Splicing Regulation at the Second Catalytic Step by Sex-lethal Involves 3′ Splice Site Recognition by SPF45

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Summary

The *Drosophila* protein Sex-lethal (SXL) promotes skipping of exon 3 from its own pre-mRNA. An unusual sequence arrangement of two AG dinucleotides and an intervening polypyrimidine (Py)-tract at the 3′ end of intron 2 is important for *Sxl* autoregulation. Here we show that U2AF interacts with the Py-tract and downstream AG, whereas the spliceosomal protein SPF45 interacts with the upstream AG and activates it for the second catalytic step of the splicing reaction. SPF45 represents a new class of second step factors, and its interaction with SXL blocks splicing at the second step. These results are in contrast with other known mechanisms of splicing regulation, which target early events of spliceosome assembly. A similar role for SPF45 is demonstrated in the activation of a cryptic 3′ ss generated by a mutation that causes human β-thalassemia.

Introduction

Removal of introns from mRNA precursors is carried out by a complex of small nuclear ribonucleoprotein particles (snRNPS) and non-snRNP polypeptides (i.e., the spliceosome) and takes place in two steps (Will and Lührmann, 2001; Hastings and Krainer, 2001). First, cleavage occurs between the upstream exon and the intron. Second, exons are ligated and the intron is released in a lariat configuration.

The 3′ end of introns is recognized in higher eukaryotes by the U2 auxiliary factor (U2AF), which is composed of two subunits of 65 and 35 kDa. $U2AF^{65}$ binds to the pyrimidine-rich (Py) tract that precedes the 3′ splice site (ss) AG, which is recognized by $U2AF^{35}$ (Zamore et al., 1992; Wu et al., 1999; Zorio and Blumenthal, 1999; Merendino et al., 1999).

Activation of the 3′ ss to undergo catalysis requires

substantial rearrangements within the spliceosome (Schwer and Guthrie, 1992; Ansari and Schwer, 1995; Sawa and Shimura, 1991; Chua and Reed, 1999a, 1999b). Work in *Saccharomyces cerevisiae*, for example, identified a DEXH helicase—Prp16p—that associates with the 3′ ss region after lariat formation and induces ATP-driven conformational rearrangements that result in replacement of Prp16p by Slu7p and Prp18p, which are protein factors important for 3′ ss activation (Schwer and Guthrie, 1991, 1992; Frank and Guthrie, 1992; Ansari and Schwer, 1995; Jones et al., 1995; Umen and Guthrie, 1995b). At least six conserved proteins and two snRNAs are known to interact with the pre-mRNA in the vicinity of the 3′ ss and/or influence 3′ ss choice (reviewed in Umen and Guthrie, 1995a; Moore, 2000). In addition, human proteins have been identified that associate with spliceosomes after the first catalytic step (Gozani et al., 1994). These proteins play a role in the second step of splicing (Gozani et al., 1994; Tronchére et al., 1997; Ajuh et al., 2000) or crosslink to the AG dinucleotide at the time of the second catalytic step (Wu and Green, 1997). However, the identity of factors that recognize and activate the AG dinucleotide during the second catalytic step remains unresolved.

The protein SPF45 was previously identified as a component of splicing complexes, with a subnuclear localization characteristic of splicing factors (Neubauer et al., 1998). Its biochemical properties and function in the splicing process, however, had not been investigated. Here we show that SPF45 directly binds to a 3′ ss AG and promotes its utilization during the second catalytic step.

Pre-mRNA splicing is often regulated during cell differentiation and development to generate mRNAs that encode distinct proteins from a single primary transcript (Graveley, 2001). Regulation is usually accomplished by promoting or repressing early events in spliceosome assembly (Smith and Valcárcel, 2000). The *Drosophila* protein Sex-lethal (SXL) is specifically expressed in female flies and controls alternative splicing of genes involved in sexual differentiation, behavior, and X chromosome dosage compensation (Schütt and Nöthiger, 2000). SXL also controls its own expression. In the absence of SXL (in male flies), exon 3 is incorporated into *Sxl* mRNAs. These mRNAs cannot encode a functional protein because exon 3 contains termination codons in-frame with the initiator ATG located in exon 2. Expression of SXL protein in female flies causes skipping of exon 3 and results in the production of an mRNA encoding full-length SXL (Figure 1A; Bell et al., 1991).

For some of the genes regulated by SXL, e.g., *transformer* (*tra*) and *male-specific-lethal 2* (*msl-2*), uridine-rich sequences that serve as SXL binding sites are located at the regulated splice sites (Sosnowski et al., 1989; Inoue et al., 1990; Kelley et al., 1995). Biochemical data are compatible with a model in which SXL blocks the access of splicing factors to these sites (Valcárcel et al., 1993; Merendino et al., 1999; Förch et al., 2001). *Sxl* autoregulation, however, is unlikely to be explained by simple splice site occlusion because most SXL bind-

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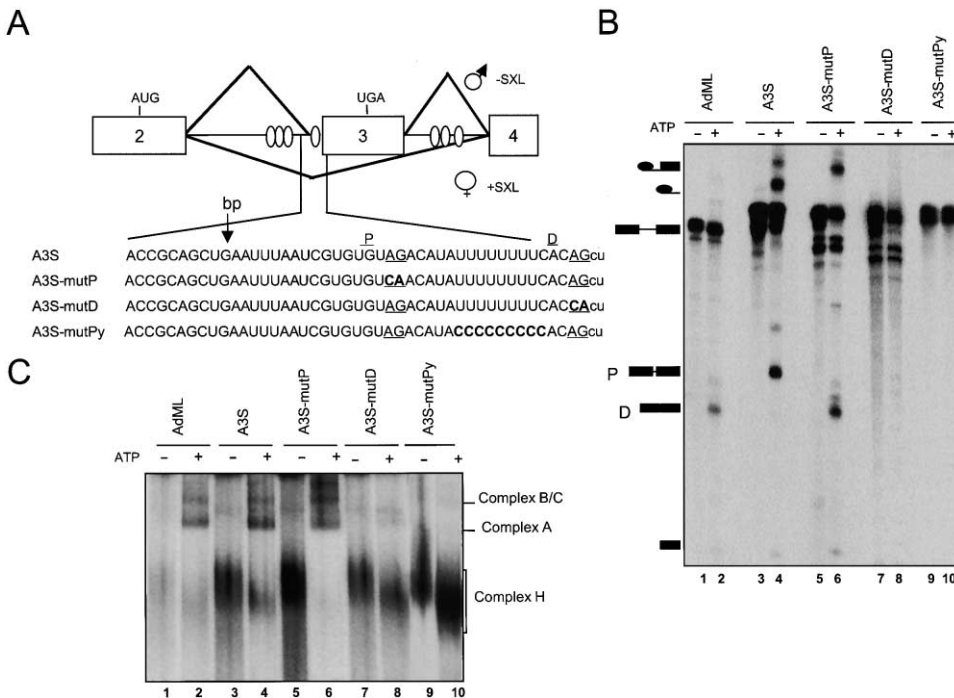[5]Present address: Theraupetic Targets S.L., Saturno 1, Tres Cantos, 28760 Madrid, Spain.

Figure 1. In Vitro Splicing of *Sxl*-Derived Pre-mRNAs

(A) Schematic representation of *Sxl* genomic region subject to sex-specific alternative splicing. Exon 3 is included in the absence of SXL (male flies) and skipped in its presence (females). Exons are represented by boxes, introns by thin lines, and patterns of alternative splicing by thick lines. Exon 3 inclusion involves splicing to the proximal AG upstream from the Py-tract (Penalva et al., 2001; see text). Ovals represent potential SXL binding sites. The positions of SXL initiator codon in exon 2 and one of several in-frame stop codons in exon 3 are indicated. The nucleotide sequence of the 3′ ss regions of RNAs analyzed in this study is also shown. AGs are underlined (P represents proximal and D distal 3′ ss). Mutations are indicated in bold case. bp indicates the nucleotide used as branch point, mapped by primer extension as described (Ruskin et al., 1986).

(B) In vitro splicing of A3S and mutant derivatives. The indicated radioactively labeled RNAs were incubated with nuclear extracts in the presence or absence of ATP as indicated. RNAs were then purified and fractionated by electrophoresis on denaturing 13% polyacrylamide gels. The positions of pre-mRNAs and products are schematically represented to the right. Boxes indicate exons; thin lines indicate introns. The positions of intermediates and products corresponding to the use of the proximal (P) and distal 3′ ss (D) is indicated.

(C) Spliceosome assembly analysis of the reactions shown in (B). The indicated radioactively labeled RNAs were fractionated on agarose-polyacrylamide gels that can resolve hnRNP complexes (H) from prespliceosomal (A) and spliceosomal (B and C) AdML complexes.

ing sites required for regulation are remote from the regulated splice sites (Sakamoto et al., 1992; Horabin and Schedl, 1993; Wang and Bell, 1994). An amino-terminal glycine-rich region of SXL has been implicated in cooperative RNA binding and shown to be important for autoregulation (Wang and Bell, 1994).

Here we report that, in contrast to regulatory mechanisms described so far, SXL inhibits the last step of the splicing process. SXL accomplishes this by interacting through its amino-terminal domain with SPF45.

Results

Sex-lethal (SXL) protein promotes skipping of exon 3 from its own pre-mRNA (Figure 1A). This exon is unusual in that it is preceded by two alternative 3′ ss AGs (Bell et al., 1991; Penalva et al., 2001). The nucleotide sequence of *Sxl* intron 2/exon 3 boundary is shown in Figure 1A. The 3′ ss AG distal (D) to the 5′ ss and its associated polypyrimidine (Py)-tract are important for exon 3 definition, because their mutation results in exon 3 skipping even in the absence of SXL protein. The proximal (P) 3′ ss, however, is preferentially used for exon ligation. Mutation of the proximal 3′ ss induces the use of the distal 3′ ss and, intriguingly, also compromises the ability of SXL to induce exon 3 skipping (Penalva et al., 2001). We set out to investigate the molecular basis for these observations.

No splicing of transcripts spanning *Sxl* exons 2–4 with either *Drosophila* or human nuclear extracts under a variety of experimental conditions was detected. This was also the case for deleted transcripts lacking segments of introns 2 and/or 3 (data not shown). Therefore, a chimeric RNA (A3S) was generated where the 3′ end of an Adenovirus (AdML) intron was substituted by the corresponding region of *Sxl* intron 2 (including both proximal and distal 3′ ss). Incubation of A3S RNA under splicing conditions resulted in the ATP-dependent accumulation of splicing intermediates and products (Figure 1B, compare lanes 3 and 4). The major spliced product corresponds in size to utilization of the proximal 3′ splice site, as confirmed by its isolation and sequencing (data not shown). This is reminiscent of the preferential use of the proximal 3′ ss in male *Drosophila* flies and in Schneider cells transfected with a *Sxl* minigene (Penalva et al., 2001). Three other features of this 3′ ss region

observed in vivo were also recapitulated in vitro using the A3S substrate. First, the distal site was utilized upon mutation of the proximal 3′ ss AG (compare lanes 4 and 6; the use of the distal site was confirmed by sequencing of the product). Second, mutation of either the distal AG or its associated Py-tract resulted in general inhibition of splicing (lanes 8 and 10). Third, consistent with the idea that the distal 3′ ss region is important for early events involved in spliceosome assembly, both mutation of the Py-tract and mutation of the distal 3′ ss AG resulted in reduction of prespliceosomal complex A formation (Figure 1C, compare lanes 4 with 8 and 10). We conclude that in vitro splicing of the A3S RNA in HeLa nuclear extracts recapitulates characteristic properties of this unusual arrangement of 3′ ss as observed for *Sxl* exon 3 in vivo.

Proteins that Interact with P and D 3′ ss

To identify factors associated with the proximal and distal splice sites, ultraviolet (UV) light-mediated crosslinking assays were carried out. To test whether $U2AF^{65}$ was associated with the Py-tract of A3S RNA, the transcript was uniformly labeled with [^{32}P]uridine, incubated with nuclear extract under splicing conditions, and irradiated with UV light, and after treatment with RNase A, immunoprecipitation was carried out using anti-$U2AF^{65}$ antibodies. $U2AF^{65}$ was crosslinked to the wild-type RNA but not to a mutant RNA in which a stretch of eight uridines at the Py-tract was substituted by cytidines (Figure 2A, compare lanes 1 and 4). This suggests that $U2AF^{65}$ interacts with the Py-tract immediately upstream from the distal 3′ ss. Mutation of the distal 3′ ss AG reduced $U2AF^{65}$ crosslinking (lane 3), while mutation of the proximal AG did not (lane 2). This suggests that the distal 3′ ss belongs to the category of AG-dependent 3′ ss, which are reliant upon interaction between $U2AF^{35}$ and the downstream 3′ ss AG to stabilize $U2AF^{65}$ binding to a Py-tract (Reed, 1989; Wu et al., 1999). Consistent with this hypothesis, a 35 kDa species was crosslinked to a A3S transcript in which a single radioactive phosphate was incorporated between the A and G at the distal 3′ ss (data not shown), and this crosslinked species could be immunoprecipitated using antibodies against $U2AF^{35}$ (Figure 2B, lane 1). The crosslinking was specific because $U2AF^{35}$ was not crosslinked when the AG was mutated to CA and the label was introduced between C and A (compare lanes 1 and 2). Taken together, the results shown in Figures 2A and 2B indicate that $U2AF^{65}$ interacts with the Py-tract at the distal 3′ ss and that $U2AF^{35}$ interacts with the distal 3′ ss AG.

To identify factors recognizing the proximal 3′ ss AG, a A3S RNA was prepared in which a single radioactive phosphate was incorporated between the A and G at the proximal 3′ ss AG. Upon incubation with nuclear extract under splicing conditions, UV light irradiation, and RNase A digestion, a crosslinked product of around 45 kDa was detected (Figure 2C, lane 1). Several results suggested that this species was relevant to the switch in 3′ ss utilization associated with exon 3 splicing and its regulation. First, crosslinking was specific because it was abolished by mutation of the proximal AG to CA (A3S-mutP, lane 2). Second, crosslinking was reduced by mutation of the distal 3′ ss AG (A3S-mutD, lane 3),

suggesting that recognition of the distal 3′ ss— presumably by $U2AF^{35}$ (Figure 2B)—is important for recognition of the proximal 3′ ss by the 45 kDa species.

Two lines of evidence indicated that the 45 kDa species was the spliceosomal protein SPF45. First, the 45 kDa species crosslinked to the proximal AG was efficiently and specifically immunoprecipitated by antibodies that recognize either a peptide region of SPF45 or the complete protein (Figure 2D, left, compare lanes 1 and 7 with lanes 4 and 10, and data not shown). As observed for the 45 kDa crosslinking product, mutation of either the proximal or the distal 3′ ss reduced SPF45 crosslinking (Figure 2D, left, compare lane 1 with lanes 2 and 3). Second, purified recombinant His-tagged SPF45 added to nuclear extracts crosslinked to the RNA specifically labeled at the proximal AG, but not to a mutant derivative in which the AG was mutated to CA, and was precipitated by nickel-agarose beads (Figure 2D, right, lanes 1 and 2). No signal was detected when recombinant SPF45 was not added to the extract (lane 3).

We conclude that the 45 kDa species is SPF45 and that it specifically recognizes the proximal 3′ ss AG in the A3S RNA. This interaction is dependent upon recognition of the Py-tract and distal 3′ ss, most likely by $U2AF^{65}$ and $U2AF^{35}$. Consistent with this, SPF45 crosslinking was compromised in nuclear extracts depleted of the U2AF heterodimer (data not shown).

SPF45 Is Important for SXL Autoregulation

Previous results have shown that mutation of the proximal AG compromises the ability of SXL to induce skipping of exon 3 from its own pre-mRNA (Penalva et al., 2001), suggesting that recognition of the proximal site facilitates *Sxl* autoregulation. If this recognition involves SPF45, interference with SPF45 activity should compromise SXL function. To test this prediction, we used double-stranded RNA (dsRNA) to interfere with endogenous SPF45 function in *Drosophila* cells. Schneider cells were transfected with dsRNA corresponding to a *Drosophila* SPF45 cDNA obtained from an embryo library, or transfected with various control dsRNAs. The levels of SPF45 mRNA were reduced at least 20-fold (data not shown). A reporter minigene containing *Sxl* exons 2–4 was cotransfected with increasing concentrations of a SXL-encoding plasmid or with empty vector. While cotransfection with limited amounts of SXL-encoding plasmid induced complete skipping of exon 3 in cells that had been treated with control dsRNA (Figure 3A, lanes 1–4), SXL-induced exon skipping was reduced in cells treated with SPF45 dsRNA (lanes 5–8). For example, while transfection with 0.004 μg/ml of SXL-encoding plasmid resulted in quantitative skipping of exon 3 in cells cotransfected with control dsRNA (lane 2), transfection with 20-fold higher concentrations of SXL-encoding plasmid resulted in less than 50% exon skipping in the presence of SPF45 dsRNA (lane 8). The effect could be specifically attributed to SPF45 expression because efficient regulation by SXL was restored by overexpression of human SPF45 in these cells (lanes 9–12). The nucleotide sequence of human and *Drosophila* SPF45 is sufficiently different so that *Drosophila* SPF45 dsRNA does not interfere with human SPF45 expression. The effects observed were not due to changes in the expression of
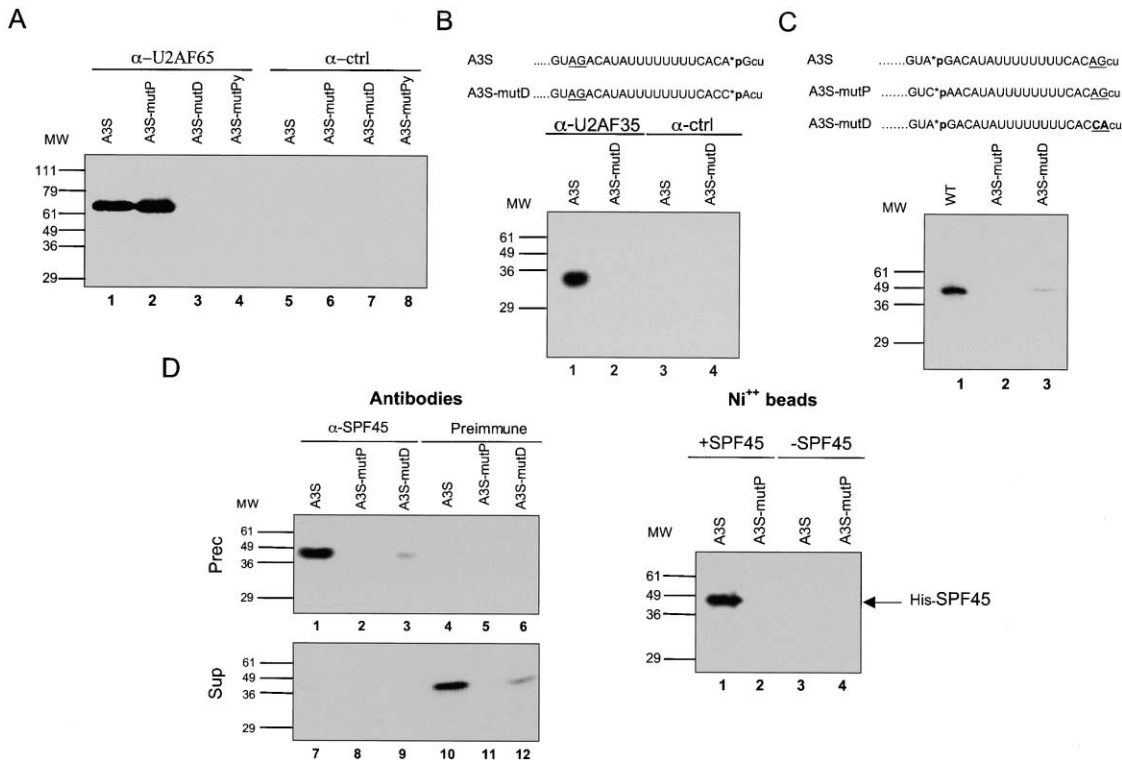
Figure 2. Identification of Factors that Bind to the 3' ss of *Sxl* Exon 3

(A) The indicated RNAs were uniformly labeled with [^{32}P]uridine, incubated with nuclear extracts in the absence of ATP, and after incubation at 30°C for 15 min, irradiated with 265 nm ultraviolet (UV) light and digested with RNase A, and the products of crosslinking were immunoprecipitated with anti-U2AF^{65} monoclonal antibodies or a control (ctrl) antibody. The sizes of molecular weight markers are indicated to the left.

(B) Crosslinking/immunoprecipitation as in (A) with RNAs site-specifically labeled at either the distal 3' ss of A3S or at an equivalent position in a mutant substrate (A3S-mutD), using antibodies against U2AF^{35} or a preimmune serum (ctrl). The nucleotide sequence and position labeled in A3S and A3S-mutD RNAs is indicated. Asterisks indicate the position of the radioactive phosphate.

(C) Pattern of polypeptides crosslinked to RNAs site-specifically labeled at the proximal 3' ss. Asterisks indicate the position of the radioactive phosphate. After UV irradiation and digestion with RNase A, crosslinked products were directly resolved on SDS-polyacrylamide gels.

(D) SPF45 crosslinking to proximal 3' ss AG. Left: products of immunoprecipitation of crosslinking reactions set up as in (C) using anti-SPF45 antibodies or preimmune serum, fractionated on denaturing SDS polyacrylamide gels. Right: precipitation with nickel agarose beads of crosslinking products corresponding to reactions set up as in (C) either in the presence or the absence of exogenously added recombinant purified His-SPF45.

SXL, because neither dsRNA-mediated interference nor overexpression of SPF45 changed the levels of SXL protein (Figure 3B). We conclude that interference with SPF45 expression compromises the ability of SXL to promote exon 3 skipping.

To test whether interference with SPF45 function influenced the relative use of the proximal and distal sites, RT-PCR assays were carried out using primers designed to distinguish between products generated by the use of each of the splice sites (Penalva et al., 2001). While the proximal site is preferentially used in control cells (as previously reported, Penalva et al., 2001), the distal site is preferentially used in cells transfected with SPF45 dsRNA (Figure 3C, compare lanes 1 and 2). Overexpression of hSPF45 increased the use of the proximal site (lane 3), arguing that the effects on 3' ss utilization were specifically related to SPF45 activity. These observations suggest that SPF45 activity correlates with the use of the proximal site.

Based on the interaction data presented in the previous section and the observation that interference with SPF45 function has effects similar to those of mutation of the proximal 3' ss AG (Figure 3; Penalva et al., 2001), we propose that both interaction with and activation of the proximal 3' ss by SPF45 are important for efficient *Sxl* autoregulation.

SPF45 Is a Second Step Factor

To analyze the function of SPF45, HeLa nuclear extracts were immunodepleted of this factor and their splicing activity was analyzed. Immunodepletion reduced the levels of SPF45 to less than 10% of mock-depleted extracts (Figure 4A). Splicing assays showed that depletion with preimmune serum resulted in a relative increase in the use of the distal site and accumulation of the corresponding lariat product (compare lane 2 in Figure 4B with lane 4 in Figure 1B). These effects may, however, be related to nonspecific (e.g., dilution) effects of the depletion procedure, because splicing assays carried out with nuclear extract preparations differing in overall protein concentration also resulted in different degrees of distal site utilization (data not shown). SPF45 depletion resulted in reduced levels of spliced mRNAs and lariat products, and a concomitant increase in the
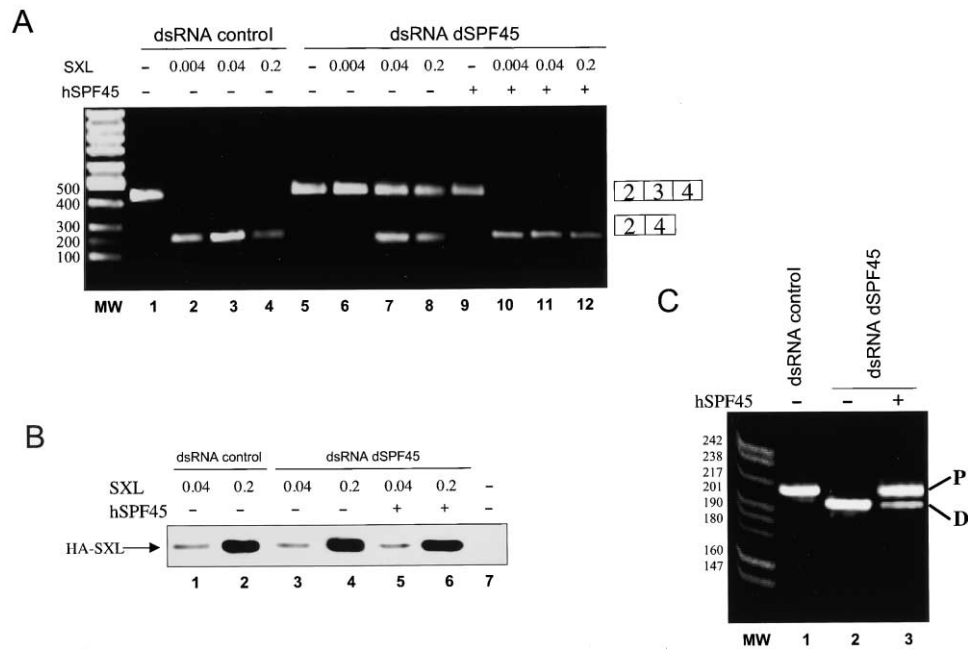
Figure 3. RNA Interference of SPF45 Compromises *Sxl* Autorregulation

(A) RT-PCR analyses of RNA isolated from Schneider cells transiently transfected with the pRmHA-234 Sxl genomic reporter and increasing concentrations (indicated in μg per 60 mm tissue culture plate) of the SXL expression plasmid pRmHA-SXL in the presence of dsRNA corresponding to dSPF45 or control dsRNA. The sizes of the products of amplification corresponding to exon 3 inclusion (2-3-4) or skipping (2-4) are indicated at the right. Cotransfection with an expression vector for human SPF45 is indicated by +. RNAs were isolated 72 hr posttransfection.

(B) Expression of HA-tagged SXL in the experiment shown in (A). Extracts from transfected cells were fractionated on SDS polyacrylamide gels, and HA-tagged SXL expression was analyzed by Western blot using anti-HA antibodies.

(C) Relative use of *Sxl* exon 3 3′ ss in Schneider cells transiently transfected with a *Sxl* reporter and dsRNA corresponding to dSPF45 or a control gene, with or without cotransfection with an expression vector for human SPF45. RT-PCR was performed using primers corresponding to exons 2 and 3 that should generate products of amplification differing by 18 bp, depending on whether the proximal or the distal 3′ splice site is used. Minigene TE234 were cotransfected in SL2 cells with 15 μg of dsRNA control or dsRNA of dSPF45 in the absence or presence of an expression vector for human SPF45.

levels of intermediates generated by the first step of splicing (Figure 4B). These effects could be partially reversed by addition of recombinant purified hSPF45 to the depleted extracts (compare lanes 3 and 4). SPF45 depletion affected utilization of the proximal but not the distal 3′ ss. Moreover, SPF45 depletion had little or no effect on splicing of the mutant A3S-mutP RNA, in which the proximal site was mutated from AG to CA (A3S-mutP) (Figure 4C). Together, these findings suggest that the proximal and distal 3′ ss show different requirements for SPF45.

SXL Inhibits A3S Splicing at the Second Catalytic Step

To investigate the possibility that SXL regulates selection of the 3′ ss at a step related to SPF45 activity, a derivative of A3S RNA was generated in which a well-characterized SXL binding site (Handa et al., 1999) was introduced both upstream and downstream of the 3′ ss region (Figure 5A, A3SU). This RNA maintained both the preferential use of the proximal 3′ ss (Figure 5B, lane 2), as well as the activation of the distal 3′ ss upon mutation of the proximal site (Figure 5B, lane 6). SXL had no effect on A3S splicing (data not shown), consistent with the expectation that splicing regulation by SXL

requires binding sites for the protein within the pre-mRNA. However, addition of SXL to splicing reactions containing A3SU RNA caused inhibition of the second step of splicing and accumulation of the first exon and lariat intermediate (Figure 5B, compare lanes 2 and 3). Importantly, SXL had no effect on splicing of the A3SU-mutP RNA, which contains SXL binding sites but lacks the proximal 3′ ss (Figure 5B, compare lanes 6 and 7). This suggests that the A3SU RNA was made refractory to SXL inhibition by preventing recognition of the proximal 3′ ss by SPF45.

We conclude that SXL inhibits A3SU splicing at a late step in the splicing process and that inhibition requires activation of the proximal 3′ ss by SPF45.

Mechanism of Regulation

To test whether SXL inhibited the association of SPF45 with the proximal 3′ ss, crosslinking and immunoprecipitation experiments were carried out essentially as in Figure 2D. The results indicate that inhibition of A3SU splicing by SXL was not related to a block of SPF45 interaction with the pre-mRNA (Figure 5C, compare lanes 1 and 2). Next we considered the possibility that SXL and SPF45 interact directly with each other. In vitro translated SPF45 was coprecipitated with recombinant
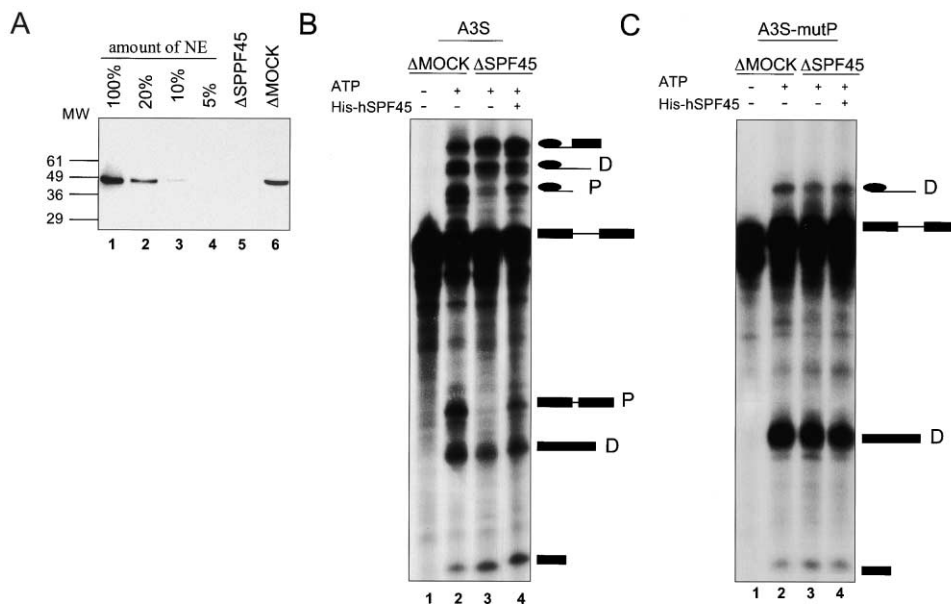
Figure 4. SPF45 Modulates the Second Catalytic Step

(A) Extent of SPF45 depletion from HeLa nuclear extracts. Western blot analyses of the levels of SPF45 in serial dilutions of nuclear extracts (NE), SPF45 immunodepleted extracts (ΔSPF45), or mock-depleted extracts (ΔMOCK). MW indicates position of molecular weight markers.

(B) Effects of SPF45 immunodepletion on splicing of A3S pre-mRNA. A3S RNA was incubated in mock-depleted extracts (ΔMOCK) or SPF45 immunodepleted extracts (ΔSPF45) in the absence or presence of ATP and in the absence or presence of recombinant purified His-hSPF45 (100 ng/μl) as indicated. RNAs were then purified and fractionated on 13% polyacrylamide gels. Positions of splicing intermediates and products are indicated on the right.

(C) Effects of SPF45 immunodepletion on splicing of A3S-mutP pre-mRNA. Splicing assays were carried out as in (B) using A3S-mutP as substrate.

purified GST-SXL using glutathione-agarose beads (Figure 5D, lane 5). To map the domains involved in the interaction (schematically represented at the top of Figure 5D), GST pull-down experiments were carried out using (1) GST-SXL and derivatives containing either only its amino-terminal glycine-asparagine-rich 94 amino acids (GST-GN) or lacking this region of the protein [GST-SXL(ΔGN)] and (2) in vitro translated SPF45 or mutants deleting the 196 amino-terminal amino acids, the central DIII domain (including a G-Patch motif), or the carboxy-terminal RRM. Only GST-SXL and GST-GN coprecipitated SPF45 as well as its amino-terminal domain (lanes 5 and 6 and 13 and 14), indicating that SXL and SPF45 interact through their amino termini.

Previous work has shown that the amino-terminal domain of SXL is important for *Sxl* autoregulation in vivo (Wang and Bell, 1994). Guided by these in vivo results and by the interaction between SXL and SPF45 described above, the activity of a SXL derivative lacking the 94 amino-terminal amino acids was tested in in vitro splicing assays. This mutant was unable to inhibit the second step of A3SU RNA splicing (Figure 5B, lane 4), thus recapitulating one additional property of *Sxl* autoregulation as observed in vivo. Crosslinking and precipitation of GST-SXL and GST-SXL(ΔGN) from the same reaction mixtures suggested that the two proteins bound similarly to the A3SU RNA (Figure 5E, compare lanes 2–4 with lanes 5–7). These results establish a correlation between the ability of SXL to interact with SPF45 and its ability to inhibit the second catalytic step. Taken together, the data are consistent with a model in which SXL inhibits splicing of SPF45-dependent substrates by

directly interacting with its amino-terminal region and thereby blocking its activity as a second step splicing factor.

SPF45 and Activation of the β[110] Thalassemic Mutation

The arrangement of 3′ ss in *Sxl* exon 3 is reminiscent of that created in β-globin pre-mRNAs by the β-thalassemic mutation β[110]. β[110] is a guanine to adenine substitution that generates a cryptic 3′ ss AG upstream from the normal 3′ ss of β-globin intron 1 (Figure 6A). Activation of the cryptic site results in a change in reading frame and therefore in an mRNA that cannot code for globin, thus giving rise to the β-thalassemic condition. Previous work indicated that activation of the cryptic site was dependent upon the integrity of the natural 3′ ss (Krainer et al., 1985; Zhuang and Weiner, 1990), similar to the dependence on the distal site for proximal site activation in *Sxl* exon 3. Given the similarities between the two systems, we hypothesized that SPF45 could be involved in activation of the β[110] cryptic 3′ ss. To test this possibility, we made use of the cell culture system described in Figure 3, where SPF45 activity could be efficiently depleted and this effect reversed by overexpression of human SPF45. Minigenes containing β-globin intron 1, with or without mutation β[110], were transfected into Schneider cells that had been cotransfected with either SPF45 dsRNA or control dsRNA. The relative use of the normal and cryptic 3′ ss was determined by RT-PCR using primers designed to allow discrimination between the use of each of the sites. The natural 3′ ss was used in cells transfected with the wild-type minigene and control
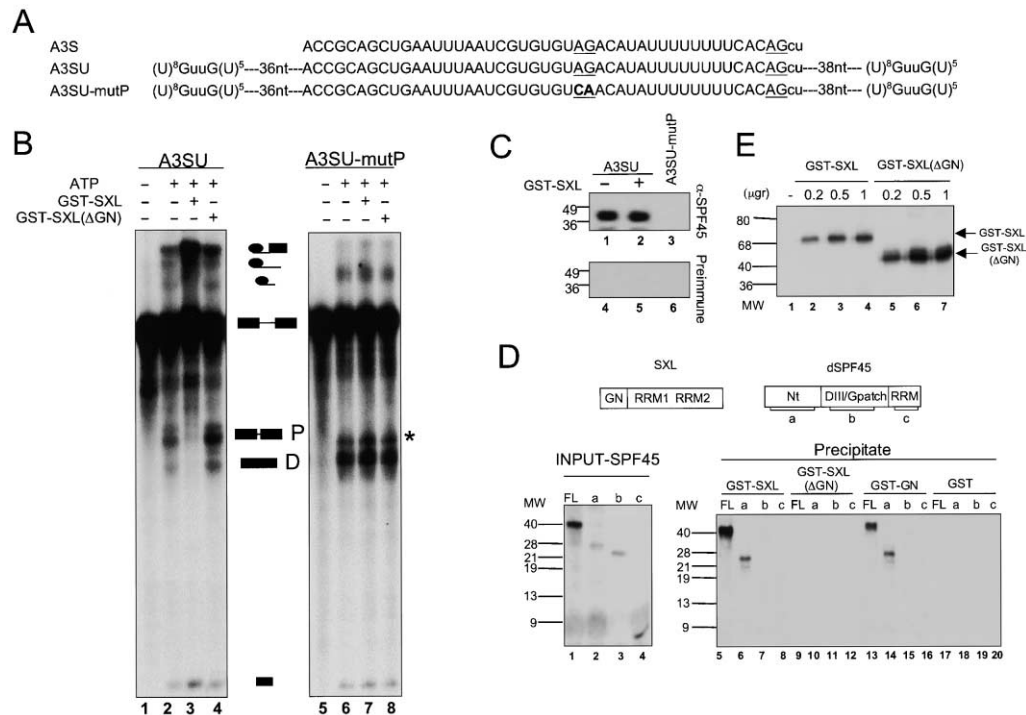
Figure 5. SXL Inhibits the Second Step of A3SU Splicing

(A) Nucleotide sequence of the 3′ end of A3SU and A3SU-mutP transcripts.

(B) In vitro splicing of AS3U and AS3U-mutP. Splicing assays were carried out in the absence or presence of ATP and recombinant purified GST-SXL (100 ng/μl) or GST-SXL(ΔGN) (200 ng/μl) as indicated. RNAs were fractionated on 13% polyacrylamide gels. Positions of splicing intermediates and products are indicated. The RNA species labeled with an asterisk most likely corresponds to the debranched linear form of the intron.

(C) Crosslinking of SPF45 to A3SU and A3SU-mutP RNAs. Reactions set up as in (B) were irradiated with UV light, and immunoprecipitation assays were carried out as in Figure 2D.

(D) Interaction between SXL and SPF45. Top: Schematic representation of the domain organization of SXL and dSPF45. a, b, and c correspond to the amino-terminal, DIII/Glycine patch and RRM domains of SPF45, respectively. Bottom left: Products of in vitro translation of ^{35}S-labeled full-length (FL) or deletion mutants of dSPF45, fractionated on SDS-polyacrylamide gels. Bottom right: Coprecipitation of in vitro translated dSPF45 and deletion mutants with various GST fusion proteins using glutathione-agarose beads. GST-GN contains SXL amino acids 1–94, while GST-SXL(ΔGN) lacks these residues compared to full-length GST-SXL.

(E) Crosslinking of GST-SXL and GST-SXL(ΔGN) to A3SU RNA. Reactions set up as in (B) with the indicated amounts of GST-SXL and GST-SXL(ΔGN) were irradiated with UV light, and the GST fusions were precipitated using glutathione-agarose beads. The precipitates were fractionated on SDS-polyacrylamide gels and autoradiographed.

dsRNA (Figure 6B, lane 1), while the cryptic 3′ ss was used in cells transfected with the minigene containing the β[110] mutation (lane 2). These results indicate that splice site selection for the β-globin constructs in Schneider cells recapitulated that reported for the endogenous gene in human cells. Consistent with an involvement of SPF45 in cryptic splice site activation, cotransfection with dSPF45 dsRNA resulted in partial activation of the normal 3′ ss (lane 3). This effect could be reversed by overexpression of hSPF45 (lane 4).

Taken together, the results suggest a common molecular basis for the switch in 3′ ss utilization observed in *Sxl* exon 3 and for the activation of a cryptic 3′ ss in β-globin by the β[110] mutation. In both cases, SPF45 plays a role in the utilization of an upstream 3′ ss AG.

Discussion

Previous work indicated that while the Py-tract and distal AG are important for *Sxl* exon 3 definition, a proximal AG is utilized as 3′ ss during catalysis. Use of the proxi-

mal site is important for regulation of exon skipping by the SXL protein. In this study we have investigated the molecular mechanisms behind these observations using a combination of in vitro assays and RNA interference in *Drosophila* Schneider cells. We provide evidence that the two subunits of U2AF bind to the Py-tract and distal AG to facilitate spliceosome assembly, and we identify the spliceosomal protein SPF45 as a second step splicing factor important for both recognition and utilization of the proximal 3′ ss of *Sxl* exon 3. The activation function of SPF45 during the second catalytic step can be inhibited by binding to SXL, thereby providing an unanticipated mechanism for regulation of splice site utilization.

SPF45 Is a Second Step Splicing Factor

The amino acid sequence and domain organization of SPF45 is different from other second step factors identified so far. SPF45 contains a carboxy-terminal RNA recognition motif (RRM) that is most closely related to that found in U2AF[35] (Birney et al., 1993). As both U2AF[35] and

A

β-globin   AUAGGCACUGACUCUCUCUGCCUAUUGGUCUAUUUUCCCUU**AG**gcu
β-globin 10, β¹¹⁰   AUAGGCACUGACUCUCUCUGCCUAUU**AG**UCUAUUUUCCCUU**AG**gcu
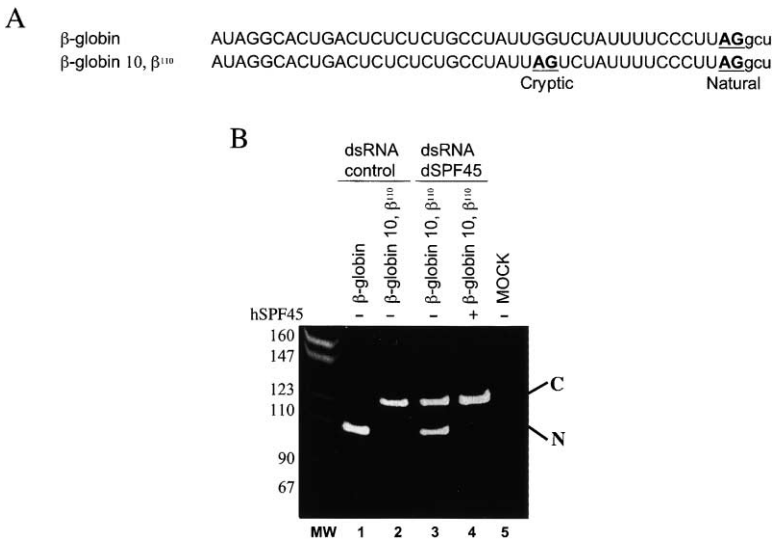   Cryptic   Natural

B

Figure 6. SPF45 Is Involved in Activation of a Cryptic 3′ ss in the β Thalassemic Mutation β¹¹⁰

(A) Nucleotide sequence of the 3′ ss region of the first intron in human β-globin and in the β-thalassemic mutation β¹¹⁰. Cryptic and natural 3′ ss are indicated.
(B) Analysis of 3′ ss AG utilization. RT-PCR analysis using primers corresponding to β-globin exons 1 and 2 predicted to generate amplification products that differ by 18 nucleotides depending on whether the natural or cryptic 3′ ss AG were utilized. The positions of the corresponding products are indicated. RNAs used in the RT-PCR reactions were purified from Schneider cells transfected with a wild-type β-globin minigene or the β¹¹⁰ mutant, in the presence of dsRNA corresponding to SPF45 or a control dsRNA, as indicated. Cotransfection with an expression vector for human SPF45 (+) or empty vector (−) is also indicated. Abbreviations: MOCK, RNA from untransfected cells; MW, positions of the molecular weight markers.

SPF45 contact 3′ ss AGs, it is conceivable that their rather unusual RRMs are domains specialized in 3′ ss AG recognition. In this context it is noteworthy that a U2AF³⁵-related protein, URP, was found to play a role in the second catalytic step in human nuclear extracts (Tronchére et al., 1997). The existence of multiple factors recognizing the 3′ ss AG may be required to increase the accuracy of exon ligation. Consistent with this idea, depletion of the human second step factor SLU7p results in catalytic activation of incorrect AGs (Chua and Reed, 1999b). Similarly, SPF45 binds to and directs catalytic activation of the proximal 3′ ss AG (Figure 7A), while either absence of SPF45 or mutation of the proximal site results in use of the distal site (Figures 7B and 7C).

Several models have been proposed for how the 3′ ss AG is specified after the first catalytic step, including (1) scanning of the first AG from the branchpoint (Smith et al., 1989, 1993; Chen et al., 2000), (2) optimal distance to the branchpoint, and (3) a combination of distance constraints and competition between 3′ ss of different strengths (Brys and Schwer, 1996; Luukkonen and Séraphin, 1997; Chiara et al., 1997; Chua and Reed, 2001). SPF45 could be an important component of these scanning/measuring mechanisms.
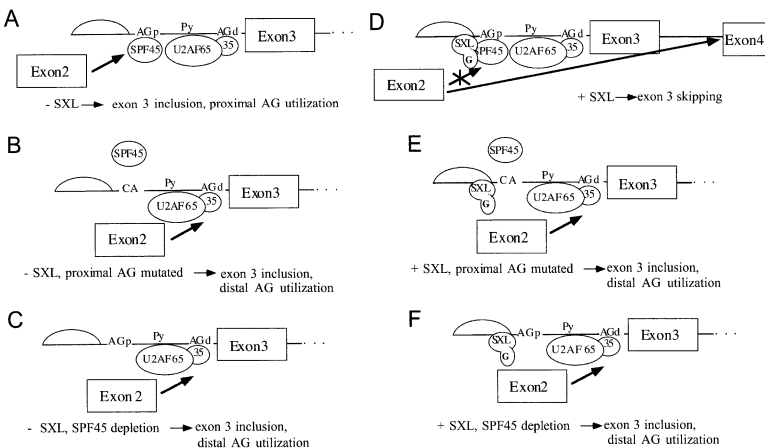

Figure 7. Model for SPF45 Function and Regulation by SXL

(A) Schematic representation of *Sxl* pre-mRNA in which intron 2 has undergone the first catalytic step of splicing, leading to accumulation of free exon 2 and intron 2 in lariat configuration. The position of the proximal (p) and distal (d) 3′ ss AG, Py-tract (Py), and binding sites for the U2AF subunits and SPF45 at the 3′ end of intron 2 are indicated. The arrow represents the second catalytic step of splicing occurring between exon 2 and the proximal AG, an event promoted by SPF45.
(B) Mutation of the proximal AG prevents interaction with SPF45 and leads to exon 3 inclusion utilizing the distal AG.
(C) Depletion of SPF45 prevents utilization of the proximal AG, which—as in (B)—leads to exon 3 inclusion utilizing the distal AG.
(D) Interaction between the glycine-rich (G) domain of SXL and SPF45 interferes with the activity of SPF45 that promotes utilization of the proximal AG during the second catalytic step (crossed arrow). The distal site is not activated in the presence of SXL, suggesting that SPF45 bound to SXL is still capable of selecting the proximal over the distal site, but fails to activate it for catalysis. As a result, exon 2 is spliced to exon 4, and exon 3 is skipped from the mature mRNA. The intron preceding exon 4 may have undergone the first catalytic step and be in lariat configuration at the time of exon 2-exon 4 ligation (not represented). SXL binding is represented for simplicity as a single binding site in the Figure, but multiple uridine-rich sequences within introns 2 and 3 contribute to *Sxl* autoregulation (Sakamoto et al., 1992).
(E) Mutation of the proximal site prevents interaction with SPF45 and disrupts SXL-mediated exon skipping, because SXL cannot prevent splicing between exon 2 and the distal 3′ ss AG.
(F) Depletion of SPF45 results in utilization of the distal 3′ ss for exon 3 inclusion. As in (E), SXL cannot inhibit splicing between exon 2 and the distal 3′ ss AG, and therefore the protein fails to promote exon 3 skipping.

Interaction of SPF45 with the proximal 3′ ss required recognition of the distal Py-tract/3′ ss by U2AF[65/35]. As SPF45 was detected in immunoprecipitates of U2AF washed under mild conditions (P. Förch, A. Bachi, M. Wilms, and J.V., unpublished observations), SPF45 may be delivered to the proximal 3′ ss region through association with complexes containing U2AF.

Here we show that different 3′ ss have different requirements for SPF45. For example, biochemical depletion of SPF45 compromised splicing of the A3S substrate but not of the A3S-mutP RNA (Figures 4B and 4C). Similarly, in vivo depletion of SPF45 levels by RNA interference reduced utilization of the proximal site in *Sxl* or of the cryptic 3′ ss in β-globin, while it did not compromise the use of the distal site in Sxl, nor the use of the 3′ ss in the downstream intron 3, nor the use of the wild-type 3′ ss in β-globin (Figures 3C and 6). We cannot completely rule out, however, that more extensive in vivo or in vitro depletion than achievable using the currently available reagents would not reveal a more general requirement for SPF45 in splicing.

SPF45 and Sxl Autoregulation

Figure 7 presents a model for *Sxl* autoregulation and the involvement of SPF45 in this process. As mentioned above, SPF45 interaction with the proximal AG facilitates proximal site utilization during the second catalytic step (Figure 7A). Interaction of SPF45 requires early recognition of the Py-tract and distal AG by the two subunits of U2AF. Either mutation of the proximal AG (Figure 7B) or depletion of SPF45 (Figure 7C) led to utilization of the distal site, without compromising exon 3 inclusion. In the presence of SXL, however, exon 3 is skipped (Figure 7D), and two lines of evidence indicate that this effect of SXL requires the interaction between SPF45 and the proximal AG. First, mutation of the proximal AG prevents SPF45 interaction, and in this situation exon 3 is included using the distal site, even in the presence of SXL (Figure 7E). This suggests that SXL bound to the pre-mRNA cannot prevent splicing of exon 2 to the distal 3′ ss AG (Figure 7E). Similarly, depletion of SPF45 also prevents proximal site utilization, and again splicing of exon 2 to the distal site is not inhibited by SXL (Figure 7F). Taken together, the data suggest that SXL causes exon 3 skipping by inhibiting the function of SPF45 as a second step splicing factor that promotes utilization of the proximal site for exon ligation (Figure 7D).

SXL does not prevent SPF45 from binding to the proximal 3′ ss. Instead, the two proteins interact through their N termini. In this way, SXL inhibits SPF45 function (Figure 7D). As the distal site is not activated in the presence of SXL, SPF45 bound to SXL is still capable of selecting the proximal over the distal site, but fails to activate it for catalysis. These observations, therefore, suggest three distinct functions for SPF45: specific recognition of the proximal 3′ ss, commitment of this site to splicing, and finally catalytic activation during the second step. The amino-terminal domain of SXL is important for *Sxl* autoregulation in vivo (Wang and Bell, 1994) and is essential for SXL to repress the second catalytic step (Figure 5B). The domain is, however, dispensable for RNA binding in our assays (Figure 5E) and for regulating splicing in vitro of other substrates in

which SXL acts at early stages of spliceosome assembly (Granadino et al., 1997, and data not shown). We conclude that SXL can regulate splicing of different genes by distinct mechanisms that have different domain requirements and that target either early or late steps in the splicing process.

Traditional models of splicing regulation target early stages of splice site recognition (Smith and Valcárcel, 2000). What could be the rationale for the selection of a mechanism of regulation that targets the second catalytic step? Male flies have a strong requirement for efficient inclusion of exon 3. Even low levels of exon skipping in the absence of SXL could result in accumulation of SXL-encoding mRNAs that could switch on the autoregulation process and establish SXL expression. In other words, the process of exon definition must be very efficient to keep a tight control over sex identity and viability. This may impose, however, serious mechanistic problems to regulators attempting to interfere with early steps of spliceosome assembly. Inhibition of the second catalytic step may allow SXL to effectively promote skipping of exon 3 after it has been recognized as an exon but before it is included in the spliced product. Dual recognition of the 3′ ss and dependence on SPF45 may provide a particularly favorable opportunity for regulation at a late step of the splicing process. Consistent with a role for SPF45 in Sxl autoregulation in vivo, initial experiments indicate that microinjection of SPF45 dsRNA, but not of control dsRNA, compromises the viability of female embryos (data not shown), which is the characteristic phenotype of *Sxl* loss of function mutations (reviewed by Cline and Meyer, 1996).

We are aware of two other pre-mRNAs where a downstream AG (which under certain circumstances can function as a 3′ ss) is required for catalytic activation of an upstream 3′ ss AG. One occurs in the polyoma virus early transcriptional unit, which gives rise to middle T or small t antigens depending on which AG of the pair is used (Ge et al., 1990). It is likely that mechanisms exist to modulate the extent of activation of each 3′ ss during viral infection. The other example is the activation of a cryptic site in β-globin by the β-thalassemic mutation β[110] (Krainer et al., 1985; Zhuang and Weiner, 1990). We showed here that interference with SPF45 function in Schneider cells resulted in partial inhibition of cryptic site activation and concomitant accumulation of correctly spliced transcripts. These results raise the interesting possibility that SPF45 could be used as a therapeutic target for treating at least one class of β-thalassemias. Finally, these results strongly suggest that the mechanism of cryptic splice site activation is likely to be similar to that observed for the activation of the proximal 3′ ss in *Sxl* exon 3, implying a more general role for SPF45 in splicing control.

Experimental Procedures

Expression of SPF45 and Antibody Production

Human SPF45 cDNA (Neubauer et al., 1998) was amplified from a human bone marrow library (Clontech) and cloned in the *Drosophila* cupper-inducible expression vector pRmHa and in the prokaryotic expression vector pET (Novagen). Human His-SPF45 was expressed in BL21 *E. coli* by inducing cultures (at OD 0.6) of cells transformed with the pRSET-SPF45 plasmid with 1 mM IPTG for 3 hr at 30°C

and purification under nondenaturing conditions by affinity chromatography on nickel beads (Talon, Clontech).

Homology searches in FlyBase identified the *Drosophila melanogaster* gene AE002927 as the putative homolog of SPF45. dSPF45 cDNA was obtained by cloning a product of PCR amplification from an embryo stage V cDNA library (Clontech) using oligonucleotides corresponding to the 5′ and 3′ ends of SPF45 ORF.

Antibodies were raised (EUROGENTECH, Belgium) in rabbits against a peptide with the sequence NH2-CPYEEDSRPRSQSSKA-COOH coupled to keyhole limpet hemacyanine or in sheep against the full-length protein (Scottish Antibody Production Unit).

Splicing Substrates and Reporters

A3S was generated by replacing the sequence CAUACUUAUCCU GUCCCUUUUUUUUCCACAG from the intron 1 of adenovirus major late transcripts in plasmid pMINX (Zillmann et al., 1988) by the sequence of Sxl intron 2 indicated in Figure 1A. Mutant derivatives were generated from this construct by PCR-based site-directed mutagenesis and confirmed by sequencing. pRmHA-TE234 and pRmHA-SXL were described in Penalva et al. (2001). pRmHa-β globin and pRmHa-β110 were generated by cloning a fragment of β globin (or its β-thalassemic variant β110) first intron and flanking exons into the copper-inducible expression plasmid pRmHA.

In Vitro Splicing Assays

HeLa nuclear extracts were prepared according to Dignam et al. (1983) or purchased from the Computer Cells Culture Center (Belgium). Site-specific labeling of pre-mRNAs was carried out as described by Moore and Sharp (1992). In vitro transcription of pre-mRNAs substrates, splicing, and spliceosome assembly assays were as described (Gebauer et al., 1998; Merendino et al., 1999). When appropriate, GST-SXL or GST-SXL(ΔGN) were preincubated with the pre-mRNA for 15 min at 30°C.

Crosslinking/Immunoprecipitation

Crosslinking and immunoprecipitation assays were performed as described in Merendino et al. (1999) on 27 μl splicing mixtures using 35 μl of anti-U2AF65 MC3 monoclonal antibody (Gama-Carvalho et al., 1997) or anti-HA monoclonal antibody control (ATCC), 20 μl of anti-U2AF35 polyclonal antiserum (Zuo and Maniatis, 1996) or a preimmune serum, and 20 μl of SPF45 antipeptide antibody or the corresponding preimmune serum. Precipitation assays of His-SPF45 were carried out using 5 μl of nickel beads (Talon resin, Clontech), and assays of GST-SXL or GST-SXL(ΔGN) used 10 μl of glutathione-agarose beads (Sigma). Precipitates were washed with 500 mM NaCl (for U2AF65, GST-SXL, or GST-SXL(ΔGN)) or 100 mM NaCl (for U2AF35 and SPF45) buffers, and the bound proteins were fractionated on SDS-10% polyacrylamide gels.

In Vitro Translation and GST Pull-Downs

In vitro transcription/translation was carried out using rabbit reticulocyte extracts (TNT, Promega), and templates were generated by PCR using appropriate primers containing a T7 promoter. GST pull-down assays were carried out in 20 μl containing 100 ng/μl GST fusion proteins and 2 μl in vitro translation products. After incubation on ice for 30 min in buffer D 0.1 M KCl, the following were added: 10 μl glutathione-agarose beads (Sigma) and 20 μl of a solution (150 mM NaCl, 10 mM Tris [pH 8.0], 0.1% NP40). The mixture incubated for one hour at 4°C on a rotating wheel. After that, 10 μg of RNase A were added and incubation at 37°C continued for 1 hr. The beads were sedimented and washed five times with 0.5 M NaCl, 10 mM Tris (pH 8.0), and 0.1% NP-40. Pellets were eluted with loading dye and fractionated on SDS-polyacrylamide gels.

Immunodepletion of SPF45 from HeLa Nuclear Extracts

Anti-SPF45 antiserum (1.5 mg) or the corresponding preimmune serum were coupled to 200 μl of protein G sepharose beads by dimethylpimelimidate (DMP)-mediated crosslinking. HeLa nuclear extracts (400 μl) adjusted to 1 M KCl were incubated with the beads for 2 hr at 4°C on a rotating wheel. The depleted extract was separated from the beads by centrifugation and dialyzed against 0.1 M KCl buffer D (Dignam et al., 1983).

Transfection and RNA Interference

Transfection of Schneider cells, RNA isolation, and RT-PCR analyses were as described previously (Penalva et al., 2001). RNA interference was achieved using 6 μg/ml of double-stranded RNA generated by in vitro transcription using T7 RNA polymerase and PCR-generated templates containing T7 promoters at both ends of the sequences corresponding to GFP (entire ORF, 717 bp) or SPF45 (ORF nucleotides 1–605). The products of transcription were heated at 65°C for 30 min and kept at room temperature for 1 hr before addition to the cultures at the time of Lipofectin-mediated transfection.

3′ ss Usage Analyses

We analyzed 15 μg of total RNA from cells transfected with pRmHa-234 or pRmHa-β/β110 globin plasmids by RT-PCR using the following primer pairs: GGTTGCTTTGCGTTACAAAAA (antisense exon 3) and AATAATAATGGTGGTTATCC (sense exon 2) for Sxl RNAs; TCCAGG GTAGACCACCAG (antisense exon 2) and TGCACCTGACTCCT GAGGA (sense exon 1) for β-globin RNAs. PCR reactions were amplified for 25 cycles of 1 min at 94°C, 1 min at 60°C, and 30 s at 72°C, followed by a final extension of 10 min at 72°C. The products of amplification were analyzed by electrophoresis on nondenaturing 6% polyacrylamide gels.

References

Ajuh, P., Kuster, B., Panov, K., Zomerdijk, J.C., Mann, M., and Lamond, A.I. (2000). Functional analysis of the human CDC5L complex and identification of its components by mass spectrometry. EMBO J. *19*, 6569–6581.

Ansari, A., and Schwer, B. (1995). SLU7 and a novel activity, SSF1, act during the PRP16-dependent step of yeast pre-mRNA splicing. EMBO J. *14*, 4001–4009.

Bell, L.R., Horabin, J.I., Schedl, P., and Cline, T.W. (1991). Positive autoregulation of sex-lethal by alternative splicing maintains the female determined state in *Drosophila*. Cell *65*, 229–239.

Birney, E., Kumar, S., and Krainer, A.R. (1993). Analysis of the RNA-recognition motif and RS and RGG domains: conservation in metazoan pre-mRNA splicing factors. Nucleic Acids Res. *21*, 5803–5816.

Brys, A., and Schwer, B. (1996). Requirement for SLU7 in yeast pre-mRNA splicing is dictated by the distance between the branchpoint and the 3′ splice site. RNA *2*, 707–717.

Chen, S., Anderson, K., and Moore, M.J. (2000). Evidence for a linear search in bimolecular 3′ splice site AG selection. Proc. Natl. Acad. Sci. USA *97*, 593–598.

Chiara, M.D., Palandjian, L., Feld Kramer, R., and Reed, R. (1997). Evidence that U5 snRNP recognizes the 3′ splice site for catalytic step II in mammals. EMBO J. *16*, 4746–4759.

Chua, K., and Reed, R. (1999a). The RNA splicing factor hSlu7 is required for correct 3′ splice site choice. Nature *402*, 207–210.

Chua, K., and Reed, R. (1999b). Human step II splicing factor hSlu7 functions in restructuring the spliceosome between the catalytic steps of splicing. Genes Dev. *13*, 841–880.

Chua, K., and Reed, R. (2001). An upstream AG determines whether

a downstream AG is selected during catalytic step II of splicing. Mol. Cell. Biol. *21*, 1509–1514.

Cline, T.W., and Meyer, B.J. (1996). Vive la difference: males vs females in flies vs worms. Annu. Rev. Genet. *30*, 637–702.

Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. *11*, 1475–1489.

Frank, D., and Guthrie, C. (1992). An essential splicing factor, SLU7, mediates 3′ splice site choice in yeast. Genes Dev. *6*, 2112–2124.

Förch, P., Merendino, L., Martinez, C., and Valcárcel, J. (2001). Modulation of msl-2 5′ splice site recognition by Sex-lethal. RNA *7*, 1185–1191.

Gama-Carvalho, M., Krauss, R.D., Chiang, L., Valcárcel, J., Green, M.R., and Carmo-Fonseca, M. (1997). Targeting of U2AF65 to sites of active splicing in the nucleus. J. Cell Biol. *137*, 975–987.

Ge, H., Noble, J., Colgan, J., and Manley, J.L. (1990). Polyoma virus small tumor antigen pre-mRNA splicing requires cooperation between two 3′ splice sites. Proc. Natl. Acad. Sci. USA *87*, 3338–3342.

Gebauer, F., Merendino, L., Hentze, M.W., and Valcárcel, J. (1998). The *Drosophila* splicing regulator sex-lethal directly inhibits translation of male-specific-lethal 2 mRNA. RNA *4*, 142–150.

Gozani, O., Patton, J.G., and Reed, R. (1994). A novel set of spliceosome-associated proteins and the essential splicing factor PSF bind stably to pre-mRNA prior to catalytic step II of the splicing reaction. EMBO J. *13*, 3356–3367.

Granadino, B., Penalva, L.O., Green, M.R., Valcárcel, J., and Sanchez, L. (1997). Distinct mechanisms of splicing regulation in vivo by the *Drosophila* protein Sex-lethal. Proc. Natl. Acad. Sci. USA *94*, 7343–7348.

Graveley, B.R. (2001). Alternative splicing: increasing diversity in the proteomic world. Trends Genet. *17*, 100–107.

Handa, N., Nureki, O., Kurimoto, K., Kim, I., Sakamoto, H., Shimura, Y., Muto, Y., and Yokoyama, S. (1999). Structural basis for recognition of the *tra* mRNA precursor by the Sex-lethal protein. Nature *398*, 579–585.

Hastings, M.L., and Krainer, A.R. (2001). Pre-mRNA splicing in the new millennium. Curr. Opin. Cell Biol. *13*, 302–309.

Horabin, J.I., and Schedl, P. (1993). Sex-lethal autoregulation requires multiple cis-acting elements upstream and downstream of the male exon and appears to depend largely on controlling the use of the male exon 5′ splice site. Mol. Cell. Biol. *13*, 7734–7746.

Inoue, K., Hoshijima, K., Sakamoto, H., and Shimura, Y. (1990). Binding of the *Drosophila* sex-lethal gene product to the alternative splice site of transformer primary transcript. Nature *344*, 461–463.

Jones, M.H., Frank, D.N., and Guthrie, C. (1995). Characterization and functional ordering of Slu7p and Prp17p during the second step of pre-mRNA splicing in yeast. Proc. Natl. Acad. Sci. USA *92*, 9687–9691.

Kelley, R.L., Solovyeva, I., Lyman, L.M., Richman, R., Solovyev, V., and Kuroda, M.I. (1995). Expression of msl-2 causes assembly of dosage compensation regulators on the X chromosomes and female lethality in *Drosophila*. Cell *81*, 867–877.

Krainer, A.R., Reed, R., and Maniatis, T. (1985). Mechanisms of human-globin pre-mRNA splicing. In Genetic Chemistry: The Molecular Basis of Heredity. The Robert A. Welch Foundation Conferences on Chemical Research, Volume XXIX, P. Berg, ed. (Houston, TX: Welch Foundation), pp. 353–382.

Luukkonen, B.G., and Séraphin, B. (1997). The role of branchpoint-3′ splice site spacing and interaction between intron terminal nucleotides in 3′ splice site selection in *Saccharomyces cerevisiae*. EMBO J. *17*, 779–792.

Merendino, L., Guth, S., Bilbao, D., Martinez, C., and Valcárcel, J. (1999). Inhibition of msl-2 splicing by Sex-lethal reveals interaction between U2AF35 and the 3′ splice site AG. Nature *402*, 838–841.

Moore, M.J. (2000). Intron recognition comes of AGe. Nat. Struct. Biol. *7*, 14–16.

Moore, M.J., and Sharp, P.A. (1992). Site-specific modification of pre-mRNA: the 2′-hydroxyl groups at the splice sites. Science *256*, 992–997.

Neubauer, G., King, A., Rappsilber, J., Calvio, C., Watson, M., Ajuh, P., Sleeman, J., Lamond, A., and Mann, M. (1998). Mass spectrometry and EST-database searching allows characterization of the multiprotein spliceosome complex. Nat. Genet. *20*, 46–50.

Penalva, L.O., Lallena, M.J., and Valcárcel, J. (2001). Switch in 3′ splice site recognition between exon definition and splicing catalysis is important for sex-lethal autoregulation. Mol. Cell. Biol. *21*, 1986–1996.

Reed, R. (1989). The organization of 3′ splice sequences in mammalian introns. Genes Dev. *3*, 2113–2123.

Ruskin, B., Pikielny, C.W., Rosbash, M., and Green, M.R. (1986). Alternative branch points are selected during splicing of a yeast pre-mRNA in mammalian and yeast extracts. Proc. Natl. Acad. Sci. USA *83*, 2022–2026.

Sakamoto, H., Inoue, K., Higuchi, I., Ono, Y., and Shimura, Y. (1992). Control of *Drosophila* Sex-lethal pre-mRNA splicing by its own female-specific product. Nucleic Acids Res. *20*, 5533–5540.

Sawa, H., and Shimura, Y. (1991). Alterations of RNase H sensitivity of the 3′ splice site region during the in vitro splicing reaction. Nucleic Acids Res. *19*, 3953–3958.

Schwer, B., and Guthrie, C. (1991). PRP16 is an RNA-dependent ATPase that interacts transiently with the spliceosome. Nature *349*, 494–499.

Schwer, B., and Guthrie, C. (1992). A conformational rearrangement in the spliceosome is dependent on PRP16 and ATP hydrolysis. EMBO J. *11*, 5033–5039.

Schütt, C., and Nöthiger, R. (2000). Structure, function and evolution of sex-determining systems in Dipteran insects. Development *127*, 667–677.

Smith, C.W., and Valcárcel, J. (2000). Alternative pre-mRNA splicing: the logic of combinatorial control. Trends Biochem. Sci. *25*, 381–388.

Smith, C.W.J., Porro, E.B., Patton, J.G., and Nadal-Ginard, B. (1989). Scanning from an independently specified branch point defines the 3′ splice site of mammalian introns. Nature *342*, 243–247.

Smith, C.W.J., Chu, T.T., and Nadal-Ginard, B. (1993). Scanning and competition between AGs are involved in 3′ splice site selection in mammalian introns. Mol. Cell. Biol. *13*, 4939–4952.

Sosnowski, B.A., Belote, J.M., and McKeown, M. (1989). Sex-specific alternative splicing of RNA from the *transformer* gene results from sequence-dependent splice site blockage. Cell *58*, 449–459.

Tronchére, H., Wang, J., and Fu, X.D. (1997). A protein related to splicing factor U2AF35 that interacts with U2AF65 and SR proteins in splicing of pre-mRNA. Nature *388*, 397–400.

Umen, J.G., and Guthrie, C. (1995a). The second catalytic step of pre-mRNA splicing. RNA *1*, 869–885.

Umen, J.G., and Guthrie, C. (1995b). Prp16p, Slu7p, and Prp8p interact with the 3′ splice site in two distinct stages during the second catalytic step of pre-mRNA splicing. RNA *1*, 584–597.

Valcárcel, 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.

Wang, J., and Bell, L.R. (1994). The Sex-lethal amino terminus mediates cooperative interactions in RNA binding and is essential for splicing regulation. Genes Dev. *8*, 2072–2085.

Will, C.L., and Lührmann, R. (2001). Spliceosomal UsnRNP biogenesis, structure and function. Curr. Opin. Cell Biol. *13*, 290–301.

Wu, S., and Green, M.R. (1997). Identification of a human protein that recognizes the 3′ splice site during the second step of pre-mRNA splicing. EMBO J. *16*, 4421–4432.

Wu, S., Romfo, C.M., Nilsen, T.W., and Green, M.R. (1999). Functional recognition of the 3′ splice site AG by the splicing factor U2AF35. Nature *402*, 832–835.

Zamore, P.D., Patton, J.G., and Green, M.R. (1992). Cloning and domain structure of the mammalian splicing factor U2AF. Nature *355*, 609–614.

Zhuang, Y., and Weiner, A.M. (1990). The conserved dinucleotide AG of the 3′ splice site may be recognized twice during in vitro splicing of mammalian mRNA precursors. Gene *90*, 263–269.

Zillmann, M., Zapp, M.L., and Berget, S.M. (1988). Gel electrophoretic isolation of splicing complexes containing U1 small nuclear ribonucleoprotein particles. Mol. Cell. Biol 8, 814–821.

Zorio, D.A., and Blumenthal, T. (1999). Both subunits of U2AF recognize the 3′ splice site in *Caenorhabditis elegans*. Nature *402*, 835–838.

Zuo, P., and Maniatis, T. (1996). The splicing factor U2AF[35] mediates critical protein-protein interactions in constitutive and enhancer-dependent splicing. Genes Dev. *10*, 1356–1368.