed two additional mutants with substitutions in nt 9–15 of the intron and the dinucleotide substitution at intron positions 7–8 in the *SPO70* intron (Figure 2A). The nt 9–15 mutant, which does not include the first 2 nt that extend base-pairing potential to snRNAs (Figure 2A), grows as well as the original mutant on copper, suggesting that splicing is not activated by an increase in base pairing to snRNAs. The nt 7–8 mutant does not grow quite as well as the nt 7–15 or 9–15 mutants on plates containing copper but grows better than wild-type, suggesting that nucleotides 7–8

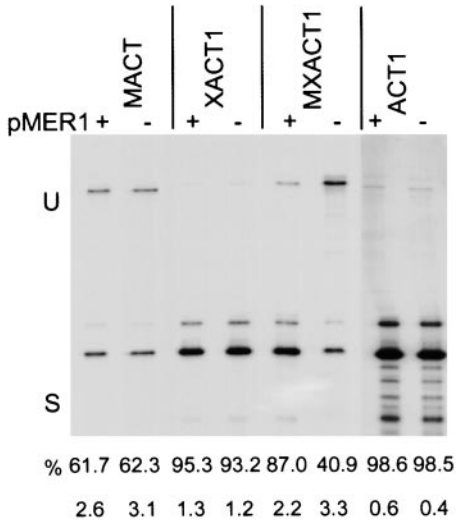

Figure 3. Mer1p-Activated Splicing of Modified Actin Intron Pre-mRNAs

MACT is the actin-*CUP1* fusion plasmid with the mutant 5′ splice site described in the text; XACT1 is the actin intron with *SPO70* enhancer sequence inserted 18 nt from the 5′ splice site; MXACT contains both the mutant 5′ splice site and the enhancer sequence at nt 18; ACT is the wild-type actin-*CUP1* fusion. The wild-type actin panel has been exposed longer to observe unspliced bands. Bands corresponding to spliced (S) and unspliced RNA (U) are indicated in the left margin. Splicing efficiency and standard deviations are reported for each RNA below each lane. Strains containing pMER1 or control vector are indicated by (+) or (-), respectively, above each lane.

also contribute to silencer function. Primer extension analysis indicates that basal splicing efficiency is increased for each mutant compared to wild-type in the absence of pMER1 (Figure 2B). Expression of mutant U1 snRNAs (Staley and Guthrie, 1999) capable of forming 1–2 additional base pairs to nt 7 and/or 9 of the *SPO70* intron does not improve its splicing efficiency (data not shown). These results suggest that weak base pairing to U1 or U6 snRNAs may only slightly contribute to inefficient basal splicing of *SPO70* pre-mRNA and that intron sequences between the 5′ splice site and enhancer act as a splicing silencer to inhibit the splicing of *SPO70* pre-mRNA.

The Splicing Enhancer Is Sufficient to Confer Mer1p-Activated Splicing to a Heterologous Intron

The above results predict that Mer1p-activated introns must contain the enhancer sequence in combination with other features that reduce its basal splicing, such as large 5′ exons, weak 5′ splice sites, or splicing silencers. If the enhancer is the only *cis*-acting element needed for Mer1p-activated splicing, it should be sufficient for Mer1p-activated splicing. To test this, we attempted to confer Mer1p-activated splicing to the constitutively spliced actin intron fused to *CUP1* (Lesser and Guthrie, 1993). In order to measure any activation of splicing, the splicing efficiency of actin had to be reduced. We changed the 5′ splice site from GU*AU*GU to GU*UC*GU, the weak but functional 5′ splice site found in the *MER2* intron (Engebrecht et al., 1991). This mutation lowers the in vivo splicing efficiency of actin from 98% to 60% but does not allow splicing activation by Mer1p (Figure 3), reinforcing the conclusion that the splice site sequence is not sufficient for Mer1p-activated splicing. The conserved element from the *SPO70* intron (the 8 conserved nt plus 9 nt flanking each end) was inserted either 18 or 50 nt from the 5′ splice site of both the wild-type and 5′ splice site mutant actin intron-*CUP1* fusion genes. The plasmids were then introduced into yeast, and *MER1*-dependent growth on copper was evident only for the construct with the enhancer inserted 18 nt from the altered 5′ splice site (plates not shown). Primer extension verifies that splicing is activated by Mer1p only when the intron includes the weak 5′ splice site and the enhancer is located closer to the 5′ splice site (Figure 3; Table 1). Splicing of an additional actin construct containing the mutant 5′ splice site and only the 8 nt consensus enhancer (no flanking sequences from *SPO70*) inserted 18 nt from the 5′ splice site is also activated by Mer1p (MXACT3, Table 1). We conclude that the 8 nt conserved element is a Mer1p-dependent splicing enhancer that is both necessary and sufficient for Mer1p-activated splicing of inefficiently spliced introns. The enhancer function is also position dependent because splicing is not activated when the enhancer is located farther from the 5′ splice site.

Nam8p Is Required for Mer1p-Activated Splicing of *SPO70* Pre-mRNA

Sporulation and splicing of *MER2* and *MER3* pre-mRNAs are impaired in yeast with null alleles of the *MRE2/NAM8* gene (Nakagawa and Ogawa, 1997; Nakagawa and Ogawa, 1999), whereas constitutively spliced introns are not affected by loss of Nam8p unless the 5′ splice site is severely weakened (Puig et al., 1999). These observations raise the question of whether Nam8p is required for splicing of *MER2* and *MER3* because their 5′ splice sites are poor matches to the consensus (Engebrecht et al., 1991; Nakagawa and Ogawa, 1999) or whether Nam8p is specifically required for Mer1p-activated splicing. Since *SPO70* has a consensus 5′ splice site (Figure 1A), it presents an opportunity to dissect the role of Nam8p in Mer1p-activated splicing from its role in activating weak 5′ splice sites. We constructed a *NAM8* deletion strain (*nam8*Δ) and measured splicing efficiency for *SPO70* and actin pre-mRNAs from yeast with or without pMER1. Splicing efficiency of *SPO70* pre-mRNA is significantly decreased in *nam8*Δ cells (Figure 4; Table 1) whereas splicing of actin pre-mRNA is not affected (discussed below). The reduction of *SPO70* basal splicing in *nam8*Δ cells is more apparent with constructs containing larger 5′ exons (Table 1), suggesting that Nam8p is important for splicing pre-mRNAs with large 5′ exons in addition to activating weak 5′ splice sites. Regardless of the basal level of splicing, Mer1p does not activate the splicing of *SPO70* without Nam8p (Figure 4A; Table 1). We conclude that Nam8p plays a direct role in Mer1p-activated splicing.

Since Nam8p has been cross-linked to the nonconserved region of introns downstream of the 5′ splice site near the location of the *SPO70* silencer (Puig et al., 1999; Zhang and Rosbash, 1999), we tested whether the activation observed for the *SPO70* efficient splicing mutant (nt 7–15) requires Nam8p. Speculating that the splicing silencer of wild-type *SPO70* reduces splicing because it cannot stably interact with Nam8p, the activating mutation might fortuitously provide a better binding site for Nam8p. This hypothesis is excluded because

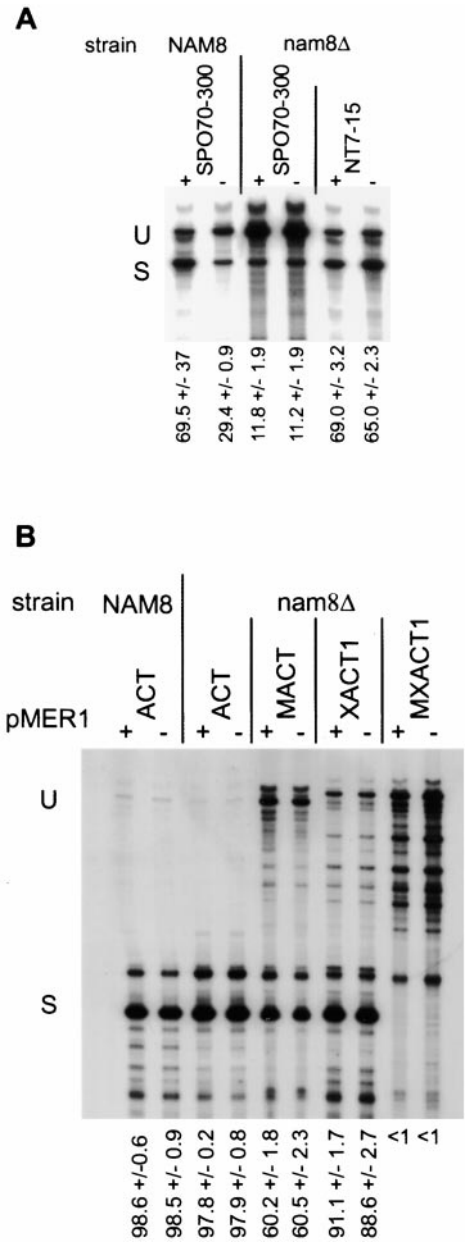

Figure 4. Nam8p Is Required for Mer1p-Activated Splicing

(A) Primer extension analysis of splicing for SPO70-300 and the silencer disruption mutant in *nam8*Δ cells.

(B) Primer extension analysis of actin and modified actin constructs in *nam8*Δ cells. Strains containing pMER1 or control vector are indicated by (+) or (-), respectively, above each lane. Splicing efficiency is reported below each lane.

efficient splicing of the nt 7–15 mutant is independent of Nam8p (Figure 4A). The mechanism by which this region of the wild-type *SPO70* intron represses splicing remains unknown; however, Mer1p and Nam8p combine to overcome its inhibitory effects.

Like the three natural Mer1p-activated introns, Mer1p-activated splicing of the modified actin intron also requires Nam8p. In *nam8*Δ cells, splicing efficiencies for actin, the actin 5′ splice site mutant, and actin with the enhancer insertion are virtually unchanged relative to the *NAM8* strain. However, no spliced product is detected for the actin intron containing the enhancer sequence and the mutant 5′ splice site in *nam8*Δ cells (Figure 4B), regardless of Mer1p. Loss of Nam8p results in many primer extension stops for actin pre-mRNAs that are not spliced efficiently. This may reflect an increased susceptibility to degradation of unspliced pre-mRNA compared to those that are efficiently spliced (Culberston, 1999). Nonetheless, even in the context of a heterologous intron, Mer1p-activated splicing is dependent on Nam8p. This observation provides further support for the conclusion that Nam8p is directly required for Mer1p-activated splicing.

A Test of Nonessential Genes Involved in Splicing Shows Only Nam8p to Be Required for Mer1p-Activated Splicing of *SPO70* Pre-mRNA

We questioned whether the requirement for Nam8p for Mer1p-activated splicing is specific or a general reflection of an inefficient splicing apparatus. To test this, we measured Mer1p-activated splicing of *SPO70* in a set of strains each lacking a single nonessential splicing factor gene. Eight deletion strains lacking a gene coding for the following proteins were tested: the U1 snRNP-associated protein Nam8p, the commitment complex protein Mud2p, the nuclear cap binding protein Cbp20p/Mud13p, the U2 snRNP-associated proteins Cus2p and Lea1p, and three proteins associated with the U4/5/6 tri-snRNP, Cdc40p, Ntc20p, and Snu66p. Primer extension analysis indicates that each strain activates splicing when Mer1p is present except the one lacking Nam8p (Figure 5). This implicates Nam8p in the specific mechanism of Mer1p-activated splicing and also indicates that the splicing factors specified by the remaining deletion strains are not likely to be directly involved in the mechanism of Mer1p activation. However, the strains with the largest splicing defects show a much greater fold activation of splicing by Mer1p than wild-type (mud2Δ, lea1Δ, snu66Δ). We do not believe this is relevant to the function of Mer1p, because clearly splicing efficiency is better when both Mer1p and the nonessential splicing factor are present. Earlier experiments indicate that Mer1p-activated splicing is more apparent for inefficiently spliced introns (Figure 3; Table 1). Of interest, two other commitment complex proteins, Cbp20p/Mud13p and Mud2p, are not required for Mer1p-activated splicing of *SPO70*, suggesting that Nam8p is specifically required for Mer1p-activated splicing and that some perturbations of the commitment complex do not lead to loss of Mer1p-activated splicing.

Recombinant Mer1p Recognizes the Enhancer In Vitro

Based on our findings that the enhancer is necessary and sufficient for Mer1p-activated splicing and based on the observation that recombinant Mer1p specifically binds *MER2* intron in vitro (Nandabalan and Roeder, 1995), we propose that the enhancer is the RNA target for Mer1p. This hypothesis was tested by gel shift analysis of actin pre-mRNAs containing either the wild-type 8 nt enhancer or the N1 variant enhancer (Figure 1) by recombinant GST-Mer1p (Figure 6). RNA containing the N1 mutant enhancer binds ~10-fold less tightly than RNA containing the wild-type enhancer. These results suggest that the enhancer is the RNA target for Mer1p and that mutation of the enhancer leads to weaker binding of Mer1p and loss of splicing activation in vivo.

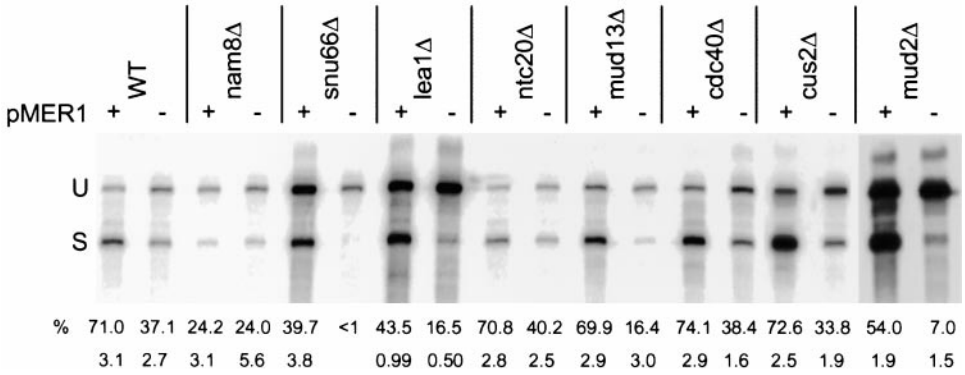

Figure 5. Mer1p-Activated Splicing in Yeast Strains Deleted of Nonessential Splicing Factor Genes

Strains are described in the text. Strains containing pMER1 or control vector are indicated by (+) or (-), respectively, above each lane. Splicing efficiency of SPO70-300 and standard deviations are reported below each lane. Deletion of *NAM8* in this strain background does not reduce splicing efficiency as much as in the KH46 background (Figure 4A).

Mer1p Is Associated with the U1 snRNP

Two observations link Mer1p function to the U1 snRNP. First, efficient splicing of *MER2* can be achieved without Mer1p by expressing mutant U1 snRNAs with extended complementarity to the 5′ splice site region (Nandabalan et al., 1993). Second, Mer1p-activated splicing requires the U1 snRNP protein Nam8p. However, an interaction between the U1 snRNP and Mer1p has not been demonstrated. To test if this interaction occurs, HA-tagged Mer1p was expressed in yeast, and splicing extracts from this strain were used for immunoprecipitation. The HA-tagged Mer1p activates splicing in vivo as well as untagged Mer1p does and can be quantitatively precipitated from extracts using protein A sepharose beads loaded with HA-specific monoclonal antibody 12CA5 (data not shown). Primer extension analysis of the coimmunoprecipitate eluted from the beads by addition of di-HA peptide indicates that Mer1p specifically precipitates a fraction of the total U1 snRNA but not U2 or U6 snRNAs (Figure 7, lane 1). U1 snRNA lacks the 8 nt consensus enhancer sequence, and its coimmunoprecipitation by Mer1p is not due to the spurious presence of an enhancer sequence within U1. Furthermore, upon dividing Mer1p into two fragments containing either the KH domain (C-terminal) or the remaining N-terminal domain, only the N-terminal domain can coimmunoprecipitate U1 snRNA (lanes 2 and 3). This supports the conclusion that the KH domain RNA binding motif does not interact with U1 snRNA. Interestingly, coimmunoprecipitation of U1 snRNA by Mer1p does not rely on Nam8p, as the same amount of U1 snRNA is precipitated from a nam8Δ strain as from the wild-type strain (lane 4). Coimmunoprecipitation of U1 snRNA by Mer1p in this assay does not require base pairing between the 5′ end of U1 snRNA and the 5′ splice site. Splicing extracts treated with a 15-mer oligonucleotide complementary to the 5′ end of U1 snRNA allow for the RNase H-mediated digestion of the 5′ end of U1 snRNA, and this shortened U1 snRNA is still immunoprecipitated by Mer1p (lane 5). Thus, we conclude that Mer1p associates with a fraction of U1 snRNP in a manner independent of Nam8p or the 5′ end of U1 snRNA.

Discussion

Our results show that a conserved intronic element found in *SPO70*, *MER2*, and *MER3* is a position-dependent splicing enhancer necessary for Mer1p-activated splicing (Figure 1; Table 1). The enhancer can impose Mer1p-regulated splicing on a heterologous intron when inserted near the 5′ splice site in combination with alterations of the intron that reduce its splicing efficiency (Figure 3; Table 1). In the absence of Mer1p, the large 5′ exon and an intronic splicing silencer sequence found adjacent to the 5′ splice site of *SPO70* are the primary causes of its poor splicing efficiency (Figure 2; Table 1). Disruption of the silencer or reduction of the 5′ exon size increases splicing efficiency of *SPO70* independently of the Mer1p or Nam8p functions required for splicing (Figures 2 and 4; Table 1). We find no situation in which Mer1p-activated splicing occurs in the absence of Nam8p (Figures 4 and 5; Table 1), unlike other nonessential splicing factors we tested (Figure 5). The enhancer sequence mediates Mer1p binding in the absence of other factors including Nam8p (Figure 6). Mer1p is associated with the U1 snRNP, and this association does not rely on Nam8p, the KH domain of Mer1p, or base pairing between the 5′ splice site and the 5′ end of U1 snRNA (Figure 7).

Cis-Acting Determinants for Mer1p-Activated Splicing

Two *cis*-acting pre-mRNA features are important for Mer1p-activated splicing. The first is the intronic splicing enhancer (Figure 1; Table 1) that is recognized by Mer1p (Figure 6). The enhancer functions when positioned near the 5′ splice site. Although the enhancer did not work at 50 nt downstream of the 5′ splice site (Table 1), we have not fully explored its distance constraints or its effect when placed within the exon or near other intron signals. A second set of features necessary for Mer1p-activated splicing confers reduced basal splicing efficiency. The means by which basal splicing efficiency is lowered can vary: large 5′ exons (Table 1; Nandabalan and Roeder, 1995), weak 5′ splice sites (Nandabalan et al., 1993; Nandabalan and Roeder, 1995), splicing silencers (Figure 2), or weak branchpoint sequences (M. S. and M. A., unpublished data). We applied these principles by successfully transporting Mer1p-activated splicing to an impaired actin intron (Figure 3).

Two models for enhancer function are consistent with data on Mer1p-activated splicing. Mer1p might bind directly to the enhancer sequence of pre-mRNA and recruit basal splicing factors to the 5′ splice site region of the intron for efficient splicing. Recently, a crystal

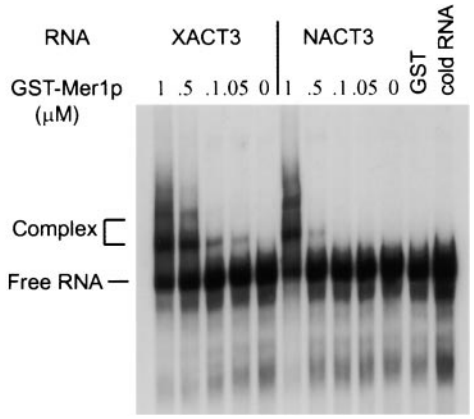

Figure 6. Mer1p Binding to Wild-Type Enhancer– or Mutant Enhancer–Containing Actin RNAs

Binding was analyzed by gel shifts. In the lane labeled GST, recombinant GST is used as a control to indicate that GST does not bind RNA. In the last lane, 1000-fold excess cold XACT3 RNA has been added to preequilibrated XACT3 RNA-GST-Mer1p (0.1 μM protein) to indicate that binding is competitive. Multiple shifted bands may be due to dimerization of the GST moieties of the fusion protein.

structure of the Nova protein KH domain bound to target RNA shows that a single KH domain interacts with as few as 4–5 nucleotides of single stranded RNA (Lewis et al., 2000), consistent with the small size of the enhancer (Figure 1A). Alternatively, Mer1p may be incorporated into the assembling spliceosome by association with a component of the basal splicing machinery, and a subsequent interaction between Mer1p and the enhancer in the pre-mRNA could activate splicing by stabilizing a labile complex or accelerating a rate-limiting step in splicing. The coimmunoprecipitation results support the latter model because Mer1p associates with the U1 snRNP in the absence of added pre-mRNA, in the absence of the KH domain of Mer1p, or after the 5′ end of U1 snRNA has been ablated. It is also possible that the Mer1p-U1 snRNP interaction is formed by other means and persists after U1 snRNP dissociates from splicing complexes.

How Do Mer1p and Nam8p Function Together to Activate Splicing?

Nam8p is an RNA binding protein that has been cross-linked to the nonconserved region of introns just downstream of the 5′ splice site (Puig et al., 1999; Zhang and Rosbash, 1999), near the position where the enhancer is found on Mer1p-activated introns. Since Mer1p efficiently binds the wild-type enhancer but not a mutated enhancer in vitro (Figure 6), it seems likely that the enhancer mutations affect Mer1p binding rather than Nam8p binding in vivo. Mutations to the enhancer are just as sensitive to *NAM8* deletion as is the wild-type intron (Table 1), supporting the interpretation that enhancer mutations do not specifically disrupt Nam8p function (in which case enhancer mutations would be insensitive to *NAM8* deletion). However, it is possible that Nam8p binds nearby the enhancer simultaneously with or after Mer1p.

A simple model for Nam8p-dependent Mer1p-activated splicing is that enhancer-bound Mer1p stimulates the Nam8p functions of the U1 snRNP and thereby accelerates a rate-limiting step or stabilizes a labile interaction. Nam8p is not likely to stabilize the interaction

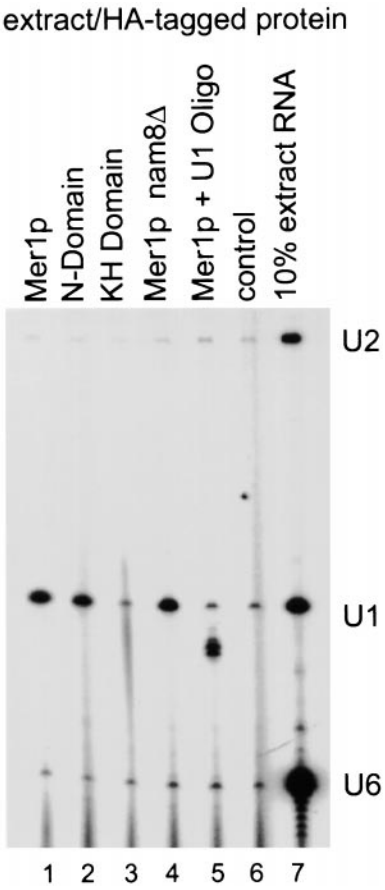

Figure 7. Mer1p Is Associated with U1 snRNA

Using anti-HA antibody (12CA5) and the indicated extracts, immunoprecipitates were analyzed by primer extension for U1, U2, and U6 snRNAs. The control sample is extract that does not contain Mer1p.

between Mer1p and the U1 snRNP because Mer1p associates with the U1 snRNP in the absence of Nam8p (Figure 7). Nam8p and Mer1p might work together to select enhancer-proximal 5′ splice sites. Although deletion of *NAM8* is synthetic lethal with cap binding complex (CBC) gene deletions (Fortes et al., 1999), Nam8p stabilizes commitment complexes formed on pre-mRNAs lacking a cap (Puig et al., 1999). Although the CBC is important for commitment complex formation (Lewis et al., 1996a, 1996b), cap-independent formation of commitment complexes and the observation that the CBC is not needed for Mer1p-activated splicing (Figure 5) suggest that Nam8p and Mer1p can activate splicing independently of CBC function in splicing. Nam8p, in concert with Mer1p, might stabilize commitment complexes formed on pre-mRNAs with inefficiently recognized 5′ splice sites and lead to increased use of the weak 5′ splice site. Currently it is not understood how Nam8p generally aids in 5′ splice site selection, but it is also possible that its specific function in Mer1p-activated splicing is only peripherally related to its role in general splicing.

Although Nam8p is not essential for splicing, its absence destabilizes the overall structure of the U1 snRNP (Gottschalk et al., 1998). Two proteins, Snu71p and Snu56p, are lost from the purified U1 snRNP in the absence of Nam8p, while the interaction of Snu65p with

U1 snRNP is stabilized (Gottschalk et al., 1998). All three of these U1 snRNP proteins are essential, and it may be difficult to test their roles in Mer1p-activated splicing directly. It is possible that the loss of Mer1p activation in the *nam8*Δ strains may be due to the destabilization of these proteins from the U1 snRNP in the absence of Nam8p. In this view, the apparent role of Nam8p in Mer1p-activated splicing would be indirect and result from alteration of the U1 snRNP. The association of Mer1p with the *nam8*Δ U1 snRNP (Figure 7) indicates that disruption of the U1 snRNP caused by the loss of Nam8p is insufficient to result in the loss of Mer1p from the U1 snRNP, despite the fact that activated splicing is blocked (Figure 4). This suggests that splicing activation by Mer1p may require stable association of Snu71p and Snu56p with the U1 snRNP. Whether splicing activation by Mer1p is critically dependent on any essential splicing factor remains to be seen. However, the role of Nam8p shows how a general accessory factor combines with a cell type-specific factor to regulate splicing during the developmental process of meiosis in yeast.

Experimental Procedures

Strains and Plasmids

Strains KH46 and KH52 were used for isolating RNA and testing splicing constructs for copper sensitivity and have the genotypes *cup1*::*ura3–52*, *trp1*, *leu2–3,112*, *his3–1*, *lys2*, *ade2–101*, *GAL*$^+$, *MAT*α. KH52 is identical to KH46 except that it is *LYS2*, *MATa*. The *mer1*Δ and *nam8*Δ strains were constructed (Rothstein, 1991; Longtine et al., 1998) in strain KH46, and the entire respective ORFs have been replaced with the *HIS3* marker. Gene disruptions were verified by Southern analysis (data not shown). The U1 deletion strain GLS008 and U1 snRNA plasmids pCG100-103 were gifts from Jonathan Staley and have been described (Staley and Guthrie, 1999). All other nonessential splicing gene deletion strains, including another *nam8*Δ strain, were purchased from Research Genetics, Inc. All *CUP1* fusion plasmids were constructed in pGAC14 (Lesser and Guthrie, 1993). Inserts were produced by PCR amplification of the gene using Vent DNA polymerase. The fragments were ligated into pGAC14 and contain the entire 5′ exon, or partial 5′ exon (Table 1), and only 30–50 bp of the 3′ exon. EcoRI-SalI fragments containing the glyceraldehyde 3-phosphate dehydrogenase promoter, gene fragment with intron, and *CUP1* gene were also subcloned from the pGAC plasmids into the same sites of the low copy pRS316 vectors for mutagenesis or attenuating copper resistance and are called 316CUP. While copper resistance is attenuated for the low copy plasmids relative to the high copy plasmids, the splicing efficiencies do not change (data not shown). The plasmids 316CUPMER2 and 316CUPMER3 contain their entire 5′ exons. Plasmid R1070 (pMER1, the *MER1* constitutive expression plasmid) and its parental vector R1130 were gifts from S. Roeder and are described by Engebrecht et al. (1991). The chimeric enhancer-actin introns were produced in the 316/CUPACT plasmid (EcoRI-SalI fragment of pGAC14 subcloned into pRS316) by ligating a synthetic oligonucleotide duplex containing the 8 nt of the *SPO70* intronic enhancer and 9 nt on either side of the enhancer into the actin intron XhoI site or Eco47 III site (XACT1 or XACT 2) or by in vitro mutagenesis to insert only the 8 nt enhancer sequence 18 nt from the actin 5′ splice site (XACT3). In vitro mutagenesis was performed as described by Kunkel et al. (1991).

RNA and Splicing Assays

RNA was isolated from yeast containing plasmids by a hot phenol method (Zavanelli and Ares, 1991) from 5 ml cultures of synthetic complete dextrose (SCD) media lacking the appropriate amino acids and/or nucleotide. Reverse transcription was performed as in Spingola et al. (1999) with a radiolableled *CUP1* primer. Splicing efficiency was quantitated with a Molecular Dynamics phosphorimager. Splicing was also assessed by growth of yeast containing *CUP1* fusion plasmids and pMER1 or its control vector by streaking transformants on SCD plates containing various amounts of cupric sulfate (from 0.1 to 0.5 mM) and incubated at 30°C for 3–5 days. Yeast transformation was performed by the LiOAc method (Hill et al., 1991).

Gel Shift Analysis

The *MER1* ORF was cloned into pGEX3x (Amersham Pharmacia Biotech) by PCR amplification of the ORF with primers containing the appropriate restriction sites for production of an in-frame fusion of GST to the N terminus of *MER1*. Recombinant GST-Mer1p was produced in *E. coli* strain XL1Blue and purified by the method of Smith and Johnson (1988). Fusion protein was diluted into 2× binding buffer (1× = 3% PEG 8000, 50 mM tris 8.0, 100 mM NaCl, 10 mM DTT, and 8 mM $MgCl_2$). Pre-mRNAs were diluted in water to a concentration of 1 nM and melted for 5 min at 95°C and snap cooled on ice. Heparin (1.25 μg/μl final) and total yeast RNA (2.5 ng/μl final) were added to the RNA. Equal volumes of 1 nM labeled RNA and protein dilutions (10 μl of each) were mixed and incubated on ice for 15 min. Five microliters of 30% glycerol and 20 mM tris 8.0 was added just prior to electrophoresis. Gels were 4% polyacrylamide (60:1 acrylamide:bis) and 0.5× TBE. Fifteen microliters of binding reaction was loaded onto the gel and electrophoresed with 0.5× TBE running buffer at 250V and ~25 mA for 3–4 hr at 4°C. Gels were dried and autoradiographed.

Coimmunoprecipitation

An N-terminal HA-tagged *MER1* expression plasmid was constructed in 2 μ plasmid and uses the glyceraldehyde-3-phosphate dehydrogenase promoter to drive transcription. The KH domain or N-terminal domain Mer1p constructs separate Mer1p at proline 181, and both protein fragments are expressed and stable in yeast extracts (Westerns not shown). Splicing extracts were produced in strains HI227 (*MATa, leu2–3,112, ura3–52, trp1, his3*δ*, lys2*δ*, pep4–3, prb1, prc1*) or *nam8*Δ by the method of Lin et al. (1985). For coimmunoprecipitation, 50 μl splicing reactions were performed as in Lin et al. (1985), except pre-mRNA was not added and the reactions were incubated for 20 min at 25°C without the addition of stop buffer. To ablate the 5′ end of U1 snRNA, extract was preincubated with 100 ng of 15-mer oligonucleotide complementary to the 5′ end of U1 snRNA for 10 min at 25°C. Splicing reactions were added to 400 μl protein A sepahrose preloaded with mAb 12CA5. First, 2 mg (dry weight) protein A sepharose was swollen in NET (50 mM tris 7.5, 0.05% NP40 and 150 mM NaCl), washed, and resuspended in 400 μl NET. Ten microliters of mAB was added (~10 μg) and bound for a minimum of 2 hr at 4°C with rotation. Beads were then washed three times with NET, resuspended with 400 μl TK150 (50 mM tris 8.0 and 150 mM KCl), and the splicing reaction was added and bound for 2 hr at 4°C with rotation. Beads were washed four times with 400 μl cold TK250, and the coimmunoprecipitate was eluted by addition of 100 μl TK250 and 0.33 mg/ml di-HA peptide (YPYDVP DYAGYPYDVPDYAG) at room temperature with occasional mixing for 15 min. This step was repeated, the eluates combined, phenol/$CHCl_3$ extracted, ethanol precipitated, and reverse transcribed as above with radiolabeled primers for U1, U2, U6 snRNAs.

Acknowledgments

We thank Shirleen Roeder, Jon Staley, and Christine Guthrie for plasmids, Doug Kellogg for HA peptide, and Takuro Nakagawa and Hideyuki Ogawa for communicating their results prior to publication. We also thank Jon Staley for stimulating discussions and Rhonda Perriman, Roland Nagel, Doug Black, and Jason Underwood for critical comments on the manuscript.

Received March 15, 2000; revised June 16, 2000.

References

Ashiya, M., and Grabowski, P.J. (1997). A neuron-specific splicing switch mediated by an array of pre-mRNA repressor sites: evidence of a regulatory role for the polypyrimidine tract binding protein and a brain-specific PTB counterpart. RNA *3*, 996–1015.

Barta, I., and Iggo, R. (1995). Autoregulation of expression of the

yeast Dbp2p "DEAD-box" protein is mediated by sequences in the conserved DBP2 intron. EMBO J. *14*, 3800–3808.

Burtis, K.C., and Baker, B.S. (1989). Drosophila doublesex gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. Cell *56*, 997–1010.

Caceres, J.F., Stamm, S., Helfman, D.M., and Krainer, A.R. (1994). Regulation of alternative splicing in vivo by overexpression of antagonistic splicing factors. Science *265*, 1706–1709.

Chabot, B. (1996). Directing alternative splicing: cast and scenarios. Trends Genet. *12*, 472–478.

Chan, R.C., and Black, D.L. (1997). The polypyrimidine tract binding protein binds upstream of neural cell-specific c-src exon N1 to repress the splicing of the intron downstream. Mol. Cell. Biol. *17*, 4667–4676.

Chou, M.Y., Rooke, N., Turck, C.W., and Black, D.L. (1999). hnRNP H is a component of a splicing enhancer complex that activates a c-src alternative exon in neuronal cells. Mol. Cell. Biol. *19*, 69–77.

Chu, S., DeRisi, J., Eisen, M., Mulholland, J., Botstein, D., Brown, P.O., and Herskowitz, I. (1998). The transcriptional program of sporulation in budding yeast. Science *282*, 699–705.

Culbertson, M.R. (1999). RNA surveillance. Unforeseen consequences for gene expression, inherited genetic disorders and cancer. Trends Genet. *15*, 74–80.

Davis, C.A., Grate, L., Spingola, M., and Ares, M., Jr. (2000). Test of intron predictions reveals novel splice sites, alternatively spliced mRNAs and new introns in meiotically regulated genes of yeast. Nucleic Acids Res. *28*, 1700–1706.

Del Gatto-Konczak, F., Olive, M., Gesnel, M.C., and Breathnach, R. (1999). hnRNP A1 recruited to an exon in vivo can function as an exon splicing silencer. Mol. Cell. Biol. *19*, 251–260.

Engebrecht, J.A., Voelkel-Meiman, K., and Roeder, G.S. (1991). Meiosis-specific RNA splicing in yeast. Cell *66*, 1257–1268.

Fortes, P., Kufel, J., Fornerod, M., Polycarpou-Schwarz, M., Lafontaine, D., Tollervey, D., and Mattaj, I.W. (1999). Genetic and physical interactions involving the yeast nuclear cap-binding complex. Mol. Cell. Biol. *19*, 6543–6553.

Gottschalk, A., Tang, J., Puig, O., Salgado, J., Neubauer, G., Colot, H.V., Mann, M., Seraphin, B., Rosbash, M., Luhrmann, R., and Fabrizio, P. (1998). A comprehensive biochemical and genetic analysis of the yeast U1 snRNP reveals five novel proteins. RNA *4*, 374–393.

Hanamura, A., Caceres, J.F., Mayeda, A., Franza, B.R., Jr., and Krainer, A.R. (1998). Regulated tissue-specific expression of antagonistic pre-mRNA splicing factors. RNA *4*, 430–444.

Hill, J., Donald, K.A., Griffiths, D.E., and Donald, G. (1991). DMSO-enhanced whole cell yeast transformation. Nucleic Acids Res. *19*, 5791.

Hodgkin, J. (1989). Drosophila sex determination: a cascade of regulated splicing. Cell *56*, 905–906.

Kunkel, T.A., Bebenek, K., and McClary, J. (1991). Efficient site-directed mutagenesis using uracil-containing DNA. Methods Enzymol. *204*, 125–139.

Lesser, C.F., and Guthrie, C. (1993). Mutational analysis of pre-mRNA splicing in Saccharomyces cerevisiae using a sensitive new reporter gene, CUP1. Genetics *133*, 851–863.

Leu, J.Y., and Roeder, G.S. (1999). Splicing of the meiosis-specific HOP2 transcript utilizes a unique 5′ splice site. Mol. Cell. Biol. *19*, 7933–7943.

Lewis, J.D., Gorlich, D., and Mattaj, I.W. (1996a). A yeast cap binding protein complex (yCBC) acts at an early step in pre- mRNA splicing. Nucleic Acids Res. *24*, 3332–3336.

Lewis, J.D., Izaurralde, E., Jarmolowski, A., McGuigan, C., and Mattaj, I.W. (1996b). A nuclear cap-binding complex facilitates association of U1 snRNP with the cap-proximal 5′ splice site. Genes Dev. *10*, 1683–1698.

Lewis, H.A., Musunuru, K., Jensen, K.B., Edo, C., Chen, H., Darnell, R.B., and Burley, S.K. (2000). Sequence-specific RNA binding by a Nova KH domain: implications for paraneoplastic disease and the fragile X syndrome. Cell *100*, 323–332.

Lin, R.J., Newman, A.J., Cheng, S.C., and Abelson, J. (1985). Yeast mRNA splicing in vitro. J. Biol. Chem. *260*, 14780–14792.

Longtine, M.S., McKenzie, A., III, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast *14*, 953–961.

Lopez, A.J. (1998). Alternative splicing of pre-mRNA: developmental consequences and mechanisms of regulation. Annu. Rev. Genet. *32*, 279–305.

Lou, H., Helfman, D.M., Gagel, R.F., and Berget, S.M. (1999). Polypyrimidine tract-binding protein positively regulates inclusion of an alternative 3′-terminal exon. Mol. Cell. Biol. *19*, 78–85.

Lynch, K.W., and Maniatis, T. (1996). Assembly of specific SR protein complexes on distinct regulatory elements of the Drosophila doublesex splicing enhancer. Genes Dev. *10*, 2089–2101.

Malone, R.E., Pittman, D.L., and Nau, J.J. (1997). Examination of the intron in the meiosis-specific recombination gene REC114 in Saccharomyces. Mol. Gen. Genet. *255*, 410–419.

Mayeda, A., and Krainer, A.R. (1992). Regulation of alternative pre-mRNA splicing by hnRNP A1 and splicing factor SF2. Cell *68*, 365–375.

Mayeda, A., Helfman, D.M., and Krainer, A.R. (1993). Modulation of exon skipping and inclusion by heterogeneous nuclear ribonucleoprotein A1 and pre-mRNA splicing factor SF2/ASF. Mol. Cell. Biol. *13*, 2993–3001.

Min, H., Chan, R.C., and Black, D.L. (1995). The generally expressed hnRNP F is involved in a neural-specific pre- mRNA splicing event. Genes Dev. *9*, 2659–2671.

Nakagawa, T., and Ogawa, H. (1997). Involvement of the MRE2 gene of yeast in formation of meiosis-specific double-strand breaks and crossover recombination through RNA splicing. Genes Cells *2*, 65–79.

Nakagawa, T., and Ogawa, H. (1999). The saccharomyces cerevisiae MER3 gene, encoding a novel helicase-like protein, is required for crossover control in meiosis. EMBO J. *18*, 5714–5723.

Nandabalan, K., and Roeder, G.S. (1995). Binding of a cell-type-specific RNA splicing factor to its target regulatory sequence. Mol. Cell. Biol. *15*, 1953–1960.

Nandabalan, K., Price, L., and Roeder, G.S. (1993). Mutations in U1 snRNA bypass the requirement for a cell type-specific RNA splicing factor. Cell *73*, 407–415.

Puig, O., Gottschalk, A., Fabrizio, P., and Seraphin, B. (1999). Interaction of the U1 snRNP with nonconserved intronic sequences affects 5′ splice site selection. Genes Dev. *13*, 569–580.

Rothstein, R. (1991). Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. Methods Enzymol. *194*, 281–301.

Siebel, C.W., Fresco, L.D., and Rio, D.C. (1992). The mechanism of somatic inhibition of Drosophila P-element pre-mRNA splicing: multiprotein complexes at an exon pseudo-5′ splice site control U1 snRNP binding. Genes Dev. *6*, 1386–1401.

Siebel, C.W., Kanaar, R., and Rio, D.C. (1994). Regulation of tissue-specific P-element pre-mRNA splicing requires the RNA-binding protein PSI. Genes Dev. *8*, 1713–1725.

Siebel, C.W., Admon, A., and Rio, D.C. (1995). Soma-specific expression and cloning of PSI, a negative regulator of P element pre-mRNA splicing. Genes Dev. *9*, 269–283.

Siomi, H., Matunis, M.J., Michael, W.M., and Dreyfuss, G. (1993). The pre-mRNA binding K protein contains a novel evolutionarily conserved motif. Nucleic Acids Res. *21*, 1193–1198.

Smith, D.B., and Johnson, K.S. (1988). Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene *67*, 31–40.

Smith, C.W., Patton, J.G., and Nadal-Ginard, B. (1989). Alternative splicing in the control of gene expression. Annu. Rev. Genet. *23*, 527–577.

Spingola, M., Grate, L., Haussler, D., and Ares, M., Jr. (1999). Genome-wide bioinformatic and molecular analysis of introns in Saccharomyces cerevisiae. RNA *5*, 221–234.

Staley, J.P., and Guthrie, C. (1998). Mechanical devices of the spliceosome: motors, clocks, springs, and things. Cell *92*, 315–326.

Staley, J.P., and Guthrie, C. (1999). An RNA switch at the 5′ splice site requires ATP and the DEAD box protein Prp28p. Mol. Cell *3*, 55–64.

Tacke, R., and Manley, J.L. (1999). Determinants of SR protein specificity. Curr. Opin. Cell Biol. *11*, 358–362.

Valcarcel, J., and Green, M.R. (1996). The SR protein family: pleiotropic functions in pre-mRNA splicing. Trends Biochem. Sci. *21*, 296–301.

Valcarcel, J., Singh, R., Zamore, P.D., and Green, M.R. (1993). The protein Sex-lethal antagonizes the splicing factor U2AF to regulate alternative splicing of transformer pre-mRNA. Nature *362*, 171–175.

Vilardell, J., and Warner, J.R. (1994). Regulation of splicing at an intermediate step in the formation of the spliceosome. Genes Dev. *8*, 211–220.

Zavanelli, M.I., and Ares, M., Jr. (1991). Efficient association of U2 snRNPs with pre-mRNA requires an essential U2 RNA structural element. Genes Dev. *5*, 2521–2533.

Zhang, D., and Rosbash, M. (1999). Identification of eight proteins that cross-link to pre-mRNA in the yeast commitment complex. Genes Dev. *13*, 581–592.