Development 130, 463-471

doi:10.1242/dev.00274

Sex-lethal splicing autoregulation in vivo: interactions between SEX-LETHAL, the U1 snRNP and U2AF underlie male exon skipping

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Accepted 29 October 2002

SUMMARY

Alternative splicing of the *Sex-lethal* pre-mRNA has long served as a model example of a regulated splicing event, yet the mechanism by which the female-specific SEX-LETHAL RNA-binding protein prevents inclusion of the translation-terminating male exon is not understood. Thus far, the only general splicing factor for which there is in vivo evidence for a regulatory role in the pathway leading to male-exon skipping is *sans-fille* (*snf*), a protein component of the spliceosomal U1 and U2 snRNPs. Its role, however, has remained enigmatic because of questions about whether SNF acts as part of an intact snRNP or a free protein. We provide evidence that SEX-LETHAL interacts with SANS-FILLE in the context of the U1 snRNP, through the characterization of a point mutation that interferes with both assembly into the U1 snRNP and complex formation with SEX-LETHAL. Moreover, we find that SEX-LETHAL associates with other integral U1 snRNP components, and we provide genetic evidence to support the biological relevance of these physical interactions. Similar genetic and biochemical approaches also link SEX-LETHAL with the heterodimeric splicing factor, U2AF. These studies point specifically to a mechanism by which SEX-LETHAL represses splicing by interacting with these key splicing factors at both ends of the regulated male exon. Moreover, because U2AF and the U1 snRNP are only associated transiently with the pre-mRNA during the course of spliceosome assembly, our studies are difficult to reconcile with the current model that proposes that the SEX-LETHAL blocks splicing at the second catalytic step, and instead argue that the SEX-LETHAL protein acts after splice site recognition, but before catalysis begins.

Key words: Splicing regulation, SXL, SNF, U1 snRNP, U2AF, *Drosophila*

INTRODUCTION

Tissue-specific control of RNA splicing is an important means of regulating gene expression in metazoans (Graveley, 2001; Lopez, 1998). Splicing is carried out by the spliceosome, a large catalytic RNA-protein machine that consists of four small nuclear ribonucleoprotein particles (snRNPs) and many non-snRNP proteins. The process of spliceosome assembly occurs in at least two stages. The intron/exon boundaries are selected first, while the chemical reactions that remove the intron and join the exons together occurs only after multiple rearrangements of the fully assembled complex (Will and Luhrmann, 2001). How these events are controlled in response to developmental and tissue-specific cues is still poorly understood.

Some of the best understood examples of regulated splicing occur in *Drosophila melanogaster*, where several tissue-specific *trans*-acting factors have been identified (Lopez, 1998). For example, the female-specific RNA binding protein SEX-LETHAL (SXL) controls the splicing pattern of the *transformer* (*tra*) pre-mRNA by binding to a sequence adjacent to the regulated 3′ splice site, thereby diverting splicing to the female-specific splice site (Granadino et al., 1997; Inoue et al., 1990; Sosnowski et al., 1989; Valcarcel et al., 1993). SXL also suppresses expression of the *male-specific-lethal-2* (*msl-2*) gene by binding similar U-rich sequences (Bashaw and Baker, 1995; Kelley et al., 1995; Zhou et al., 1995). However, the mechanism by which SXL regulates *msl-2* processing is more complex than for *tra* because it includes both translational repression and splicing inhibition (Bashaw and Baker, 1997; Forch et al., 2001; Gebauer et al., 1999; Gebauer et al., 1998; Kelley et al., 1997; Merendino et al., 1999). These two examples demonstrate that SXL is capable of controlling expression of its target pre-mRNAs by diverse mechanisms.

In addition to controlling expression of *tra* and *msl-2*, SXL also positively regulates its own expression to insure the continuous production of SXL protein exclusively in females (Bell et al., 1991; Keyes et al., 1992; Sakamoto et al., 1992). Tight control of *Sxl* expression is crucial because the presence or absence of SXL protein determines three major cell fate decisions: somatic sexual differentiation, germline development and X-chromosome dosage compensation (Cline and Meyer, 1996; Schutt and Nothiger, 2000). Thus, misregulation can result in sex-specific lethality, sex

transformations or ovarian tumors. *Sxl* expression is controlled in two phases. The early phase is defined as the short period of time in early embryogenesis when *Sxl* expression is first turned on in females; at this stage, expression is controlled at the level of transcription (Keyes et al., 1992). The late phase begins less than 1 hour later, when transcripts are detected in both males and females (Keyes et al., 1992; Salz et al., 1987; Salz et al., 1989). Expression, however, remains sex specific because the pre-mRNAs are differentially spliced such that protein-encoding mRNAs are produced only in females (Bell et al., 1988; Samuels et al., 1991). The mRNAs produced in males all include the third exon, which contains several in-frame stop codons.

Sxl regulates its own expression through a mechanism by which the female-specific SXL protein prevents inclusion of the translation-terminating male specific exon (Bell et al., 1991; Horabin and Schedl, 1993; Sakamoto et al., 1992). Autoregulation has been linked to several essential SXL-binding sites that are located in the introns on both sides of the regulated male exon. Because these sites are located at some distance from the 5′ and 3′ intron/exon boundaries, it has been suggested that SXL promotes exon skipping by interacting with and inactivating components of the general splicing machinery (Horabin and Schedl, 1993). Indeed, recent studies carried out in tissue culture cells suggest that SXL interacts with the general splicing factor SPF45 at the male-exon 3′ splice site to block inclusion at the second catalytic step of splicing (Lallena et al., 2002). However, although blocking the 3′ splice site is by itself sufficient to ensure that the male exon is skipped in transient tissue culture assays (Lallena et al., 2002; Penalva et al., 2001; Sakamoto et al., 1992), studies using similar splicing constructs expressed in transgenic animals have shown that blocking the male exon 5′ splice site is also required for male-exon skipping (Horabin and Schedl, 1993). Thus, the model proposed by Lallena et al. (Lallena et al., 2002) does not provide a complete explanation for how SXL operates in the fly.

To date, the only general splicing factor for which there is in vivo evidence for a regulatory role in the pathway leading to male-exon skipping is *sans-fille* (*snf*), a protein component of the spliceosomal U1 and U2 snRNP particles (Flickinger and Salz, 1994; Polycarpou-Schwarz et al., 1996; Stitzinger et al., 1999a). SNF was identified as a regulator of *Sxl* splicing because, in females, the viable snf^{1621} mutation disrupted the establishment of the *Sxl* autoregulatory splicing loop in the germline, resulting in the accumulation of *Sxl* mRNAs spliced in the male mode, leading to female sterility (Bopp et al., 1993; Oliver et al., 1993). Since that time, the analysis of multiple *snf* alleles, including the lethal null allele, has reinforced the view that *snf* functions as a co-repressor of *Sxl* splicing in both the germline and the soma (Albrecht and Salz, 1993; Cline et al., 1999; Flickinger and Salz, 1994; Hager and Cline, 1997; Salz, 1992; Salz and Flickinger, 1996). Furthermore, a central role for SNF in *Sxl* splicing regulation is supported by its co-fractionation with SXL (Deshpande et al., 1996; Samuels et al., 1998). That SNF is an integral snRNP protein has led to a model in which SXL blocks male-exon use by interfering with snRNP function (Deshpande et al., 1996; Salz and Flickinger, 1996). However, obtaining evidence in favor of this model has proven to be difficult (Cline et al., 1999), raising the possibility that SNF acts outside of the snRNP in a manner analogous to the way its human counterpart, U1A, inhibits its own polyadenylation (Boelens et al., 1993; Klein Gunnewiek et al., 2000).

In this study, we characterize a viable *snf* mutation that interferes with both SXL complex formation, and assembly into the U1 snRNP. This analysis clarifies the role of *snf* in *Sxl* autoregulation, and suggests that SXL interacts with SNF in the context of the U1 snRNP. Consistent with this, we provide compelling in vivo evidence to link other U1 snRNP components to *Sxl* autoregulation by first showing that in embryonic extracts SXL can form an RNase-resistant complex with these factors, and then providing genetic evidence that supports the biological relevance of these physical interactions. Interestingly, we find that the interaction between the U1-70K protein and SXL does not require SNF, suggesting that SXL can interact with the U1 snRNP through several means. Using similar genetic and biochemical approaches, we also link SXL to the heterodimeric splicing factor U2AF. Together, these studies point specifically to a mechanism by which SXL antagonizes splicing during the early steps of spliceosome assembly by associating with these key splicing factors at both ends of the male exon.

MATERIALS AND METHODS

Fly strains

The *snf* alleles used in this study are: snf^{148}, snf^{1210}, snf^{e8H}, snf^{1621} and the $P\{w^+, snf^{5MER}\}$transgenic allele (Flickinger and Salz, 1994; Salz, 1992; Stitzinger et al., 1999a; Swan et al., 2001). $P\{w^+, otu::Sxl\}$ has been described previously (Hager and Cline, 1997). For the genetic interaction assays, the following null mutations were used: Sxl^{7B0} (Salz et al., 1987), $U2af50^{X15}$ (Rudner et al., 1998), $U2af38^{\Delta E18}$ (Rudner et al., 1996) and $U1\text{-}70K^{1}$ (S. M. M., unpublished). Descriptions of marker mutations and balancers not listed here or in the text are described on FlyBase (http://www.flybase.org). All crosses were carried out on standard *Drosophila* medium at room temperature (22°C).

Immunoprecipitation and GST pull down experiments

Immunoprecipitation, western blot analysis, RNA isolation from the RNA-protein complexes and northern blot analysis were carried out as previously described (Stitzinger et al., 1999a).

For the GST pull-down assays, GST-tagged SXL protein was purified from *E. coli*, using standard methods. The concentration of the GST fusion protein was determined, and 60-80 μg of recombinant protein was combined with 20 μl glutathione Sepharose 4B beads (Amersham Pharmacia Biotech AB) and incubated for 1 hour 30 minutes at 4°C. After washing three times with 400 μl PBS to remove the unbound protein, the GST::SXL loaded beads were combined with 100-150 μl embryonic extracts prepared from 400 μl 3- to 8-hour-old embryos homogenized in 1ml PBS containing protease inhibitor cocktail (Roche). For experiments in which the extracts were pretreated with RNase, 100 μl RNase A (10 mg/ml) and 50 μl RNase T1 (100,000 units/ml) were added to 1 ml of extract and incubated for 30 minutes at 30°C. The extract/bead mixture was incubated overnight at 4°C and then washed three times with 400 μl PBS. To analyze the proteins selected in the pull-down assays, 25 μl SDS loading buffer was added to the beads and 20 μl loaded onto a 12.5% SDS-polyacrylamide gel and analyzed by western blot analysis using the following antibodies: anti-SNF (Flickinger and Salz, 1994; Habets et al., 1989), anti-U2AF38 (Rudner et al., 1996) and anti-U2AF50 (Rudner et al., 1998). A rabbit polyclonal antibody was raised against amino acids 1-213 of the *Drosophila* U1-70K protein by standard

methods (Covance). Antibody binding was visualized using ECL (Amersham Life Sciences). To analyze the RNAs selected in the pull-down assays, the RNAs were isolated and analyzed by Northern blot analysis as described previously (Stitzinger et al., 1999a).

To generate a homogeneous population of mutant embryos for the GST pull-down assays, embryos were collected as follows. For snf^{1621} and snf^{148}, embryos were collected from *snf/snf; P{w+; otu::Sxl}* females crossed to *snf* males. For snf^{e8H}, embryos were collected from snf^{e8H}/snf^{e8H} females crossed to snf^{e8H} males (snf^{e8H} is a fertile allele of *snf*). For snf^{5MER}, which is a transgenic allele of *snf*, embryos were collected from snf^{J210}/snf^{J210}; $P\{w^+; snf^{5MER}\}/P\{w^+; snf^{5MER}\}$; $P\{w^+; otu::Sxl\}$ females crossed to snf^{J210}; $P\{w^+; snf^{5MER}\}/P\{w^+; snf^{5MER}\}$ males.

RT-PCR analysis

The reporter construct and the sequences of the PCR primers used to amplify the RNA expressed from the reporter constructs have been described previously (Horabin and Schedl, 1993). For the RT-PCR analysis, two procedures were used. In Fig. 3, RNA from adults or isolated ovaries was purified by standard methods. Reverse transcription was carried out using the 'Superscript First-Strand Synthesis System for RT-PCR' (Gibco BRL) using 1-3 μg of RNA primed with random hexamers. The PCR reactions were performed in a 100 μl volume with 2 μl of the RT reaction, the Z1 *lacZ* primer and the *Sxl*-specific primer using the 'Expand High Fidelity PCR system' (Roche). The PCR conditions were as follows: 95°C for 3 minutes; followed by 10 cycles of 95°C for 45 seconds, 62°C for 2 minutes, 68°C for 45 seconds. This was followed by 15 cycles of 95°C for 45 seconds, 62°C for 2 minutes, 68°C for 2 minutes 30 seconds, and a single final step at 68°C for 7 minutes. A 0.01% aliquot of the first amplification reaction was then reamplified in a 100 μl volume using the *Sxl*-specific primer and the Z2 *lacZ* primer internal to the one used in the first amplification reaction and 10 μCi ^{32}P dCTP (3000 Ci/mmol, NEN) to label the products. The PCR conditions were as follows: 94°C for 3 minutes; followed by 16 cycles of 95°C for 45 seconds, 61°C for 2 minutes, 72°C for 1 minutes 30 seconds; and a single step at 72°C for 5 minutes. Each PCR reaction (15 μl) was loaded on a 5% polyacrylamide gel and amplified fragments were quantified using a phosphorimager. In Fig. 4, RNA was isolated from either adults or from embryos and the RT-PCR reaction was carried out, using the same primers and the conditions described elsewhere (Stitzinger, 1999b).

RESULTS

The snf^{148} mutation compromises both SXL complex formation and U1 snRNP incorporation in vivo

The observation that SNF can form an RNase-sensitive complex with SXL in whole cell extracts suggested that SNF plays a central role in *Sxl* splicing autoregulation and is likely to do so as part of a snRNP (Deshpande et al., 1996). As a first step towards determining whether this physical association is essential for *Sxl* splicing autoregulation (and whether it involves free SNF, SNF within the U2 snRNP or SNF within the U1 snRNP), we surveyed the protein-encoding *snf* mutations in our collection for a mutation that no longer interacts with SXL (Fig. 1A,B). Although our collection includes both viable and lethal alleles of *snf*, the only lethal allele is a deletion of the entire open reading frame (the null allele, snf^{J210}), whereas the protein-encoding mutations are all viable.

Complex formation was assayed by pull down experiments in which a GST::SXL fusion protein was expressed in bacteria, purified, bound to glutathione sepharose beads and incubated with extracts made from either wild-type or mutant embryos (Fig. 2). The presence or absence of SNF in complexes formed on the beads was determined by western blot analysis. Using this assay, we found that the GST::SXL fusion protein, but not GST alone, could pull down SNF from extracts made from wild-type embryos. As in similar studies (Deshpande et al., 1996), we found that this interaction is sensitive to RNase digestion (see Fig. 4), and thus unlikely to be direct. To test whether GST::SXL could pull down the mutant SNF proteins, we made embryonic extracts from a homogeneous population of embryos whose only source of SNF protein (both maternal and zygotic) was the mutant protein. As illustrated in Fig. 2, we found that GST::SXL was capable of selecting the SNF^{1621}, SNF^{e8H} and SNF^{5MER} mutant proteins from mutant extracts. By contrast, GST::SXL could not pull down the SNF^{148} protein. We have therefore identified a mutation that disrupts the association between SXL and SNF.

snf^{148} is an uncharacterized mutation that was isolated in a genetic screen for X-linked female-sterile alleles (Swan et al., 2001). Sequencing of the snf^{148}-coding region revealed a single missense mutation that changes a conserved asparagine at position 12 to an aspartic acid (Fig. 1B). According to the information from the crystal structures of the human U1A protein bound to its RNA target sequence in the U1 snRNA (Oubridge et al., 1994) and human U2B″ bound to the U2 snRNA (Price et al., 1998), the conserved N12 residue contacts the RNA directly, suggesting that the substitution of an aspartic acid at this position might disrupt assembly of SNF^{148} into U1 snRNPs and/or U2 snRNPs. As in previous studies, we assayed incorporation by testing whether U1 and U2 snRNAs can be immunoprecipitated from whole cell extracts with antibodies directed against SNF. Extracts from wild-type and mutant flies were incubated with anti-SNF antibodies and the immunoprecipitated complexes tested for the presence of U1 and U2 snRNAs by northern blot analysis. As illustrated in Fig. 1C, both U1 and U2 snRNAs were immunoprecipitated from extracts made from wild-type, snf^{1621} and snf^{e8H} animals. In extracts made from snf^{148} mutant animals, however, the SNF specific antibody precipitated U2 snRNA without precipitating significant amounts of U1 snRNA.

Thus, we have identified a single point mutation in the N-terminal RRM of SNF that compromises both SXL complex formation and U1 snRNP incorporation, without having an apparent effect on U2 snRNP incorporation. The fact that snf^{148} animals are viable indicates that the stable association of SNF with the U1 snRNP complex is not crucial for U1 snRNP function in vivo. Indeed, recent biochemical studies have also suggested that SNF is dispensable for U1 snRNP function (Labourier and Rio, 2001).

Sxl splicing autoregulation is disrupted in snf^{148} mutant females

We had anticipated that a lack of association between SNF and SXL would cause a major perturbation of splicing autoregulation, resulting in the accumulation of *Sxl* mRNAs spliced in the male mode, an outcome known to result in female lethality. Instead, we found that snf^{148} mutant females were simply sterile.

To examine the snf^{148} mutant phenotype in more detail, ovaries from both wild-type and mutant females were fixed and

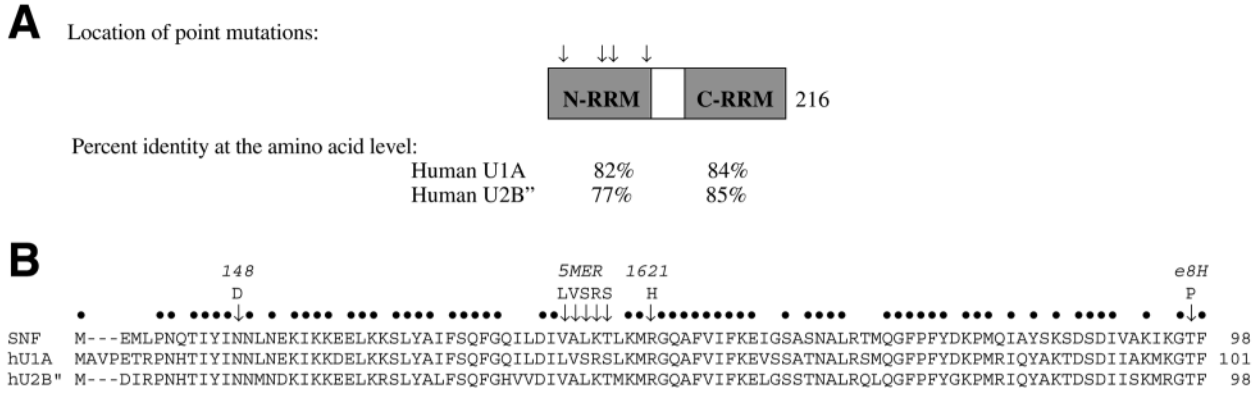

Fig. 1. Impact of *snf* mutations on snRNP assembly. (A) Schematic representation of SNF, the *Drosophila* U1A/U2B″ protein. SNF contains two RRM domains (for RNA recognition motif) separated by a short linker region. The arrows indicate the location of the mutations used in this study. As indicated, the sequence of the N- and C-terminal RRM motifs share significant sequence identity with both the human U1A and U2B″ proteins. (B) Amino acid sequence alignment of the N-terminal RRM domain from SNF and the human U1A and U2B″ proteins and the amino acid substitutions associated with each *snf* allele used. Identical amino acids are indicated by black dots above the sequence. (C) snRNP incorporation was tested by immunoprecipitation of SNF from extracts made from adult flies of the indicated genotype followed by northern blotting to detect U1 and U2 snRNAs in RNA extracted from the precipitated fractions.

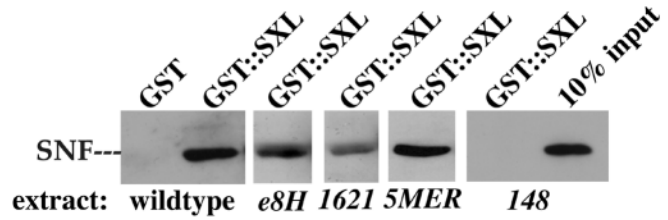

Fig. 2. Impact of *snf* mutations on SXL-SNF complex formation. SXL/SNF complex assembly was tested by GST pull-down assays. Equal amounts of GST::SXL fusion protein, or GST alone, bound to glutathione sepharose beads were incubated with embryonic extracts of the indicated genotype followed by western blotting using an antibody directed against SNF. Because a substantial amount of maternally produced SNF protein is supplied to the embryo, we carried out crosses (described in the Materials and Methods) to obtain a homogeneous population of embryos whose only source of SNF protein (both maternal and zygotic) is the mutant protein. The lane marked 10% input, is a control in which the amount of snf^{148} extract loaded corresponds to ~10% of the material applied to the glutathione affinity beads.

stained with SNF antibodies as well as DAPI to visualize the nuclei. In wild-type ovaries, each ovariole consists of a series of egg chambers, each of which contains 15 polyploid nurse cells and an oocyte (Fig. 3A). As in the soma, SNF is localized to the nucleus in the early stages of oogenesis, including the region at the tip of the ovariole, called the germarium (Fig. 3C). Egg chambers from snf^{148} mutant females, however, are filled with many small nuclei (Fig. 3B). This defect, which appears to be identical to the ovarian phenotype of other female-sterile alleles of *snf*, is called an ovarian tumor phenotype. This experiment also demonstrates that the SNF^{148} mutant protein retains its ability to localize to the nucleus (Fig. 3D), eliminating the trivial explanation that the female-sterile mutant phenotype is a result of SNF protein mislocalization.

Because a common characteristic of the *snf* ovarian tumor mutant phenotype is the presence of male-specific *Sxl* RNA products, we examined the *Sxl* splicing pattern in isolated ovarian tissue from snf^{148} mutant animals. In these studies, we used the *Sxl* reporter construct described elsewhere (Horabin and Schedl, 1993), which contains *Sxl* genomic sequences from exon L2 to the middle of exon L4 fused to *lacZ*-coding sequences and faithfully reproduces the endogenous splicing pattern (Fig. 3E). When *Sxl* spliced products were analyzed by semi-quantitative RT-PCR in wild-type adults, expression of this reporter construct mimics the sex-specific regulated splicing of the endogenous locus: In males, the reporter was spliced to include exon L3, generating an L2-L3-L4 product, while in females the reporter was spliced to exclude exon L3, generating an L2-L4 spliced product that is 200 bp shorter (Fig. 3F, lane 1 and 2). Similarly, we found that the male-specific exon is consistently skipped in isolated ovarian tissue (lane 4). By contrast, two products were detected in ovaries isolated from snf^{148} mutant animals. One corresponds to the L2-L3-L4 male-specific product and the other corresponds to the L2-L4 female-specific product (lane 5). Thus, we conclude that the *Sxl* male-exon is not reliably skipped in snf^{148} mutant ovaries. Importantly, we could rescue the mutant females to fertility by expression of a transgenic copy of the *Sxl* cDNA under control of a germline-specific promoter *P{otu::SxlcDNA}*, demonstrating that the perturbation of *Sxl* splicing is responsible for the sterile phenotype (data not shown).

The viability of snf^{148} mutant females could be explained if the maternally produced SNF protein provided by their heterozygous mothers was sufficient for the successful establishment of the *Sxl* autoregulatory loop in the mutant embryos. To test this possibility, we assessed the viability of

snf^{148} mutant animals whose only source of SNF protein, both maternal and zygotic, was derived from the mutant allele. To generate these animals, we bypassed the sterility of the *snf* mutant females by expression of the germline-specific transgene *P{otu::SxlcDNA}*. Previous studies have shown that this transgene drives *Sxl* expression exclusively in the germline, and does not interfere with our assessment of the effects of *snf* on zygotic *Sxl* expression (Salz and Flickinger, 1996). Contrary to our expectations, the survival rate of *snf*^{148} mutant females was equivalent to *snf*^{148} mutant males (n>500), and comparable with the survival rate of animals from heterozygous mothers. Moreover, in accordance with their viable phenotype, only a small amount of male-specific *Sxl* product was detectable in RNA isolated from these females (Fig. 3F, lane 3). Thus, we conclude that the *snf*^{148} mutation does not have a major effect on either *Sxl* splicing in embryos or female viability in an otherwise wild-type background.

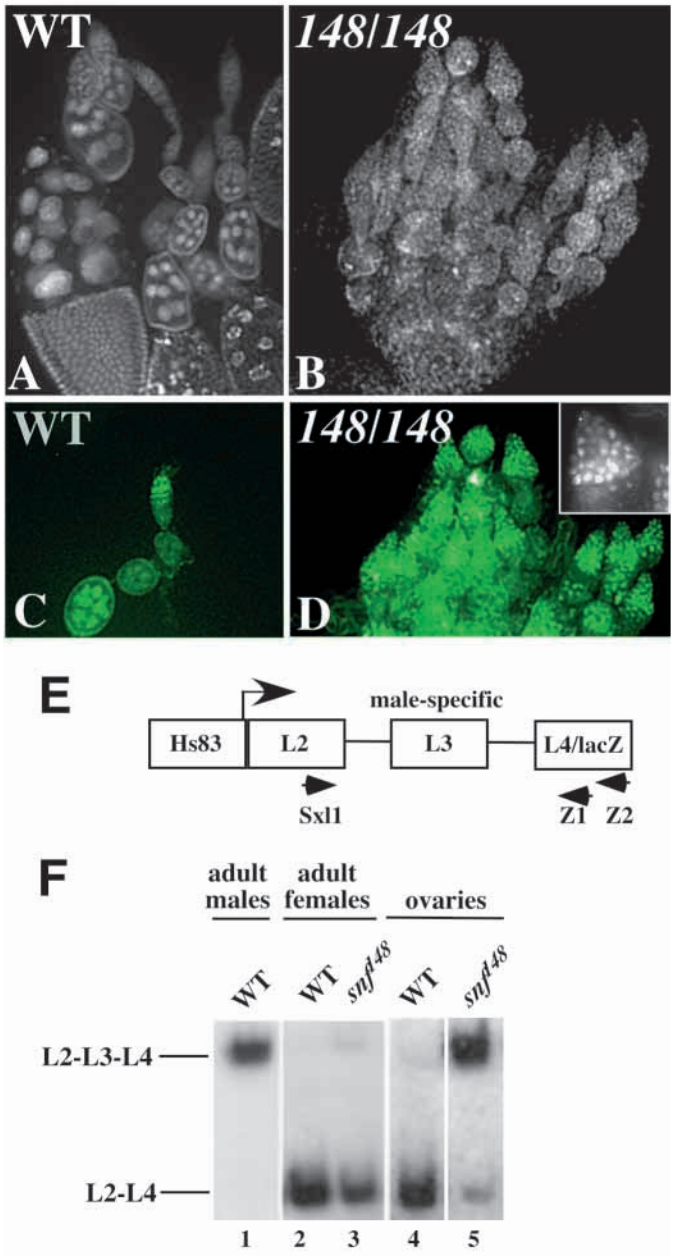

Fig. 3. Analysis of the *snf*^{148} mutant phenotype. (A) Wild-type ovarioles stained with the nuclear dye DAPI. (B) Homozygous *snf*^{148} mutant ovaries stained with DAPI contain tumorous egg chambers that are filled with large numbers of undifferentiated cells. (C) Wild-type ovarioles stained with an antibody directed against SNF, illustrating that SNF localizes to the nucleus. (D) Homozygous *snf*^{148} mutant ovaries stained with an antibody directed against SNF. The magnification of a single mutant egg chamber (insert) illustrates that this mutation does not alter the nuclear localization of SNF. (E) Diagram of the reporter construct that mimics *Sxl* splicing in all tissues. The arrows below the construct show the positions of the nested PCR primer sets used for RT-PCR. (F) To analyze the RNAs produced by the *Sxl* reporter construct, total RNA was isolated from either adults or isolated ovaries of the indicated genotype, and analyzed by semi-quantitative RT-PCR.

Although our data indicate that *snf* is dispensable for the establishment of *Sxl* autoregulation in the embryo, they do not imply that *snf* has no role in *Sxl* autoregulation. In fact, its involvement is evident when we test for female-viability in a genetically sensitized background. For example, when *snf* mutant females (*snf*^{148}/*snf*^{148}; *P{otu::SxlcDNA}*) were crossed to males carrying the normally recessive null allele of *Sxl* (*Sxl*^{7B0}), only 6% of her *Sxl*^{7BO}/+ daughters survive.

SXL associates with U1 snRNP particles in whole cell extracts

The finding that mutation of a single residue in the N-terminal RRM of SNF interferes with both SXL complex formation and assembly into the U1 snRNP suggests that SXL associates with SNF in the context of an intact U1 snRNP. To address this possibility further, we tested whether SXL can physically associate with the U1 snRNP in embryonic extracts. Using GST pull-down assays, we found that both the U1 snRNA (data not shown) and U1-70K protein (Fig. 4A) could be selected from embryonic extracts by GST::SXL but not by GST alone, thus demonstrating that SXL can associate with the intact U1 snRNP particle. Interestingly, of the two U1-70K isoforms observed in whole cell extracts, only the more rapidly migrating U1-70K species was identified in the pull-down experiments. As U1-70K is known to be phosphorylated (Tazi et al., 1993; Woppmann et al., 1990), we suspected that the more rapidly migrating form might be dephosphorylated. Consistent with this notion, we found that phosphatase treatment of the embryonic extracts prior to western blot analysis resulted in a single U1-70K species with a similar mobility to the protein detected in the pull down experiments (data not shown). Thus, U1-70K phosphorylation may modulate SXL complex formation.

To determine whether the interaction between SXL and U1-70K is mediated by RNA present in the extract, we pretreated the extract with RNase prior to performing the GST pull-down assays. The results show that, in contrast to the SXL/SNF interaction, the interaction between SXL and U1-70K is resistant to RNase digestion (Fig. 4A). Thus, the SXL/U1-70K interaction is unlikely to be mediated by an RNA, although we cannot exclude the possibility that a bridging RNA (e.g. U1 snRNA) was protected from the nuclease.

The difference in RNase sensitivity between the SXL/SNF and SXL/U1-70K complex under these conditions suggests that the association between U1-70K and SXL is not mediated by SNF. To test this directly, pull-down assays were used to determine if GST::SXL could select U1-70K from *snf*^{148}

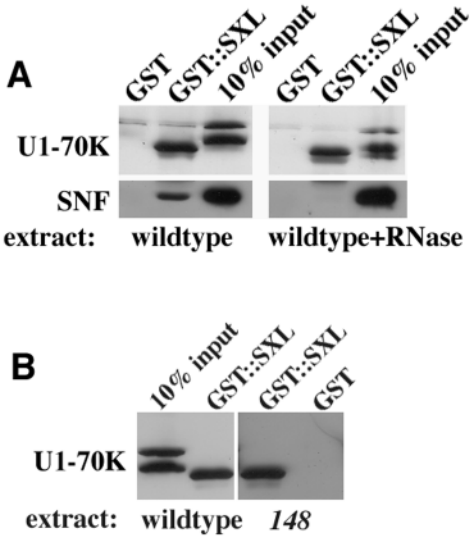

Fig. 4. SXL associates with the U1 snRNP particle in embryonic extracts. (A) The SXL/U1-70K association is more robust than the SXL/SNF association. The ability of SXL to associate with SNF and U1-70K in whole cell extracts was tested by GST pull-down assays, followed by western blotting. The RNase sensitivity of these interactions was tested by pre-treating the embryonic extracts with a combination of RNaseA and RNase T1. (B) SXL associates with U1-70K in *snf*^{148} mutant extracts. GST pull-down experiments were carried out as in Fig. 2, with extracts made from wild-type and a homogenous population of embryos whose only source of SNF protein is the mutant SNF^{148} protein.

mutant extracts. As illustrated in Fig. 5B, U1-70K was selected from these mutant extracts, demonstrating that the association between U1-70K and SXL does not depend on SNF.

Mutations in U1-70K enhance *Sxl* mutations, resulting in female-lethality and *Sxl* splicing defects

The data presented thus far indicate that SXL can physically associate with the U1 snRNP. If SXL works together with the U1 snRNP to promote male-exon skipping, each component of the U1 snRNP might, therefore, have an independent effect on the function of the SXL-blocking complex. This predicts that simultaneously lowering the available SNF and U1-70K protein in the embryo might have an additive effect, which will reduce the efficiency of male-exon skipping, resulting in female-lethality.

To test this idea, we asked whether we could detect a maternal-effect genetic interaction phenotype between null alleles of *snf* and *U1-70K* (Fig. 5A). In control crosses, we find that *snf*^{J210}/+ mothers provide a sensitized genetic background for these assays because only 81% of her *Sxl*^{7B0}/+ daughters survive. Reducing the dose of *U1-70K*, however, has no effect, as the viability of *Sxl*^{7B0}/+ daughters from *U1-70K*^{1}/+ mothers was not significantly reduced (data not shown). However, when the mothers were heterozygous for both *snf*^{J210} and *U1-70K*^{1}, there was a significant reduction in viability, with only 34% of the expected *Sxl*^{7B0}/+ daughters surviving.

To determine whether this synergistic female lethality can be correlated with an increase in male-exon inclusion, we assayed splicing using an X-linked *Sxl* reporter construct linked to the same chromosome as the *Sxl*^{7B0} mutant allele (Fig. 5B). As a consequence of this genetic linkage, the *Sxl*^{7B0}/+ female progeny will carry the reporter construct and their male siblings will not. In control experiments, when the spliced products were analyzed by RT-PCR, only the female-spliced product was detectable in embryos from *snf*^{J210}/+ mothers (lane 3). By contrast, we observed a significant amount of the male-spliced product in embryos from *snf*^{J210}/+; *U1-70K*^{1}/+ mothers (lane 5), consistent with the synergistic female-lethal phenotype. These additive gene-dose effects, therefore, are consistent with a model in which SXL works together with the U1 snRNP to block male-exon inclusion.

Genetic and physical interactions between U2AF and SXL

While the preceding data implicate components bound to the male exon 5′ splice site in *Sxl* splicing autoregulation, recent studies have suggested that additional components bound to the male-exon 3′ splice site are also important (Penalva et al., 2001). Moreover, in studies carried out in HeLa cells, the heterodimeric splicing factor U2AF was crosslinked to the intron on the upstream side of the *Sxl* male exon (Lallena et

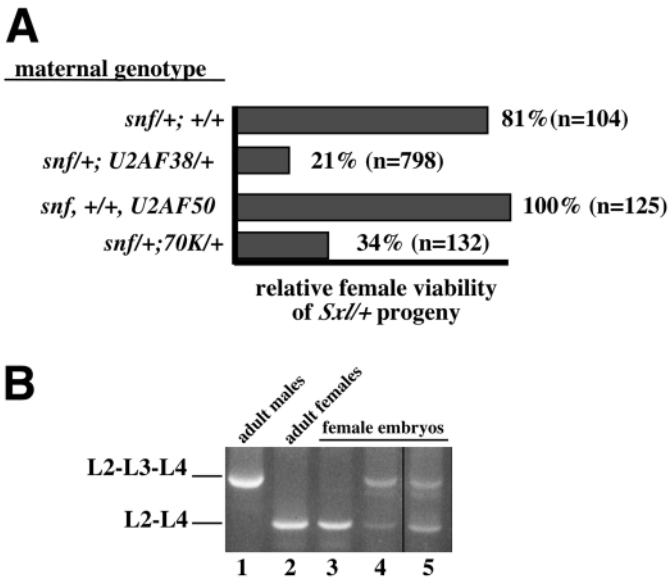

Fig. 5. Mutations in the genes encoding the U2AF38 and U1-70K splicing factors are dosage-sensitive maternal modifiers of *Sxl* splicing autoregulation. (A) Synergistic genetic interactions between splicing factors leads to female-lethality. In these assays females of the indicated maternal genotype were mated to *Sxl*^{7BO}/Y males and the resulting male and female progeny scored. The viability of the female progeny, all of which are heterozygous for *Sxl* (*Sxl*^{7B0}/+), was assessed by comparing the number of females recovered to the number of males recovered. (B) *Sxl* splicing pattern in *Sxl*^{7B0}/+ female embryos. Splicing was assayed by an RT-PCR based assay, in which RNA was isolated from a pool of embryos in which only the *Sxl*^{7B0}/+ embryos carried the reporter construct. This pool of embryos was collected from the experimental adult females crossed to males carrying an X-chromosome which carries both *Sxl*^{7B0} and a copy of the *Sxl* reporter construct described in Fig. 3E. Lanes 3-5: embryos were collected from *snf*^{J210}/+ control mothers (lane 3); *snf*^{J210}/+; *U2af38*$^{\Delta E18}$/+ mothers (lane 4); and *snf*^{J210}/+; *U1-70K*^{1}/+ mothers (lane 5). Controls include: lane 1, splicing of the reporter construct in adult males; and lane 2, splicing of the reporter construct in adult females.

al., 2002). To obtain functional evidence to support a role for U2AF in *Sxl* autoregulation, we used the genetic and biochemical assays described in the previous section.

First, we showed that loss-of-function mutations in the small subunit of U2AF (*U2af38*$^{\Delta E18}$) and *snf* exert synergistic effects on the viability of *Sxl*^{7B0}/+ females and on *Sxl* splicing (Fig. 5A). Only 21% of the expected *Sxl*^{7B0}/+ heterozygous females were recovered from *snf*^{J210}/+; *U2af38*$^{\Delta E18}$/+ mothers. Furthermore, we found that the female lethality was correlated with *Sxl* splicing defects, as a significant amount of male-spliced product is detected in *Sxl*/+ female embryos collected from *snf*^{J210}/+; *U2af38*$^{\Delta E18}$/+ mothers (Fig. 5B, lane 4). Together, these in vivo findings indicate that the small subunit of U2AF plays an active role in *Sxl* splicing autoregulation.

Interestingly, we did not detect a genetic interaction with null mutations in the large subunit of U2AF (*U2af50*^{X15}). However, as dose-sensitive interactions are detectable only if the gene product being assayed is present in limiting quantities, the failure to detect a genetic interaction may simply mean that reducing the level of this core splicing factor is not sufficient to compromise *Sxl* splicing significantly. Thus, while detection of a genetic interaction provides compelling evidence for the active involvement of the small subunit of U2AF in *Sxl* splicing regulation, the failure to detect an interaction does not argue against a role for the large subunit of U2AF.

Finally, to support the genetic role of U2AF in *Sxl* splicing autoregulation, we tested whether the U2AF heterodimer could be selected from embryonic extracts by GST::SXL in pull-down assays. As illustrated in Fig. 6, our data indicate that both subunits of U2AF associate with SXL. This association is robust, as we found that GST:: SXL could pull down both the large and small subunit from extracts pretreated with RNase (data not shown). Moreover, we could pull down both subunits of U2AF from *snf*^{148} mutant extracts, demonstrating that the association between these two proteins does not require a prior association between SXL and SNF. Together with the genetic interaction data, these physical associations suggest that SXL blocks use of the male-exon 3′ splice site by associating with the U2AF heterodimer.

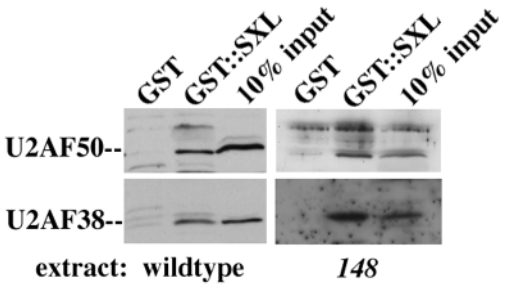

Fig. 6. SXL associates with both U2AF subunits in embryonic extracts. GST pull-down experiments were carried out as in Fig. 2, with extracts made from wild-type and a homogenous population of embryos whose only source of SNF protein is the mutant SNF^{148} protein.

DISCUSSION

Although *Sxl* pre-mRNA splicing has long served as a model example of a regulated splicing event, the mechanism by which the male-specific exon is efficiently skipped in females is still poorly understood. The results of these studies not only provide critical insight into the mechanism that underlies *Sxl* autoregulation in vivo, they highlight the value of looking at splicing factors and their target pre-mRNAs in their natural context.

The *Sxl* male exon is unusual in that it contains two 3′ AG dinucleotides separated by a short polypyrimidine tract (Bell et al., 1988). Interestingly, although the upstream 3′ splice site is used almost exclusively for exon ligation in tissue-culture cells, both 3′ splice sites are required for SXL-mediated male-exon skipping (Penalva et al., 2001). Moreover, crosslinking studies in HeLa cell extracts have shown that the U2AF heterodimer binds to the downstream 3′ splice site and the intervening polypyrimidine tract (Lallena et al., 2002), suggesting that U2AF may play an active role in *Sxl* regulation. We validate these biochemical data by demonstrating that the SXL protein can associate with the *Drosophila* U2AF orthologs. More importantly, our genetic data provide compelling support for the biological relevance of these interactions by demonstrating that in females, the small subunit is important for both *Sxl* male-exon skipping and female viability. In addition to demonstrating a role for U2AF in *Sxl* autoregulation, this genetic result is notable because previous studies have failed to find RNA splicing defects associated with small subunit mutations (Burnette et al., 1999; Rudner et al., 1996). Whether our success reflects substrate-specificity or sensitivity of our assay remains to be determined.

In addition to controlling the use of the male exon 3′ splice site, our studies suggest that SXL controls the use of the male-exon 5′ splice site by interacting with the U1 snRNP. We were able to establish this connection in three ways. First, we find that mutation of a single residue in the N-terminal RRM of SNF compromises both complex formation with SXL and assembly into the U1 snRNP, thus suggesting that the two events are linked. Second, we demonstrated that, in addition to SNF, SXL can associate with other integral U1 snRNP components, including the U1-70K protein and the U1 snRNA in whole cell extracts. Finally, our genetic interaction data provide evidence that U1-70K, like SNF, is important for the successful establishment of the *Sxl* autoregulatory splicing loop in females.

Although our discovery that SNF is an snRNP protein was the first clue that SXL might act by associating with components of the general splicing machinery, the role of SNF has remained enigmatic. We clarify the role of SNF by demonstrating that its contribution to the function of the U1 snRNP is not absolutely essential for viability of either sex, and that SXL can associate with the U1 snRNP through a SNF-independent mechanism. Nevertheless, our in vivo analysis continues to support a role for *snf* in *Sxl* splicing autoregulation by demonstrating that *Sxl* splicing defects are detectable under specific conditions. Interestingly, the phenotypic consequences of these *Sxl* splicing defects are more severe in the germline than in the soma. One possible explanation for this difference is that the requirements for *Sxl* splicing autoregulation are fundamentally different in the two tissue types. We think, however, that it is more likely that the mechanism is the same, but that the additional interaction with the U1 snRNP provided by SNF becomes critical when SXL protein levels are low. This

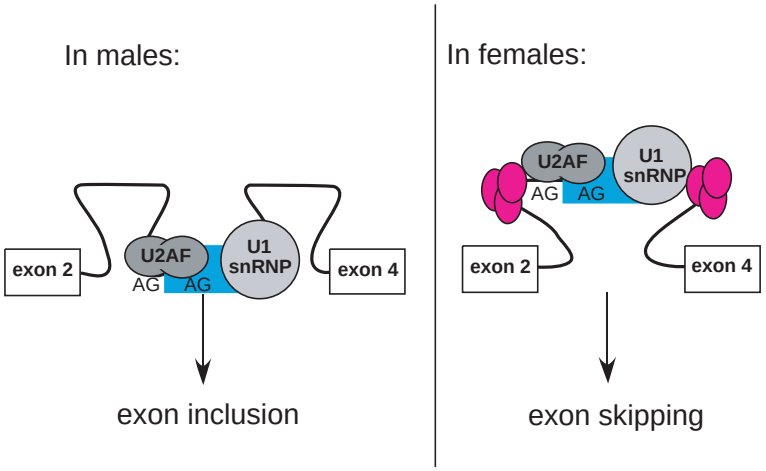

Fig. 7. How SXL promotes exon-skipping. In females, the SXL protein (pink ovals) binds to several sites within the introns located both upstream and downstream of the male-exon (blue rectangle), and associates with both U2AF and the U1 snRNP to block the 3′ and 5′ splice sites from being used. Formation of this dead-end complex guarantees that the male-exon will be skipped, and that exon 2 is spliced to exon 4. In males, where there is no SXL protein, U2AF and the U1 snRNP are free to assemble into an active spliceosome and exon 3 is included in the mature transcript. AG indicates the location of the two male exon 3′ splice sites.

hypothesis is based on the fact that, in the germline, the majority of SXL protein is cytoplasmic, and thus low levels of nuclear SXL protein are the norm (Bopp et al., 1993). By contrast, in other tissues, the SXL protein accumulates in the nucleus, enabling the SXL-U1 snRNP complex to form even when SNF is not stably associated with the U1 snRNP. Our finding that these *snf* mutant females rarely survive if they are also heterozygous for *Sxl*, provides additional support for the idea that SNF function is only critical when SXL protein levels are low.

Together, our studies argue that interactions between SXL, the U1 snRNP and U2AF underlie the mechanism by which SXL promotes skipping of the male exon. Based on these studies, we propose a model in which SXL acts not by preventing assembly of the U1 snRNP or U2AF onto the pre-mRNA, but instead interacts with the U1 snRNP bound to the male-exon 5′ splice site, and U2AF at the male-exon 3′ splice site, to form complexes that block these general splicing factors from assembling into a functional spliceosome (Fig. 7). These 5′ and 3′ SXL blocking complexes might function independently or they might interact across the exon to form a larger inhibitory complex. Furthermore, because we have been unable to demonstrate that SXL interacts directly with either U1-70K or U2AF, we speculate that one or more bridging proteins are required to link SXL to the general splicing machinery.

Although our in vivo approach cannot directly address when in the pathway of spliceosome assembly SXL acts, biochemical studies have shown that during the course of spliceosome assembly, U2AF and the U1 snRNP are only transiently associated with splicing substrates, and are released before the formation of a functional spliceosome. Therefore, based on our studies, it seems reasonable to propose that SXL acts by blocking splicing after splice site recognition but before catalysis begins. Our data are therefore difficult to reconcile with the recent model presented by Lallena et al. (Lallena et al., 2002), which proposes that SXL blocks splicing after spliceosome assembly, at the second catalytic step of the reaction. Using RNA interference in *Drosophila* tissue culture cells, Lallena et al. (Lallena et al., 2002) demonstrate that efficient male exon skipping depends on the presence of SPF45, a protein that is known to be required for the second step of splicing. Together with studies that show that SPF45 can bind to the upstream 3′ splice site of the *Sxl* male exon and physically interact with SXL, these data point to a role for SPF45 in *Sxl* splicing regulation. However, the primary evidence that SXL blocks the splicing reaction during the second step rests on the results of in vitro splicing assays in which SXL was shown to inhibit splicing of a chimeric splicing substrate that contains only a small region of the intronic region required for successful autoregulation in vivo (Horabin and Schedl, 1993). We suspect that by looking at this 48 bp region, which contains a dispensable SXL-binding site in addition to the two potential 3′ splice sites, out of context, Lallena et al. (Lallena et al., 2002) have uncovered a failsafe mechanism that comes into play when SXL-mediated splicing regulation is otherwise compromised. Additional studies investigating the function of SPF45 in vivo will be required to determine the importance of this second step blocking mechanism and should provide insight into whether multiple mechanisms are needed to drive efficient regulated exon skipping.

We thank J. Wise for critical reading of the manuscript, and B. Suter, D. Rio, L. Bell, J. Horabin and T. Cline for reagents. This work was supported by NSF grant MCB9904565 (H. K. S.), NIH grants RO1-GM61039 (H. K.S.) and RO1-GM37991 (S. M. M.). A. N. was partially supported by HD07104, a Developmental Biology Training grant from the NIH.

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