RS domains contact splicing signals and promote splicing by a common mechanism in yeast through humans

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Serine–arginine (SR) proteins are general metazoan splicing factors that contain an essential arginine–serine-rich (RS) domain. We have previously found that mammalian spliceosome assembly involves a series of sequential interactions between RS domains and two splicing signals: the branchpoint and the 5′ splice site. Here we study how RS domains are directed to specifically contact splicing signals, and how this interaction promotes splicing. The yeast *Saccharomyces cerevisiae* lacks SR proteins. However, we show that tethering a mammalian RS domain to a yeast actin pre-mRNA rescues splicing of certain branchpoint or 5′ splice site mutants in which U snRNA base-pairing has been decreased. Conversely, on a mammalian pre-mRNA, a normally essential SR protein becomes dispensable when the complementarity of a splicing signal to a U snRNA is increased. We find that in the absence of other splicing factors an RS domain tethered to a pre-mRNA selectively contacts a double-stranded RNA region and enhances RNA–RNA base-pairing. Significantly, all of these activities require phosphorylation of the RS domain. Based on these results, we propose that RS domains selectively contact splicing signals because, due to transient U snRNA base-pairing, they are partially double-stranded. The RS domain–splicing signal interaction, in turn, promotes (or stabilizes) base-pairing between the U snRNA and pre-mRNA substrate, thereby enhancing splicing. Our results reveal a common mechanism of RS domain function in yeast through humans.

Serine–arginine (SR) proteins are general metazoan splicing factors that function through binding to exonic splicing enhancers (ESEs) (Graveley 2000; Hastings and Krainer 2001; Black 2003). SR proteins, as well as several other mammalian splicing factors, contain an arginine–serine-rich (RS) domain required to promote pre-mRNA splicing. How RS domains function in constitutive and alternative pre-mRNA splicing has remained an important question in the field.

We have developed an RNA–protein cross-linking procedure to identify the target of RS domains (Shen and Green 2004; Shen et al. 2004). Using this approach, we have found that multiple RS domains are required for splicing, and have identified distinct roles for the different RS domains in splicing complex assembly (Shen and Green 2004; Shen et al. 2004). Two RS domains, one from the essential splicing factor U2AF[65] and a second from an SR protein, are required for prespliceosome as-

sembly. These two RS domains contact the branchpoint sequentially: First, the RS domain from polypyrimidine (Py)-tract-bound U2AF[65] contacts the branchpoint in complex E and, subsequently, the RS domain from the SR protein contacts the branchpoint in the prespliceosome. A third RS domain, provided by another SR protein, is required for mature spliceosome assembly and contacts the 5′ splice site.

SR proteins are present in a variety of splicing factors in all metazoans as well as in *Schizosaccharomyces pombe*, although they are apparently absent from *Saccharomyces cerevisiae* (Graveley 2000; Kaufer and Potashkin 2000; Allemand et al. 2002; Sanford et al. 2003). The splicing machinery has, in general, been highly conserved, and it is therefore puzzling that SR proteins are not found in *S. cerevisiae*. A possible explanation for the absence of SR proteins in budding yeast relates to the fact that *S. cerevisiae* branchpoints and 5′ splice sites match the splicing signal consensus sequences significantly better than do their mammalian and *S. pombe* counterparts (e.g., see Lim and Burge 2001). The degree of similarity between the branchpoint and 5′ splice site and their consensus sequences is directly related to their po-

tential for base-pairing with U2 and U1 snRNAs, respectively. We have proposed that SR proteins contact splicing signals and promote their interaction with U snRNAs (Valcarcel et al. 1996; Shen and Green 2004; Shen et al. 2004). We therefore considered the possibility that because *S. cerevisiae* branchpoints and 5′ splice sites strongly match the consensus, their ability to base-pair extensively with U snRNAs could obviate the requirement for SR proteins in this organism.

In this study, we first test this hypothesis by asking whether an RS domain can promote splicing of a yeast pre-mRNA bearing a splicing signal mutation that disrupts U snRNA base-pairing. The results of these experiments provide insight into two questions regarding RS domain function: How are RS domains directed to specifically contact splicing signals? How does the RS domain–splicing signal interaction promote splicing? We then pursue these questions further through RNA–protein and RNA–RNA cross-linking experiments in mammalian splicing extracts.

Results

### In vitro splicing of yeast actin branchpoint mutants promoted by an RS domain

To direct RS domains to yeast pre-mRNAs, we adopted a previously described protein fusion strategy (Graveley and Maniatis 1998; Shen and Green 2004; Shen et al. 2004). Chimeric proteins comprising an MS2 RNA-binding domain fused to the RS domain of various mamma-

lian splicing factors were tested for their ability to promote splicing of yeast pre-mRNAs containing an MS2-binding site positioned near the branchpoint or 5′ splice site.

We first asked whether an RS domain could function at a yeast pre-mRNA branchpoint. We constructed a wild-type yeast actin pre-mRNA derivative, actin-MS2(E2), containing an MS2-binding site in exon 2, 64 nucleotides (nt) downstream from the branchpoint. As expected, the actin-MS2(E2) pre-mRNA was spliced in a yeast whole-cell extract (WCE) (Fig. 1A). Addition of a control MS2 protein or the MS2–RS domain fusion protein, MS2–(ASF)RS, had no appreciable effect on splicing of the wild-type actin-MS2(E2) pre-mRNA. We next analyzed a series of previously characterized yeast branchpoint mutants in the actin-MS2(E2) background. Consistent with previous studies (Jacquier et al. 1985; Vijayraghavan et al. 1986; Parker et al. 1987), none of the five branchpoint mutants were spliced in a yeast WCE. Addition of an MS2–(ASF)RS fusion protein enabled splicing of two branchpoint mutants, actin-MS2(E2)/1U-A and actin-MS2(E2)/2A-U, whereas the control MS2 protein (Fig. 1A) and an MS2 fusion protein lacking an RS domain [MS2–(ASF)RRM1] (Supplementary Fig. 1A) had no effect. MS2–ASF(RS) did not promote splicing of these same branchpoint mutants if the actin pre-mRNA substrate lacked an MS2-binding site (Supplementary Fig. 1B).

Previous studies have revealed relatively nonstringent sequence requirements for function of the RS domains of U2AF[65] (Valcarcel et al. 1996) and SR proteins (Philipps et al. 2003; Shen and Green 2004; Shen et al. 2004). To determine whether this was also the case for RS domains

Figure 1. In vitro splicing of yeast actin branchpoint mutants promoted by an RS domain. (*A*) A chimeric protein in which the MS2 RNA-binding domain was fused to the RS domain of ASF/SF2 was analyzed for its ability to support splicing of the wild-type actin-MS2(E2) pre-mRNA substrate or mutant derivatives (shown on the *left*; the branchpoint adenine is underlined). In vitro splicing reactions were performed in yeast WCE to which no protein (–) or recombinant, purified MS2 or MS2–(ASF)RS protein was added, as indicated. Identities of the spliced products are shown on the *right*. (*B*) MS2–(U2AF[65])RS and MS2–(U2AF[35])RS were analyzed for their ability to rescue splicing of the actin-MS2(E2)/1U-A and actin-MS2(E2)/2A-U mutant substrates. (*C*) UV cross-linking analysis of the RS domain–pre-mRNA interaction with the wild-type actin-MS2(E2) (*top*), actin-MS2(E2)/1U-A (*middle*), and actin-MS2(E2)/5A-U (*bottom*) substrates labeled with a single ^{32}P-G at the 5′ splice site (5′ss), branchpoint (BP), Py-tract (Py), 3′ splice site (3′ss), or MS2-binding site (MS2), indicated in the schematic diagram (*bottom*). Molecular weight markers are shown on the *left*, and the positions of the 26-kDa ^{32}P-tagged MS2–TF–(ASF)RS and 8-kDa ^{32}P-tagged (ASF)RS TEV-cleaved product are indicated on the *right*.
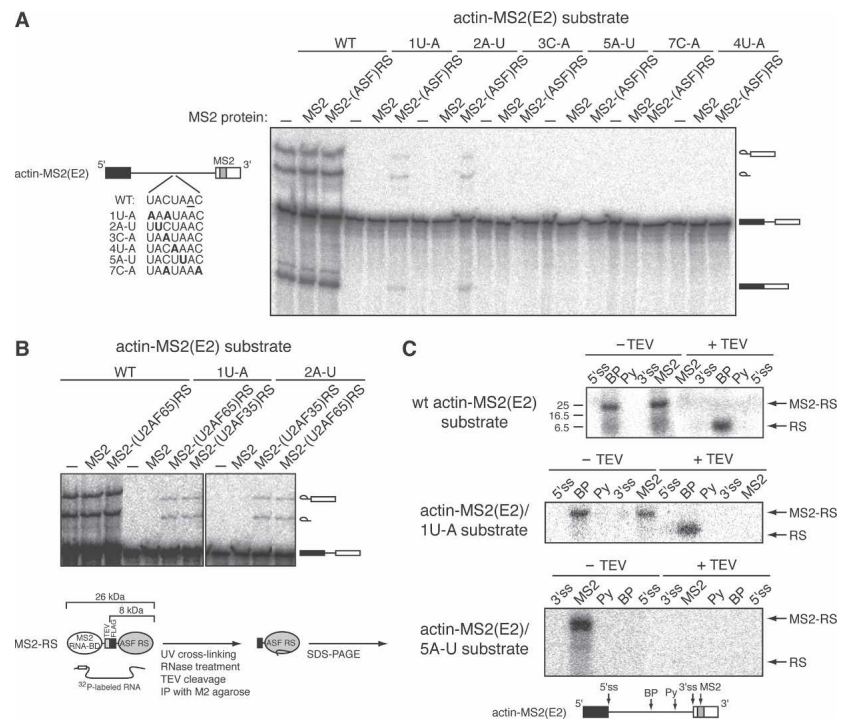
that promote splicing of yeast pre-mRNA mutants, we analyzed two other MS2–RS domain fusion proteins, MS2–(U2AF[65])RS and MS2–(U2AF[35])RS. The results of Figure 1B show that like MS2–(ASF)RS, MS2–(U2AF[65])RS and MS2–(U2AF[35])RS promoted splicing of actin-MS2(E2)/1U-A and actin-MS2(E2)/2A-U. Thus, unrelated, heterologous RS domains can support splicing when directed to the vicinity of a yeast pre-mRNA splicing signal.

We have developed an ultraviolet light (UV) cross-linking assay to detect interactions between RS domains and splicing signals (Shen and Green 2004; Shen et al. 2004). To identify the site in the yeast actin-MS2(E2) pre-mRNA contacted by the RS domain, we used an MS2–(ASF)RS derivative containing a TEV protease cleavage site and Flag epitope inserted between the MS2 RNA-binding and RS domains (Shen et al. 2004). The fusion protein was added to a yeast WCE containing wild-type actin-MS2(E2) or actin-MS2(E2)/1U-A that had been ^{32}P-labeled at the 5′ splice site, branchpoint, Py-tract, 3′ splice site, or MS2-binding site. The reaction mixture was irradiated with UV light to induce RNA–protein cross-links, and following RNase treatment, TEV protease cleavage and immunoprecipitation with an anti-Flag antibody, ^{32}P-tagged polypeptides were fractionated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and detected by PhosphorImager analysis.

Figure 1C shows that in the absence of TEV cleavage a 26-kDa ^{32}P-tagged polypeptide, the expected size of the intact MS2–(ASF)RS fusion protein, was detected when the ^{32}P-label was at the branchpoint or MS2-binding site but not at other positions of the wild-type actin-MS2(E2) or actin-MS2(E2)/1U-A substrates. Upon TEV cleavage,

an 8-kDa ^{32}P-tagged polypeptide, the expected size of the RS domain, was detected only when the RNA substrate was labeled at the branchpoint. These results indicate that when bound near the branchpoint, the RS domain of MS2–(ASF)RS specifically contacted the branchpoint, analogous to the results obtained with higher eukaryotic pre-mRNAs (Shen and Green 2004; Shen et al. 2004). Significantly, even though MS2–(ASF)RS did not increase splicing of the wild-type actin-MS2(E2) pre-mRNA, the MS2–(ASF)RS RS domain specifically contacted the branchpoint. By contrast, no interaction between the RS domain and branchpoint was detected with the actin-MS2(E2)/5A-U mutant, which did not undergo splicing upon addition of MS2–(ASF)RS.

In vitro splicing of yeast actin 5′ splice site mutants promoted by an RS domain

Figure 2 presents a similar analysis of previously characterized 5′ splice site mutants (Jacquier et al. 1985; Vijayraghavan et al. 1986) using a yeast actin pre-mRNA derivative, actin-MS2(I), containing an MS2-binding site in the intron, 15 nt downstream from the 5′ splice site. Once again, splicing of the wild-type actin-MS2(I) pre-mRNA was unaffected by addition of MS2–(ASF)RS (Fig. 2A). Addition of MS2–(ASF)RS rescued splicing of two 5′ splice site mutants, actin-MS2(I)/5G-A and actin-MS2(I)/6U-C, whereas the control MS2 protein (Fig. 2A) and an MS2 fusion protein lacking an RS domain [MS2–(ASF)RRM1] (Supplementary Fig. 2A) had no effect. Moreover, MS2–ASF(RS) did not promote splicing of these same 5′ splice site mutants if the actin pre-mRNA substrate lacked an MS2-binding site (Supplementary
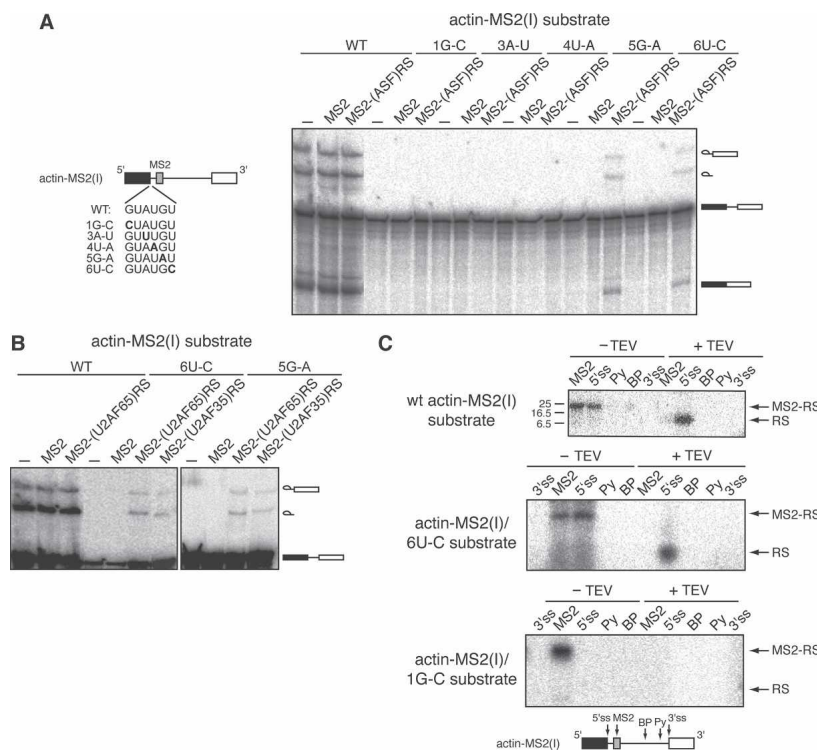
Figure 2. In vitro splicing of yeast actin 5′ splice site mutants promoted by an RS domain. (*A*) In vitro splicing analysis of the wild-type actin-MS2(I) pre-mRNA substrate and mutant derivatives (shown on the *left*), performed as described in Figure 1A. (*B*) MS2–(U2AF[65])RS and MS2–(U2AF[35])RS were analyzed for their ability to rescue splicing of the actin-MS2(I)/6U-C and actin-MS2(I)/5G-A mutant substrates. (*C*) UV cross-linking analysis of the RS domain–pre-mRNA interaction with the wild-type actin-MS2(I) (*top*), actin-MS2(E2)/6U-C (*middle*), and actin-MS2(E2)/1G-C (*bottom*) substrates, which were labeled with a single ^{32}P-G at the 5′ splice site (5′ss), MS2-binding site (MS2), branchpoint (BP), Py-tract (Py), 3′ splice site (3′ss), as shown in the schematic diagram (*bottom*).

Fig. 2B). Figure 2B shows that addition of MS2–(U2AF^{65})RS or MS2–(U2AF^{35})RS also rescued splicing of the actin-MS2(I)/5G-A and actin-MS2(I)/6U-C mutants. The UV cross-linking experiment shown in Figure 2C shows that the RS domain of MS2–(ASF)RS specifically contacted the 5′ splice site of the wild-type actin-MS2(I) and actin-MS2(I)/6U-C mutant, but not that of the actin-MS2(I)/1G-C mutant, which was not rescued by MS2–(ASF)RS.

In vivo splicing of yeast actin branchpoint and 5′ splice site mutants promoted by an RS domain

We next tested whether the RS domain could also promote splicing of the yeast pre-mRNA mutants in vivo. We constructed yeast strains expressing a wild-type or mutant actin-MS2(E2) or actin-MS2(I) pre-mRNA derivative. Each strain also expressed an MS2–(ASF)RS domain fusion protein, the control MS2 protein, or no MS2 derivative. Splicing of the pre-mRNAs was measured by an RNase protection assay.

The results shown in Figure 3 show, as expected from previous studies (Seraphin et al. 1988), that in the absence of an MS2 derivative the wild-type actin-MS2(E2) and actin-MS2(I) pre-mRNAs were spliced, whereas the mutants were not. Identical results were obtained in yeast strains expressing the control MS2 protein. In strains in which splicing did not occur, there was a large accumulation of unspliced pre-mRNA.

Consistent with the in vitro data, expression of MS2–(ASF)RS had no effect on splicing of the wild-type actin pre-mRNA. However, expression of MS2–(ASF)RS, MS2–(U2AF^{65})RS, or MS2–(U2AF^{35})RS restored splicing of the branchpoint mutant, actin-MS2(E2)/1U-A, and the 5′ splice site mutant, actin-MS2(I)/6U-C, to levels equivalent to that of the wild-type pre-mRNA. We note that although the MS2-RS domain fusion proteins rescued splicing of the actin-MS2(E2)/1U-A and actin-MS2(I)/

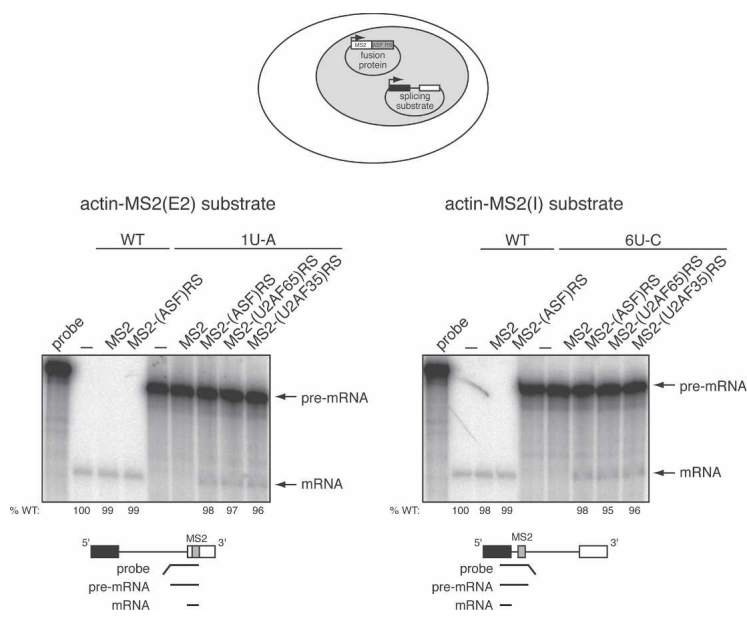6U-C mutants, there was no obvious decrease in the levels of unspliced pre-mRNA.

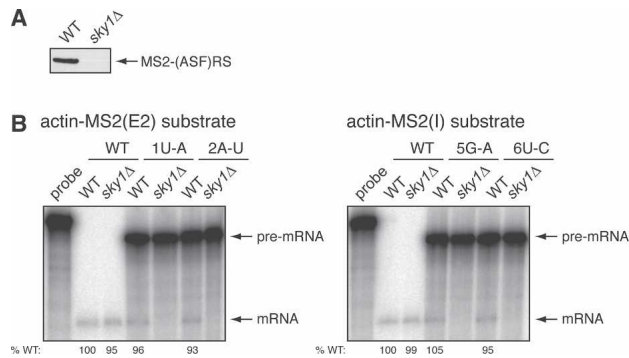### Promotion of yeast in vivo splicing by an RS domain requires the SR protein kinase, Sky1p

It is well established that the ability of an SR protein to promote splicing of a mammalian pre-mRNA is dependent on phosphorylation of the RS domain (Graveley 2000; Sanford et al. 2003). Previous studies have shown that *S. cerevisiae* contains a protein kinase, Sky1p, which can phosphorylate mammalian RS domains (Siebel et al. 1999). Moreover, an RS domain expressed in a wild-type yeast strain is phosphorylated but is nonphosphorylated in a *sky1-Δ* deletion mutant strain (Yeakley et al. 1999), indicating that Sky1p is the only kinase in *S. cerevisiae* that can phosphorylate RS domains.

To determine whether phosphorylation of the RS domain was required for its ability to promote splicing in *S. cerevisiae*, we repeated the in vivo splicing experiment in a *sky1-Δ* strain. Figure 4A shows, as expected, that MS2–(ASF)RS was phosphorylated in a wild-type strain but not in the *sky1-Δ* strain, as evidenced by immunoblotting with the mAb104 antibody, which recognizes phospho-epitopes in the RS domains of SR proteins (Zahler et al. 1992). Figure 4B shows that in contrast to the results in a wild-type strain, the MS2–(ASF)RS fusion protein failed to rescue splicing of the actin pre-mRNA 5′ splice site and branchpoint mutants in the *sky1-Δ* strain. Thus, as in mammals, an RS domain must be phosphorylated to promote splicing in *S. cerevisiae*.

An SR protein can be rendered dispensable by increasing the complementarity of a higher eukaryotic splicing signal to a U snRNA

The experiments described above indicated that an RS domain enhanced splicing in yeast only when the complementarity between the splicing signal and U

Figure 3. In vivo splicing of yeast actin branchpoint and 5′ splice site mutants promoted by an RS domain. Splicing of the pre-mRNAs was measured by an RNase-protection assay in yeast strains expressing a wild-type or mutant actin-MS2(E2) (*left*) or actin-MS2(I) pre-mRNa (*right*) derivative. Each strain also expressed an MS2–(ASF)RS domain fusion protein, the control MS2 protein, or no MS2 derivative. RNase protection assays used a probe that spanned the exon–intron junction (shown *below*), which allowed for differentiation of the spliced and unspliced products. Identities of the unspliced (pre-mRNA) and spliced (mRNA) products are indicated on the *right*. The amount of spliced product was quantitated relative to the amount of product obtained with a wild-type pre-mRNA substrate in the absence of an MS2 fusion protein (percent wild type: % WT).

Figure 4. Promotion of yeast in vivo splicing by an RS domain requires the SR protein kinase, Sky1p. (*A*) Immunoblot analysis of MS2–(ASF)RS phosphorylation in wild-type yeast and a *sky1-Δ* deletion strain. (*B*) In vivo splicing assays in wild-type or *sky1-Δ* deletion strains coexpressing MS2–(ASF)RS and a wild-type or mutant actin-MS2(E2) (*left*) or actin-MS2(I) pre-mRNA (*right*) derivative. The amount of spliced product was quantitated relative to the amount of product obtained with wild-type substrate in wild-type yeast (percent wild type: % WT).

snRNA was disrupted. This finding raised the possibility that an RS domain normally essential for splicing a mammalian pre-mRNA substrate may become dispensable if the complementarity between the splicing signal and U snRNA was increased. We have previously shown that the RS domain of an SR protein contacts the 5′ splice site in the mature spliceosome (Shen and Green 2004). Psoralen cross-linking experiments indicated that this RS domain–5′ splice site interaction promoted base-pairing between U6 snRNA and the 5′ splice site. We therefore tested whether increasing the complementarity of the 5′ splice site to U6 snRNA would render the SR protein dispensable.

We constructed a dsx-MS2 derivative, dsx-MS2(U6 mut), bearing a 2-nt substitution at the 5′ splice site that increases complementarity to U6 snRNA (Fig. 5A). Consistent with our previous results, Figure 5B shows that in an S100 extract, which lacks SR proteins, splicing of the wild-type dsx-MS2 pre-mRNA required addition of both an MS2–(ASF)RS fusion protein, which contacts the branchpoint (Shen et al. 2004), and the SR protein, ASF/SF2, which contacts the 5′ splice site (Shen and Green 2004). By contrast, splicing of dsx-MS2(U6 mut) required the MS2–RS fusion protein, but occurred even in the absence of the SR protein. As expected, an MS2 fusion protein lacking an RS domain [MS2–(ASF)RRM1] failed to support splicing in the presence or absence of ASF/SF2 (Supplementary Fig. 3). Thus, improving complementarity of the 5′ splice site to U6 snRNA obviates the need for one of the multiple SR proteins normally required for mature spliceosome assembly.

An RS domain tethered to RNA selectively contacts
a double-stranded RNA region in the absence
*of other proteins*

We next performed a series of experiments to determine how the RS domain is directed to specifically contact the splicing signal. We have previously shown that the interaction between the RS domain of an SR protein and the branchpoint is dependent on U2 snRNA (Shen et al. 2004). This observation raised the possibility that the RS domain is directed to contact the branchpoint because it is partially double-stranded due to U2 snRNA base-pairing.

To test this possibility we first asked whether an RS domain tethered to RNA would selectively contact a double-stranded RNA region. In the first experiment we analyzed the interaction between MS2–(ASF)RS and the dsx-MS2 branchpoint. UV cross-linking experiments were performed with dsx-MS2 site-specifically labeled at the branchpoint in the presence or absence of a molar excess of a 15-nt RNA oligonucleotide complementary to the branchpoint region (BP-oligo). The results of Figure 6A (left) show that an interaction between the RS domain and the branchpoint was only detected upon addition of the BP-oligo and HeLa nuclear extract (NE).

HeLa NE contains several protein kinases that phosphorylate RS domains at serine residues (Graveley 2000; Sanford et al. 2003). Our previous analysis of RS domain mutants indicated that phosphorylation is required for the RS domain to contact splicing signals and promote spliceosome assembly and splicing (Shen et al. 2004). We therefore considered the possibility that phosphorylation of the RS domain was the basis of the NE requirement. Consistent with this possibility, Figure 6A (left) shows that a micrococcal nuclease-treated NE (MNase-NE) supported the RS domain–RNA interaction, indicating that the essential factor was a protein and not a U snRNP.

As an additional test of whether a protein kinase was involved, we asked whether the RS domain–branchpoint interaction was ATP-dependent. Standard HeLa NE contains low levels of ATP, which can be depleted by pre-
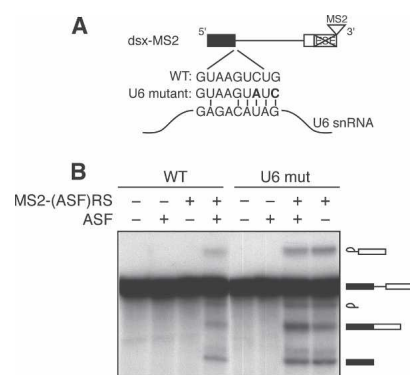
Figure 5. An SR protein can be rendered dispensable by increasing the complementarity of a mammalian splicing signal to a U snRNA. (*A*) Schematic diagram showing the sequence of the wild-type dsx-MS2 5′ splice site and the mutant derivative that increases complementarity to U6 snRNA [dsx-MS2(U6 mut)]. The sequence of the base-pairing region of U6 snRNA is shown; mutated nucleotides are indicated in bold. (*B*) In vitro splicing analysis of the wild-type dsx-MS2 pre-mRNA substrate and dsx-MS2(U6 mut) mutant derivative in the presence of the MS2–(ASF)RS fusion protein and/or the ASF/SF2 protein. Splicing reactions were performed in S100 extract.
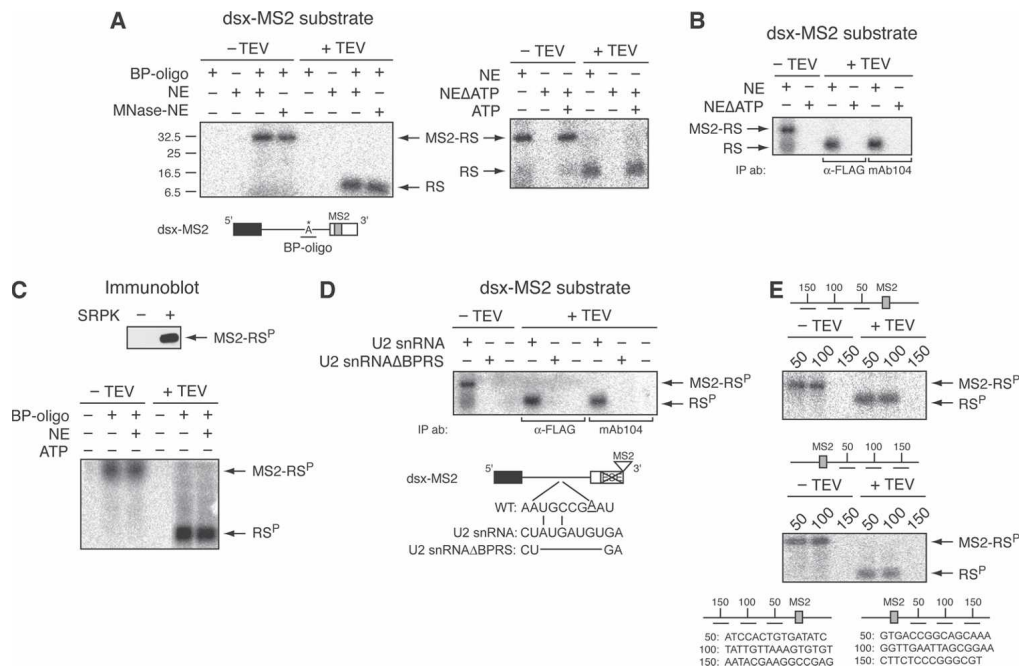
Figure 6. An RS domain tethered to RNA selectively contacts a double-stranded RNA region in the absence of other proteins. (*A*, *left*) UV cross-linking analysis of the ability of the RS domain to selectively contact a double-stranded RNA region. The dsx-MS2 pre-mRNA substrate was site-specifically labeled at the branchpoint (indicated by the asterisk) and incubated with the MS2–(ASF)RS fusion protein in the presence or absence of a molar excess of a 15-nt RNA oligonucleotide complementary to the branchpoint region (BP-oligo indicated by a line). Splicing reactions were performed in the presence of HeLa nuclear extract (NE) or micrococcal nuclease-treated nuclear extract (MNase-NE). (*Right*) UV cross-linking analysis in the presence of NE or ATP-depleted NE (NEΔATP), and in the presence or absence of exogenously added ATP. (*B*) UV cross-linking analysis in the absence of exogenously added ATP, and in the presence of NE or ATP-depleted NE (NEΔATP). Immunoprecipitations were performed with an anti-Flag antibody or the mAb104 antibody, as shown. (*C*, *top*) Immunoblot analysis of MS2–(ASF)RS phosphorylation following expression of MS2–(ASF)RS in a bacterial strain coexpressing the RS domain protein kinase SRPK. (*Bottom*) UV cross-linking analysis of the ability of phosphorylated MS2–(ASF)RS to contact a double-stranded RNA in the absence of exogenously added ATP and in the presence and absence of nuclear extract. (*D*, *top*) UV cross-linking analysis of the ability of phosphorylated MS2–(ASF)RS to contact RNA in the presence of U2 snRNA or a mutant derivative lacking the branchpoint recognition sequence (U2 snRNAΔBPRS). (*Bottom*) Schematic diagram showing the sequences of the dsx-MS2 branchpoint (the branchpoint adenine is underlined), the base-pairing region of U2 snRNA, and the nucleotides deleted in the U2 snRNAΔBPRS mutant. (*E*) Analysis of the sequence, distance, and orientation dependence of the interaction between the RS domain and the double-stranded RNA region. RNA containing an MS2-binding site and lacking natural intron sequences was site-specifically labeled at ~50, 100, or 150 nt either upstream (*top*) or downstream (*bottom*) from the MS2-binding site. Each reaction mixture contained an RNA oligonucleotide complementary to the site-specifically labeled region (indicated by a line), and the ability of the phosphorylated MS2–(ASF)RS to contact the double-stranded RNA region was analyzed by UV cross-linking analysis. (*Bottom*) Sequences of the RNA oligonucleotides.

incubation at 30°C (e.g., see Newnham and Query 2001). Figure 6A (right) shows that a ^{32}P-labeled (ASF)RS domain was not detected in NE depleted of ATP (NEΔATP), but was observed upon addition of exogenous ATP, indicating that ATP was required to support the RS domain–branchpoint interaction. Moreover, the ^{32}P-labeled (ASF)RS domain could be immunoprecipitated by the mAb104 antibody, indicating that it was phosphorylated when cross-linked to the branchpoint (Fig. 6B).

We next asked whether the requirement for NE could be bypassed by the addition of a phosphorylated RS domain. An RS domain can be phosphorylated when expressed in a bacterial strain coexpressing the RS domain protein kinase SRPK (Yue et al. 2000). We produced MS2–(ASF)RS in a bacterial strain coexpressing SRPK, and confirmed it was phosphorylated by immunoblot analysis with the mAb104 antibody (Fig. 6C, top). The

UV cross-linking experiment shown in Figure 6C (bottom), which was performed in the absence of exogenously added ATP, shows that the phosphorylated MS2–(ASF)RS RS domain was not dependent on NE for interaction with the branchpoint.

U2 snRNA contains a branchpoint recognition sequence (BPRS) (Staley and Guthrie 1998) that is partially complementary to the dsx-MS2 branchpoint. We predicted that U2 snRNA could function like the BP-oligo and, through base-pairing with the dsx-MS2 branchpoint, form a partially double-stranded RNA region that directs interaction with the RS domain. Consistent with this prediction, the UV cross-linking results shown in Figure 6D show that analogous to the BP-oligo, U2 snRNA enabled the phosphorylated MS2–(ASF)RS RS domain to interact with the branchpoint. To confirm that base-pairing between U2 snRNA and the branchpoint

was required to direct interaction with the RS domain, we analyzed a U2 snRNA mutant lacking the branch-point recognition sequence (U2 snRNAΔBPRS; Valcarcel et al. 1996). Figure 6D shows that U2 snRNAΔBPRS failed to promote an interaction between the phosphorylated MS2–(ASF)RS RS domain and the branchpoint.

We next investigated whether the interaction between the RS domain and the double-stranded RNA region was constrained by sequence, distance, or relative orientation. We derived an RNA substrate from a bacterial plasmid that lacked any natural intron sequences but contained an MS2-binding site. The RNA was site-specifically labeled at one of three positions located ~50, 100, or 150 nt either upstream or downstream from the MS2-binding site. Each reaction mixture contained a 14–16-nt RNA oligonucleotide complementary to the site-specifically labeled region. The UV cross-linking assay of Figure 6E shows that the phosphorylated MS2–(ASF)RS RS domain contacted the double-stranded RNA region when it was located 50 or 100 nt either upstream or downstream from the MS2-binding site but not when it was position 150 nt from the MS2-binding site. The most likely interpretation of these collective results is that the interaction between a phosphorylated RS domain and a double-stranded RNA region is sequence independent but distance constrained.

The RS domain–splicing signal interaction promotes RNA base-pairing

We have previously shown that following binding to the Py-tract the U2AF[65] RS domain contacts the branch-point and promotes base-pairing with U2 snRNA in the absence of other splicing factors (Valcarcel et al. 1996). To determine whether the RS domain of the MS2–RS fusion protein functioned similarly, we performed psoralen cross-linking experiments. Reaction mixtures containing a uniformly ^{32}P-labeled dsx-MS2 pre-mRNA, unlabeled in vitro synthesized U2 snRNA, the phosphorylated MS2–(ASF)RS fusion protein and psoralen were irradiated with UV light to induce RNA–RNA cross-links and the RNA species fractionated on a denaturing polyacrylamide gel. Figure 7A shows that a cross-linked RNA product (arrow) was obtained upon addition of U2 snRNA and psoralen. The amount of cross-linked species was increased by addition of the MS2–ASF(RS) but not the control MS2 protein. As expected, following addition of the U2 snRNAΔBPRS mutant a psoralen cross-linked product was not detected, verifying a requirement for the U2 snRNA branchpoint recognition sequence.

To confirm that the psoralen cross-link occurred between U2 snRNA and the branchpoint, we performed RNase H protection experiments using a uniformly ^{32}P-labeled dsx-MS2 pre-mRNA. We reasoned that the pre-mRNA region that contained the psoralen cross-link would be resistant to oligonucleotide-directed RNase H degradation. Figure 7B (left) shows that DNA oligonucleotides complementary to the branchpoint, Py-tract, 3′ splice site or MS2-binding site all efficiently directed RNase H-mediated cleavage of the dsx-MS2 pre-mRNA.
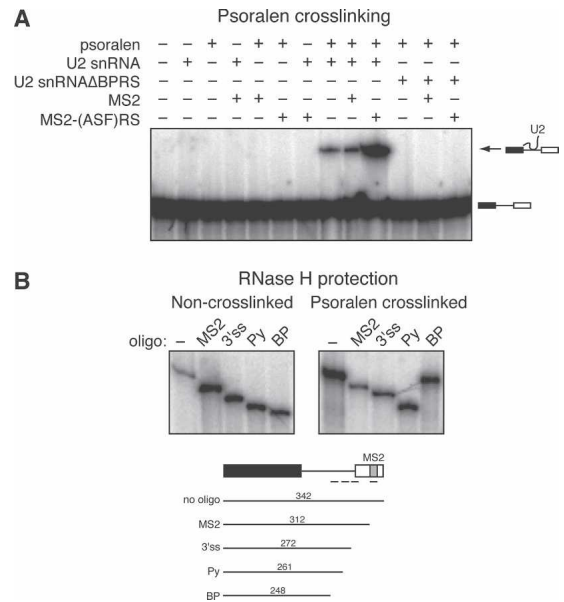
Figure 7. The RS domain–splicing signal interaction promotes RNA base-pairing. (*A*) Psoralen cross-linking experiment. Reaction mixtures containing a uniformly ^{32}P-labeled dsx-MS2 pre-mRNA; unlabeled in vitro synthesized U2 snRNA; or U2 snRNAΔBPRS, the MS2–(ASF)RS fusion protein, and psoralen were irradiated with UV light to induce RNA–RNA cross-links and the RNA species were fractionated on a denaturing polyacrylamide gel. The cross-linked RNA product is indicated by the arrow. (*B*) The position of the U2 snRNA–RNA contact was mapped by RNase H protection assays. Uniformly labeled dsx-MS2 pre-mRNA mixed with unlabeled U2 snRNA (*left*) or the eluted RNA–RNA cross-linked band (*right*) was incubated with a ~20-nt DNA oligonucleotide complementary to the branch-point, Py-tract, 3′ splice site, or MS2-binding site, and subjected to RNase H treatment in the absence (*left*) or presence (*right*) of psoralen cross-linking. Shown *below* is a schematic diagram depicting the dsx-MS2 substrate, the positions of the DNA oligonucleotides, and the expected sizes of the larger of the two RNase H-generated fragments. The portion of the gel containing the larger of the two cleavage products is shown.

On the psoralen cross-linked dsx-MS2 substrate, only the branchpoint olignonucleotide was unable to direct RNase H cleavage (Fig. 7B, right), indicating, as expected, that the psoralen cross-link maps to the pre-mRNA branchpoint.

Discussion

In this study, we performed a series of experiments using yeast and mammalian splicing systems to ask how RS domains are directed to specifically contact splicing signals and how these interactions promote splicing. The results of these experiments lead us to propose that RS domains selectively contact splicing signals because, due to transient U snRNA base-pairing, they are partially double-stranded. The RS domain–splicing signal interaction, in turn, promotes (or stabilizes) base-pairing between the U snRNA and the pre-mRNA, thereby en-

hancing splicing. Our results illustrate how formation of short RNA helices, which are essential for assembly and catalytic activation of the spliceosome (Staley and Guthrie 1998), can be modulated by protein splicing factors.

Mammalian RS domains can function in yeast

We have shown that certain yeast 5′ splice site and branchpoint mutants that disrupt U snRNA base-pairing (Seraphin et al. 1988; Siliciano and Guthrie 1988) and are not normally spliced (Jacquier et al. 1985; Vijayraghavan et al. 1986; Parker et al. 1987), can undergo splicing when a mammalian RS domain is directed to the vicinity of the mutated splicing signal. These yeast results are reminiscent of splicing of a typical mammalian intron, in which complementarity between the splicing signal and the U snRNA is imperfect and multiple RS domains are required for splicing (Shen and Green 2004; Shen et al. 2004). As with mammalian introns (Shen and Green 2004; Shen et al. 2004), we found that the RS domain directly contacts the yeast splicing signal.

In our experiments, the RS domain rescued splicing of some but not all yeast branchpoint and 5′ splice site mutants. Even for those mutants that were rescued, the RS domain did not restore splicing to wild-type levels. A likely explanation for this finding is that the branchpoint and 5′ splice site are recognized multiple times during splicing, and that the RS domain is unable to promote all of these interactions. For example, although contact between the RS domain and the mutant splicing signal is proposed to increase RNA–RNA base-pairing, it is not expected to facilitate a protein interaction. Thus, the RS domain would not promote a step (or steps) in which a protein, such as branchpoint-binding protein (BBP)/SF1, rather than a U snRNA, recognizes the splicing signal (Berglund et al. 1997). In support of the idea, we note that even compensatory U1 and U2 snRNA mutants do not rescue all splicing signal mutations to wild-type levels (e.g., see Seraphin et al. 1988; Siliciano and Guthrie 1988).

The RS domain had no effect on splicing of the wild-type yeast actin pre-mRNA. Nonetheless, the RS domain contacted the branchpoint and 5′ splice site of the wild-type pre-mRNA. This is consistent with our previous studies showing that if splicing complex assembly occurs, the RS domain can contact the splicing signal (Shen and Green 2004; Shen et al. 2004), presumably due to formation of a double-stranded RNA region (see below). Conversely, mutants that are unable to undergo splicing or splicing complex assembly fail to support the RS domain–splicing signal interaction.

The failure of the RS domain to enhance splicing of the wild-type yeast pre-mRNA is also consistent with the proposal that RS domains function by promoting base-pairing. Because the complementarity between the wild-type yeast splicing signal and the U snRNA is already extensive, the RS domain is not expected to further stimulate splicing. The inability of an RS domain to enhance splicing of the wild-type yeast pre-mRNA helps explain why *S. cerevisiae* lacks SR proteins. Consistent with this model, we found that in a mammalian pre-mRNA, increasing the complementarity between the 5′ splice site and U6 snRNA renders the SR protein dispensable. Our results show that the apparent discrepancy between the yeast and higher eukaryotic splicing machinery does not in actuality reflect differences in pathways or mechanisms, underscoring the evolutionary conversation of the splicing process.

The yeast results presented here strongly support the proposed protein–RNA interaction model for RS domain function. An alternative proposal is that RS domains function through interactions with RS domains of other splicing factors including SR proteins, U1 snRNP 70K and U2AF[35] (Wu and Maniatis 1993; Kohtz et al. 1994; Graveley 2000). Although our results do not rule out this possibility, we note that in yeast these other splicing factors are either absent (SR proteins, U2AF[35]) (for reviews, see Graveley 2000; Sanford et al. 2003) or lack an RS domain (U1 snRNP 70K) (Smith and Barrell 1991).

RS domains have an intrinsic affinity for double-stranded RNA regions and can promote RNA–RNA base-pairing

What is the basis for the selective interaction of the RS domain with double-stranded RNA? The negative charge density of double-stranded RNA is higher than that of single-stranded RNA, which could explain the selective interaction with the positively charged RS domain. Our results also suggest that the interaction of the RS domain with RNA is constrained by distance, as expected for a simple, bimolecular reaction, and is consistent with previous studies demonstrating a distance constraint for splicing enhancer function (Graveley et al. 1998).

We found that the interaction between the RS domain and the branchpoint promoted U2 snRNA–branchpoint base-pairing in the absence of other splicing factors. We propose that this occurs in the same way that basic peptides accelerate nucleic acid hybridization (Feughelman et al. 1955): The basic amino acid side chains interact with and neutralize the negatively charged phosphates, thereby facilitating pairing of the two RNA strands. According to this model, the RS domain–RNA interaction could facilitate the RNA annealing reaction by either increasing the on rate (i.e., promoting formation of the RNA duplex) or decreasing the off rate (i.e., stabilizing the RNA duplex). The results presented here are analogous to our previous finding that binding of U2AF[65] to the Py-tract through its RNA recognition motif (RRM) positions the RS domain to contact the upstream branchpoint (Valcarcel et al. 1996). Contact of the U2AF[65] RS domain with the branchpoint, in turn, promotes base-pairing with U2 snRNA in the absence of other proteins. It has been previously reported that addition of excess SR proteins can compensate for loss of U1 snRNP function (Crispino et al. 1994; Tarn and Steitz 1994). The mechanism we propose may be relevant to this observation. For example, the excess SR proteins may facilitate the U2 snRNA–branchpoint interaction, thereby promoting pre-spliceosome assembly in the absence of U1 snRNP.

RS domain function requires phosphorylation

We have found that all in vitro and in vivo RS domain activities are dependent on phosphorylation of the RS domain. Our results fit in well with numerous previous studies in which phosphorylation of the RS domain was shown to be required for promotion of complex assembly and splicing (Graveley 2000; Sanford et al. 2003). Based on the proposed model, we were surprised that phosphorylation, which introduces negative charge, was required for the RS domain to contact the double-stranded RNA region. There are several plausible explanations for this finding. For example, a reduction of the positive charge of the RS domain may be important for proper discrimination between single- and double-stranded RNA; if the net positive charge is too high, the RS domain may interact with and be trapped on single-stranded pre-mRNA regions, preventing the specific interaction with the splicing signal. Consistent with this idea, the U2AF[65] RS domain, which is directly positioned to contact the branchpoint through binding to the adjacent Py-tract, does not require phosphorylation for splicing-related activities (Valcarcel et al. 1996). An alternative possibility is that phosphorylation induces a conformational change of the RS domain that facilitates interaction with double-stranded RNA. The experimental approaches described here can be used to test these models.

Materials and methods

Pre-mRNA substrates

For in vitro studies, plasmids encoding the wild-type actin-MS2(E2) and actin-MS2(I) pre-mRNAs were constructed from plasmid SP65-actin (Vijayraghavan et al. 1986) by inserting an MS2-binding site 15 nt downstream from the 3′ splice site or 5′ splice site, respectively. Mutant derivatives were made by site-directed mutagenesis. For in vivo studies, actin-MS2(E2) and actin-MS2(I) pre-mRNAs and their mutant derivatives were constructed from plasmid pYAHB2 (Vijayraghavan et al. 1986). The plasmid encoding dsx-MS2(U6 mut) was constructed by site-directed mutagenesis of a plasmid encoding dsx-MS2 (Shen et al. 2004).

Protein expression and purification

MS2, MS2–(ASF)RS, MS2–(U2AF[35])RS, MS2–(U2AF[65])RS, and MS2–TEV-Flag–(ASF)RS fusion proteins were expressed in *Escherichia coli* strain BL21 and purified as described previously (Dauksaite and Akusjarvi 2002). For expression of the fusion proteins in vivo in yeast, plasmids encoding MS2, MS2–(ASF)RS, MS2–(U2AF[35])RS, and MS2–(U2AF[65])RS were constructed by inserting the fusion protein coding region into the backbone of a yeast expression plasmid based on the Cytotrap vector (Stratagene), which had been modified to remove the hSos gene. The His6-tagged ASF/SF2 protein was expressed and purified as described previously (Caceres and Krainer 1993). In all of the experiments involving yeast or mammalian extracts (nuclear or S100), the RS domains were phosphorylated, as evidenced by reactivity with the mAb104 antibody. Phosphorylated MS2–(ASF)RS was produced in an *E. coli* strain BL21 co-expressing the protein kinase SRPK, as described previously (Yue et al. 2000).

In vitro and in vivo splicing assays

For in vitro splicing reactions in yeast WCE, the protease-deficient strain EJ101 was grown in YPD medium at 30°C to an A_{600} of ~3.0, and cells were harvested and WCE was prepared as described previously (Cheng et al. 1990). Splicing assays were performed essentially as described previously (Cheng et al. 1990) in a solution containing 60 mM potassium phosphate (pH 7.0), 3 mM $MgCl_2$, 2 mM ATP, 1 mM spermidine, 3% polyethylene glycol 8000, 0.4 nM labeled pre-mRNA substrate, 40% yeast WCE, and 0.2 μM purified recombinant MS2, MS2–(ASF)RS, MS2–(U2AF[35])RS, or MS2–(U2AF[65])RS; the reaction mixture was incubated for 20 min at 23°C. After protease K treatment, proteins were removed by phenol-chloroform extraction, and RNA was ethanol-precipitated and analyzed on 8% polyacrylamide/8 M urea gels.

For in vivo splicing reactions, plasmids expressing pre-mRNA substrates and MS2–RS fusion proteins were cotransformed into yeast haploid strain FY23. Cells were grown at 30°C in selective medium (–Leu –Trp) to log phase and total RNA was extracted using Trizol reagent. Splicing of the pre-mRNAs was measured by RNase protection assay as described previously (Huang and Carmichael 1996). To distinguish between endogenous and ectopically expressed actin, the RNA probe was designed to hybridize to the region of the ectopically expressed actin pre-mRNA that contains the MS2-binding site. The probe spanned the exon–intron junction, which allowed for differentiation of the spliced and unspliced products. The actin-MS2(E2) probe is 135 nt, and expected sizes of protected fragments are 120 nt for the unspliced product and 50 nt for the spliced mRNA, whereas the actin-MS2(I) probe is 136 nt, and expected sizes of protected fragments are 121 nt for the unspliced product and 50 nt for the spliced mRNA. Uniformly labeled RNA probes were synthesized by in vitro transcription using T7 RNA polymerase in the presence of [α-^{32}P]UTP. The probes were mixed with 10 μg of total RNA in a buffer lacking Mg^{2+} and hybridized overnight. The hybridization products were digested with an RNase T1/T2 mixture in a buffer lacking Mg^{2+} for 2 h at 37°C, and the resulting products were resolved on 8% denaturing polyacrylamide gels. Signals were visualized by PhosphorImager (Fujifilm FLA-5000 imaging system), and the amount of spliced product was quantified by ImageGuage version 4.22 software.

In vitro splicing reactions in S100 extract were performed as described previously (Graveley and Maniatis 1998). Protein concentrations in the splicing reactions were generally as follows: MS2–(ASF)RS, 0.2 μM; and ASF/SF2, 0.5 μM.

UV cross-linking assay

Site-specific labeling of pre-mRNA substrates and UV cross-linking in both yeast WCE and HeLa nuclear extract were performed as described previously (Shen et al. 2004), except in experiments presented in Figure 6, A and B, in which the assays were performed in 6% HeLa nuclear extract. The sequence of the BP-oligo is 5′-GAGACATTCGGCATT-3′. To deplete ATP, HeLa nuclear extract was preincubated for 30 min at 30°C.

Yeast strain construction

The *sky1-Δ∷URA3* strain was constructed from the starting haploid strain FY23 using a PCR-based approach. Sequences of the primers were as follows: Sky-1 (5′-GAGGTTGAAGAGA TAGAGTAAAGAAGAAGTGTAGACATTAATGTCGAAAGC

TACATATAAG-3′) and Sky-2 (5′-AAAAGTAAAAGGCAA GGGCAAAATAAAGGTATAAAGGTAATTAGTTTTGCTG GCCGCATC-3′). Genotypes were verified by PCR.

Psoralen cross-linking and RNase H protection assays

Psoralen cross-linking reactions were carried out as described previously (Zhu et al. 2003) in the presence of uniformly labeled dsx-MS2 pre-mRNA and unlabeled U2 snRNA or U2 snRNAΔBPRS (Valcarcel et al. 1996). The final concentration of MS2 or MS2–(ASF)RS protein was 0.2 µM. Twenty micrograms of 4′-aminomethyl-4.5′, 8-trimethyl psoralen (Sigma) was used. The reaction mixtures were incubated for 10 min at 30°C, and UV-irradiated (365 nm) for 10 min at 4°C to generate RNA–RNA cross-links. Products were analyzed on a 5% denaturing polyacrylamide gel.

For the RNase H protection assay, the RNA–RNA cross-linked band was eluted and mixed with 0.1 pmol of DNA oligonucleotide in the presence of 0.1 M NaCl; the mixture was hybridized by incubating for 5 min at 80°C, followed by gradual cooling to room temperature. RNase H and was added to the reaction mixture and the reaction was incubated for 1 h at 37°C (Forch et al. 2003). For noncross-linked samples, RNase H treatment was performed on labeled pre-mRNA incubated with unlabeled U2 snRNA and DNA oligonucleotide. Products were separated on a 5% denaturing polyacrylamide gel.

Acknowledgments

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