The *Drosophila* sex determination gene *snf* encodes a nuclear protein with sequence and functional similarity to the mammalian U1A snRNP protein

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Alternative splicing controls the expression of many genes, including the *Drosophila* sex determination gene *Sex-lethal*. Previous studies have suggested that *snf* plays a role in regulating *Sex-lethal* splicing. Here, we demonstrate that *snf* is an integral component of the machinery required for splice site recognition. We have cloned *snf* and found that it has sequence homology to the mammalian U1A and U2B* snRNP proteins. Moreover, we establish that *snf* encodes a *Drosophila* protein shown previously to have functional similarity to U1A. Finally, with the isolation and analysis of a null mutation, we demonstrate that *snf* is an essential gene. These studies provide the first demonstration, in a multicellular organism, that mutations in a U1 snRNP protein alter splicing in vivo.

[Key Words: *snf* gene; *Drosophila*; sex determination; snRNP protein; pre-mRNA splicing]

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Control of RNA splicing has proven to be a common means of regulating differential gene expression (for review, see McKeown 1990; Green 1991). Although much is known about the biochemistry of the splicing reaction in vitro, the mechanisms regulating alternative splice site choices remain largely speculative. To further our understanding of the mechanisms of splicing regulation, we have chosen to use the *Drosophila* sex determination system because of its accessibility to genetic techniques (for review, see Belote 1992; Burris and Wollner 1992; McKeown and Madigan 1992; Steinmann-Zwicky 1992; Cline 1993; Cronmiller and Salz 1994). By focusing on the identification of mutations that disrupt specific alternative splicing events, we hope to identify products that play a crucial role in splicing regulation irrespective of whether they are structural or regulatory components of the splicing machinery. In particular, our approach has been to identify and characterize mutations that disrupt the sex-specific splicing pattern of *Sex-lethal* (*Sxl*). *Sxl* is a binary switch gene that regulates the three major aspects of sexual cell fate: somatic sexual differentiation, germ-line development, and X-chromosome dosage compensation (for review, see Cline 1993; Cronmiller and Salz 1994). If *Sxl* is “on”, the female developmental pathway is selected; alternatively, if *Sxl* is “off”, male development ensues. Because of its role in the vital process of dosage compensation, loss-of-function mutations result in female-specific lethality and gain-of-function mutations result in male-specific lethality. Although the on/off regulation of *Sxl* is controlled primarily at the level of RNA splicing, female-specific expression is initially regulated at the level of transcription (Keyes et al. 1992). Once female-specific expression is initiated, a switch from the female-specific promoter to the nonsex-specific–promoter results in the production of nonsex-specific pre-mRNAs (Salz et al. 1989; Keyes et al. 1992). These pre-mRNAs are differentially spliced such that only the female-specific mRNA contains a large open reading frame [ORF] (Bell et al. 1988; Samuels et al. 1991). One of the first splicing factors to be identified as necessary for the on/off regulation of *Sxl* was *Sxl* itself (Cline 1984; Bell et al. 1991; Keyes et al. 1992). *Sxl* is believed to control its own splicing by the *SXL* protein binding its own pre-mRNA to block recognition of the male-specific exon (Sakamoto et al. 1992; Horabin and Schedl 1993a,b). In addition to *Sxl* itself, genetic studies have identified two other genes that regulate *Sxl* splicing: *fl(2)d* (Granadino et al. 1990, 1991, 1992) and *snf* (Oliver et al. 1988; Steinmann-Zwicky 1988; Salz 1992; Albrecht and Salz 1993; Bopp et al. 1993; Oliver et al. 1993). Although genetic studies have suggested that *fl(2)d* is required throughout development to maintain the female-specific splicing pattern of *Sxl*, no molecular data are available to elucidate further its role in splicing regulation.

In this paper we investigate the molecular mechanism by which *snf* regulates the sex-specific splicing of *Sxl*. *snf* was first identified as a positive regulator of *Sxl* in the germ line based on the analysis of a single allele, *snf*1601 (Oliver et al. 1988, 1993; Steinmann-Zwicky 1988; Salz...
Females homozygous for this allele are sterile because the germ-line components of the ovary form ovarian tumors that express Sxl in the male-specific mode (Bopp et al. 1993; Oliver et al. 1993). In contrast to its role in germ-line sex determination, the role of the snf gene in somatic sex determination can only be inferred from a female-lethal synergistic interaction between snf and Sxl (Oliver et al. 1988; Steinmann-Zwicky 1988; Salz 1992). Analysis of this female-lethal interaction has suggested that snf is utilized to establish the female-specific splicing pattern of the Sxl mRNA (Albrecht and Salz 1993).

Here, we demonstrate that snf is a U1 snRNP protein and is therefore an integral component of the machinery required for splice site recognition. We have cloned the snf gene and found that it encodes a nuclear protein with homology to the mammalian U1A and U2B” small nuclear ribonucleoprotein particle (snRNP) proteins. Moreover, we have established that snf encodes a Drosophila protein shown previously to have functional similarity to the U1 snRNP-specific U1A protein. Finally, with the isolation and analysis of a null mutation, we show that snf function results in embryonic lethality: A complete loss of snf function results in embryonic lethality. Taken together, these results strongly suggest that snf plays a direct role in alternative splice site selection. Although biochemical studies will be required to establish the exact role of snf in splicing, the data presented here underscore the importance of snRNP proteins in regulating alternative splicing events in vivo.

Results

Identification of the snf genomic region

As the first step toward cloning the snf gene, we screened for P-element insertions that failed to complement snf1621. Because P elements are thought to transpose preferentially into genomic regions close to their original insertion sites (Tower et al. 1993), we mobilized a PlacW element, located in 4F1,2, within the coding sequence of the closely linked deadhead (dhd) gene (Salz et al. 1994). Although females homozygous for this P-element insertion, dhdP8, are female-sterile, dhdP8 fully complements snf1621 and is therefore not allelic to snf.

Of ~700 male-viable derivative lines established after mobilization of the P-element insertion in dhdP8, one new P-element insertion, snfPR110, failed to complement both snf1621 and dhdP8. Excision of the P element within snfPR110 did not revert either the snf or the dhd mutant phenotypes, suggesting that snfPR110 had both a P-element insertion and an additional chromosomal rearrangement. Cloning of the genomic DNA flanking the P-element insertion site in snfPR110 revealed that snfPR110 contained both a P-element insertion and a deletion of ~3-kb of genomic DNA on the 5' side of dhd (Fig. 1). Another P-element-derived deletion, snfIA2, was characterized at the molecular level and found to be identical to snfPR110; however, the P element had excised from snfIA2 (Fig. 1). These data located the sequences required for snf function to within ~3 kb of dhd.

The snf gene encodes a 1.0-kb non-sex-specific transcript

Genomic DNA fragments from the region 5' of dhd were used as probes on Northern blots to identify a single 1-kb non-sex-specific RNA adjacent to dhd (Salz et al. 1994 and data not shown). The possibility that snf encodes a non-sex-specific transcript is consistent with our understanding of snf function, as genetic studies had shown that snf mutations can affect Sxl expression in both

Figure 1. Molecular map of the region surrounding snf. The extent of the deletions in the region and the position of inserted P elements are indicated at the top of the diagram by heavy lines and inverted triangles, respectively. snfPR110 was identified in a screen to mobilized the P element within dhdP8 [see Materials and methods]. snfIA2 and snfI210 are derivatives of snfPR110 [see Materials and methods]. (C) Exons; (thin lines) intervening sequences. Restriction enzyme sites are indicated by (B) BamHI, (E) EcoRI, (K) KpnI, (Xb) Xbal, and (X) Xhol. Arrows labeled P1–P7 show the location and orientation of primers used for PCR amplification. The location of the 4.8-kb Xhol fragment that rescues dhd mutations is indicated (lower right). In the expanded view of the snf locus (lower left), the small open boxes represent untranslated regions.
males and females [Steinmann-Zwicky 1988, Salz 1992]. To determine whether this RNA corresponds to snf, several corresponding cDNAs were isolated from a head cDNA library (Itoh et al. 1986), one of these cDNAs, KKE-14, was characterized further. When used as a probe on Northern blots, KKE-14 detected a 1-kb non-sex-specific RNA that is missing in homozygous snf/IA2 animals but not in dhdP8 animals [Fig. 2]. Instead, a 0.9-kb RNA was detected in both snf/IA2 and snf/PR110 animals but not in dhdP8 animals. Southern blot and RNase protection analysis demonstrate that the deletions associated with snf/PR110 and snf/IA2 remove the second exon of this transcriptional unit and break within the intron [Figs. 1 and 3]. Thus, it is likely that the 0.9-kb transcript produced by these deletion alleles is a truncation of the wild-type message. This finding, in conjunction with the finding that snf1621 is a missense mutation within the coding region of this 1-kb RNA (see below), identifies this mRNA as a transcript of the snf gene.

snf encodes a RRM protein with homology to two similar snRNP proteins: U1A and U2B"

The insert within the KKE-14 cDNA, as well as the corresponding genomic region, was sequenced and found to have an ORF of 216 amino acids that would produce a protein with an apparent molecular mass of 28 kD [Fig. 3]. The predicted protein contains two RNA recognition motifs (RRMs), one at the amino terminus and the other at the carboxyl terminus [for review, see Kenan et al. 1991]. This protein shows extensive overall similarity to two closely related human snRNP proteins, U1A and U2B" [Habets et al. 1987; Sillekens et al. 1987]. U1A and U2B" are two highly similar RNA-binding proteins that bind different target RNAs. U1A binds U1 snRNA, and U2B" binds U2 snRNA. Between SNF and these two human proteins, the amino acid identity is 72% and 70%, respectively [Fig. 4]. For just the amino terminal RRM domain, which is responsible for the snRNA-binding specificity of U1A and U2B", the identity is even stronger: 82.5% with U1A and 79% with U2B" [Scherly et al. 1990a,b; Bentley and Keene 1991; Jessen et al. 1991; Kenan et al. 1991; Tsai et al. 1991]. Finally, the amino-terminal RRM domain of the SNF protein also shows extensive homology to U1A in Xenopus (69% identity), to U2B" in potato (54% identity), but not to U1A in yeast (14.5% identity) [Kenan et al. 1991; Scherly et al. 1991; Simpson et al. 1991; Liao et al. 1993]. Interestingly, even though the function of the carboxy-terminal RRM is not known, it too is highly conserved between Drosophila, human, Xenopus, and potato [Fig. 4B].

We also found that the SNF protein is identical to a previously cloned Drosophila gene, D25, reported to have functional similarity to U1A [Harper et al. 1992]. Although Harper et al. (1992) did not publish a cytological location for D25, D25 must be snf because the sequences of the cDNAs are virtually identical. We found only five differences between the sequence of the snf cDNA and that published by Harper et al. (1992); none of these differences results in any change in the coding potential [Fig. 3].

snf1621 contains a single point mutation within the amino-terminal RRM

To establish further that we have cloned the snf gene, we characterized the molecular lesion associated with snf1621 by amplifying and sequencing the genomic DNA from this mutant allele and its parent chromosome. The snf1621 mutation resulted from a G to A transition at position 209, substituting a histidine for an arginine residue in the amino terminal RRM [at position 49, Fig. 4B]. As illustrated in Figure 4B, this arginine is conserved in both U1A and U2B" proteins. Moreover, in vitro structure/function studies have shown that this residue is crucial for human U1A to bind its target RNA [Nagai et al. 1990; Jessen et al. 1991].

SNF is a nuclear protein recognized by an antibody specific for mammalian U2B"

Previously, a monoclonal antibody (mAb 4G3) directed against the human U2B" was shown to detect a Drosophila nuclear antigen that is similar in size to the predicted 28-kD SNF protein [Habets et al. 1989; Amero et al. 1992; Bopp et al. 1993]. Is this antigen SNF? The epitope for mAb 4G3 has been mapped to the carboxy-terminal RRM of U2B" [Habets et al. 1989]. Thus, if mAb 4G3 was specific for SNF, then the 28-kD protein would not be detected in extracts from either snf/IA2 or snf/PR110 flies because both of these alleles carry deletions that remove the second exon of the gene, including
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tgcccagctt gtagaacgag ccattgtggc gctctcaaat aatcatttgg -835
tttggcagct ttggcagag cagctccttg ctactgttgc attccctcct -755
tgctcctcct ttcttctctgc atctgttgta cagctcctgc cgcggcctta -675
ggcggccct ggcggccccg cgcggccgta gcggcgccgg ggcggccgta -595
gcggccaatgcc gcggccaatgcc gcggccaatgcc gcggccaatgcc gcggccaatgcc -515
ggcggcctgag gcggcctgag gcggcctgag gcggcctgag gcggcctgag -435
tgcgaggtgc gcggccgca gc ggccgca gcggccgca gcggccgca gcggccgca -355
ggcggccgca gcggccgca gcggccgca gcggccgca gcggccgca gcggccgca -275
tgcgaggtgc gcggccgca gcggccgca gcggccgca gcggccgca gcggccgca -195
agttagggagg gcggccgca gcggccgca gcggccgca gcggccgca gcggccgca -115
agttagggagg gcggccgca gcggccgca gcggccgca gcggccgca gcggccgca -35

Figure 3. Nucleotide and deduced amino acid sequence of the snf genomic locus. The genomic nucleotide sequence corresponding to the snf cDNA is indicated by uppercase letters, the flanking and intervening nucleotide sequences are in lowercase. The putative translation product in the single-letter amino acid code with the RRM domains in bold is presented below the nucleotide sequence.

The exact location of the breakpoint is indicated by an arrow, and the location of the snf1621 missense mutation is shown. In addition, the approximate location of the snf1PR110 and snfA2 mutations are indicated (†). There are no nucleotide differences between the D25 cDNA (Harper et al. 1992; GeneBank accession number M89775) and the corresponding regions of this genomic sequence. On the other hand, the snf cDNA that we isolated, AKE-14, has 5 nucleotide differences from this sequence that do not result in an alteration of coding potential. These three of these differences are illustrated; however, a dinucleotide insertion (GA) at nucleotide 37 present in the snf cDNA of AKE-14 is not shown. The genomic sequence reported here has been assigned GenBank accession number L29521.

Figure 5A, mAb 4G3 recognizes a 28-kD protein in wild-type animals but not in either snfA2 or snf1PR110 animals. Similarly, no cross-reacting antigen was detected in whole-mount immunohistochemical staining of tissues from snfA2 homozygous animals (Fig. 6C,E). The failure
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**A**

| MAVPETRPHHTYINNLNEKIKDEKLKKSLYAIIFSQQQGQLDILVDVLKLRQMQAFVIPKEVSSATNALSNNQGFPYDKPKRIIQYAKTDSSDI1AKMKGT |
| M---EMLPNQHTIYINNLNEKIKKEELKKSLYAIIFSQQQGQLDIVALKLKLRQMQAFVIPKEIAGSASNLRTMQGFPYDKPKMIAYSKSDSIVAKIK GT |
| M---DIRPNHTIYINNLNEKIKKEELKKSLYAIIFSQQQGQLDIVALKLKLRQMQAFVIPKEIAGSASNLRTMQGFPYDKPKMIAYSKSDSIVAKIK GT |

| FVERDRKREKRKPKQETPATKKAQGGGAT\[GAQGGMPPMTQARIMHHMGQPYMGMIPGLAPGQIGAMPQQLMGQMPAQP |
| FKERPKKVKPPKPAPG-TDE-KKKKKKK---PSSAE-NSNPNAQ- |
| FADKEKKKEKKKAKTVEQTATTTTNNKPKQGQPNSANQG-NSTPNPQV |

| LSENPFPNHILFLTNPESTNEMSSLNLFQFPGKEVRVLPGRFVIAFVPNDEAGAQAGARALQFKITQNAMKISFAKK |
| -TRQPNNQILFLTNPESTNEMSSLNLFQFPGKEVRVLPGRFVIAFVPNDEAGAQAGARALQFKITFPTHAMKITFAPK |
| P-DYPPPPIILFLNPESTNEMSSLNLFQFPGKEVRVLPGRFVIAFVPNDEAGAQAGARALQFKITFPTHAMKITFAPK |

**B**

### N-terminal RRM:

 Generating homology to the U1A and U2B’ snRNP proteins. (A) Comparison of the 28-kD snf translation product with the human U1A and U2B’ snRNP proteins. The predicted SNF protein contains two RMM motifs, one at the amino terminus and one at the carboxyl terminus. Amino acid identities are denoted by vertical lines; the lack of a corresponding residue is represented by dashes. Amino acids are identified by their single-letter code and the RRM (as defined by Kenan et al. 1991) are in boldface type. (B) A detailed comparison of the amino acid sequences for the amino-terminal and carboxy-terminal RRM of SNF with the human U2B’ and U1A proteins, the potato U2B’ protein, and the *Xenopus* U1A protein (Habets et al. 1987; Sillekens et al. 1987; Scherly et al. 1991; Simpson et al. 1991). Also indicated are the different components of the RRM: RNP-1, RNP-2, and 5-MER. [For a complete description of these motifs, see Kenan et al. (1991).] The amino acids are identified by their single-letter code and identical amino acids are represented by a dash.

Figure 4. *snf* encodes a protein with homology to the U1A and U2B’ snRNP proteins. (A) Comparison of the 28-kD *snf* translation product with the human U1A and U2B’ snRNP proteins. The predicted SNF protein contains two RMM motifs, one at the amino terminus and one at the carboxyl terminus. Amino acid identities are denoted by vertical lines; the lack of a corresponding residue is represented by dashes. Amino acids are identified by their single-letter code and the RRM (as defined by Kenan et al. 1991) are in boldface type. (B) A detailed comparison of the amino acid sequences for the amino-terminal and carboxy-terminal RRM of SNF with the human U2B’ and U1A proteins, the potato U2B’ protein, and the *Xenopus* U1A protein (Habets et al. 1987; Sillekens et al. 1987; Scherly et al. 1991; Simpson et al. 1991). Also indicated are the different components of the RRM: RNP-1, RNP-2, and 5-MER. [For a complete description of these motifs, see Kenan et al. (1991).] The amino acids are identified by their single-letter code and identical amino acids are represented by a dash.
The sex determination gene \(snf\) is a snRNP protein

![Figure 5](image)

**Figure 5.** The SNF protein is recognized by an antibody directed against the mammalian U2B⁺ throughout development. (A) Western transfer of protein extracts from homozygous adult wild-type, \(snf^{IA2}\), \(snf^{PR110}\), \(snf^{ebf}\), and \(snf^{IA2}\) females probed with mAb 4G3. (B) Developmental profile of the SNF protein. Proteins were extracted from wild-type adults and staged embryos and larvae. The ages of the embryos are in hours after egg deposition (25°C). The Western blot contains equal amounts of protein in all lanes except for the 0- to 30-min embryo collection, which contained about half the amount of protein [data not shown]. The locations of protein size standards [Bio-Rad] [in kD] are indicated at left.

To detect a protein in the 3' deletion mutations establishes that mAb 4G3 is specific for SNF protein.

To determine the temporal distribution of the SNF protein, we probed Western blots containing protein extracts from different stages of development with mAb 4G3 and found the protein present in all stages examined [Fig. 5B]. To examine more precisely the subcellular localization of SNF, mAb 4G3 was used to stain whole-mount embryos and larval tissues. We found that mAb 4G3 staining is localized to the nucleus in all tissues throughout most of development [Fig. 6B, D; data not shown]. For example, we found staining localized to the polytene nuclei of salivary glands from third-instar larvae [Fig. 6D]. These results are consistent with those obtained previously by Amero et al. [1992] who found that mAb 4G3 detected an antigen that is associated with many sites throughout the polytene chromosomes. As might be expected, we found that the staining of chromosomes is not limited to the salivary gland. During the early embryonic mitotic divisions, we also observed staining to condensed chromosomes [data not shown]. Together with the developmental Western analysis, these data clearly demonstrate that SNF is a nuclear protein that is expressed throughout development.

Because genetic data have demonstrated a requirement for maternal \(snf\) function, we examined the expression of SNF in early embryos and during oogenesis. When Western blots containing protein extracts from newly laid eggs [0-30 min of development] were probed with mAb 4G3, we found that the 28-kD SNF protein was present [Fig. 5B]. In contrast to most of development, whole-mount antibody staining of these early embryos revealed that SNF was non-nuclear, mAb 4G3 staining was homogeneously distributed throughout these syncytial embryos [Fig. 6A]. Several mitotic divisions later, SNF was restricted to the nuclei [Fig. 6B; data not shown]. To determine whether SNF is likely to be among the proteins made by the nurse cells and deposited into the oocyte, we examined the distribution of mAb 4G3 antigens in the ovary. All nuclei in both the germ-line and the somatic components of the ovary were found to be stained by mAb 4G3. Furthermore, we found high levels of staining in the cytoplasm of the nurse cells, suggesting that the SNF protein is deposited into the developing egg.
The null phenotype of snf is a non-sex-specific lethal

Although previous genetic studies have shown that mutations in snf disrupt the establishment of the Sxl female-specific splicing pattern in both the germ line and the soma [Albrecht and Salz 1993; Bopp et al. 1993; Oliver et al. 1993], the most extreme allele isolated until now, snf<sup>1621</sup>, is associated only with female sterility, suggesting that snf may only be essential in the female germ line (Gans et al. 1975; Salz 1992). However, based on genetic criteria, this allele appears to retain some function (Gollin and King 1981). Similarly, the 3' deletion mutant allele, snf<sup>1A2</sup>, is not a candidate for a null allele because it retains significant amounts of snf gene activity [see below].

To determine unequivocally the null phenotype, we screened directly for deletions of the entire snf-coding sequence in a manner that was independent of phenotype. The P element in snf<sup>PR110</sup> was remobilized and 347 balanced stocks were established on the basis of eye color reversion. Each stock was then screened for deletions of the first snf exon by a combination of PCR and Southern analysis. Of 335 homozygous viable stocks, none contained deletions of the remaining snf exon. In contrast, 12 lethal derivatives all appeared to contain larger deletions. One of these deletion derivatives, snf<sup>1210</sup>, appeared to contain a breakpoint within the first exon. Sequencing revealed that snf<sup>1210</sup> does delete the entire coding sequence of snf, starting at nucleotide 21 of the 5'-untranslated region and extending into, but not beyond, the dhd-coding sequence [Figs. 1, 3].

Animals homozygous for snf<sup>1210</sup> were found to be embryonic lethals [data not shown]. Two lines of evidence demonstrated that the lethal phenotype is attributable to lack of snf function. First, the lethality mapped by both recombination and deficiency mapping to the same interval as the snf locus. Within this region there are only two other known lethals, l(1)4Fa and l(1)4Fb, both of which complement snf<sup>1210</sup> [Salz 1992, H. Salz, unpubl.]. Second, the lethality is not attributable to the simultaneous deletion of both dhd and snf because the lethality is not rescued by a transgene carrying a copy of dhd<sup>+</sup>.

Genetic characterization of the new snf alleles

Although snf<sup>1210</sup> is a non-sex-specific lethal, its function in germ-line sex determination is still evident when one examines the ovaries of viable combinations of snf mutations. For instance, snf<sup>1210</sup>/snf<sup>1A2</sup> [in the presence of a transgene carrying a copy of dhd<sup>+</sup>; see Fig. 1] and snf<sup>1210</sup>/snf<sup>1621</sup> females produced ovarian tumors.

Surprisingly, and in contrast to either snf<sup>1210</sup>/snf<sup>1A2</sup> females or snf<sup>1621</sup> homozygous females, females homozygous for snf<sup>1A2</sup> are fertile [in the presence of a transgene carrying a copy of dhd<sup>+</sup>; see Fig. 1]. The observation that the snf<sup>1A2</sup> deletion mutation is homozygous viable suggests that the carboxyl terminus of the protein is not essential for function. In vitro structure/function analysis of the mammalian U1A protein have shown previously that a fragment containing only the amino-terminal RRM binds U1 snRNA with the same affinity as the native protein [Jessen et al. 1991; Tsai et al. 1991].

The role of snf in somatic sex determination can be assessed by the strength of a female-lethal synergistic interaction with Sxl [Oliver et al. 1988; Steinmann-Zwicky 1988; Salz 1992]. When assessing the function of snf<sup>1621</sup>, we found that the lethal synergistic interaction it displayed with Sxl was surprisingly weak when compared with the Sxl–snf<sup>1621</sup> lethal synergistic interaction [Table 1; cf. crosses A and D]. Similarly, the lethal synergistic interaction snf<sup>1A2</sup> displays with Sxl, is clearly weaker than the Sxl–snf<sup>1621</sup> lethal synergistic interaction [Table 1; cf. crosses B and C to A]. The Sxl–snf<sup>1A2</sup> lethal synergism was only detectable when mothers were homozygous for snf<sup>1A2</sup> [cf. crosses B and C].

Because snf<sup>1621</sup> produces a stronger phenotype than loss-of-function mutations, it can be classified as a gain-of-function mutation. Gain-of-function mutations, like snf<sup>1621</sup>, whose mutant phenotypes are suppressed by an extra copy of the wild-type gene, are thought to disrupt the processes in which they normally participate [Salz 1992; Albrecht and Salz 1993; H. Salz, unpubl.]. The similarity in phenotype between snf<sup>1621</sup> and the viable loss-of-function mutations further suggests that complexes containing the the Sni<sup>1621</sup> protein interfere with the establishment of the Sxl autoregulatory loop by functional inactivation of U1 snRNPs. Taken together, these results demonstrate that snf<sup>1621</sup> is an antimorphic or dominant-negative mutation [Muller 1932, Herskowitz 1987].

Discussion

In Drosophila, the choice between male and female development requires that Sxl activity be tightly controlled throughout development. In females, Sxl maintains its activity state by an autoregulatory feedback loop in which SXL protein directs the female-specific splicing pattern of its own pre-mRNA [Cline 1984; Bell et al. 1991; Keyes et al. 1992; Sakamoto et al. 1992; Horabin and Schedl 1993a,b]. Although previous studies had shown that snf also participates in the establishment of the Sxl autoregulatory loop, it remained to be determined whether snf was directly involved in female-specific splicing [Albrecht and Salz 1993; Bopp et al. 1993; Oliver et al. 1993]. Our finding that the SNF protein is localized to the nucleus and has extensive homology to biochemically characterized RNA-binding proteins strongly suggests that SNF directly influences splicing regulation. Furthermore, by characterizing an unambiguous snf null mutation, we show that the function of snf is not limited to sex determination: A complete loss of snf function results in embryonic lethality.

snf encodes a U1 snRNP protein

We show here that snf encodes a nuclear protein with strong homology to two highly similar snRNP proteins,
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**Table 1. Female-lethal synergism between \textit{snf} and \textit{Sxl}**

| Cross | Maternal genotype | Percent relative viability$^a$ genotype, and the number of progeny \([n]\) recovered from each cross$^b$ |
|-------|-------------------|-------------------------------------------------------------|
|       | \textit{snf} \textit{+}/FM7 | \textit{Sxl}, \textit{sis-a}/FM7 females | \textit{Sxl}, \textit{sis-a}/FM7 females | nonbalancer males \([n]\) | control males \([n]\) |
| A     | \textit{snf}\textit{1621}/FM7 | 1.3 | 19 | 106 | \textit{snf}\textit{1621}/Y |
|       |                     |     |     |     | \textit{FM7}/Y | \textit{FM7}/Y |
| B     | \textit{snf}\textit{1A2}/FM7 | 261 | 230 | 259 | \textit{snf}\textit{1A2}/Y |
|       |                     |     |     |     | \textit{FM7}/Y | \textit{FM7}/Y |
| C     | \textit{snf}\textit{1A2}, \textit{snf}\textit{1A2} | 55 | 131 | 184 | \textit{snf}\textit{1A2} |
|       |                     |     |     |     | \textit{FM7}/Y | \textit{FM7}/Y |
| D     | \textit{snf}\textit{210}/FM7 | 122 | 131 | 184 | \textit{snf}\textit{210} |
|       |                     |     |     |     | \textit{FM7}/Y | \textit{FM7}/Y |
| E     | \textit{dhdP}8/FM7 | 248 | 232 | 184 | \textit{dhdP}8/Y |
|       | \textit{snf} \textit{+}/FM7 |     |     |     | \textit{FM7}/Y | \textit{FM7}/Y |
|       |                     |     |     |     | \textit{FM7}/Y | \textit{FM7}/Y |

The lethal synergistic interaction between \textit{snf} and \textit{Sxl} is enhanced by the addition of a mutation at another sex determination locus, \textit{sisterless-a} \([\text{sis-a}]\) as described in Salz (1992).

$^a$Relative viability is defined as the number \([n]\) of experimental animals relative to the number of control males recovered from each cross. To facilitate comparisons of relative viability between the different crosses, balancer males are the most appropriate control males. However, because these males are not as viable as nonbalancer males our estimates of viability are overestimates.

$^b$cm \textit{Sxl} \textit{+} \textit{sis-a}/Y males were crossed to the following females: [A] \textit{w} \textit{snf}\textit{1621} \textit{ct}/FM7\textit{c}; [B] \textit{w} \textit{snf}\textit{1A2}/FM7\textit{c}; [C] \textit{w} \textit{snf}\textit{1A2}/\textit{w} \textit{snf}\textit{1A2} \textit{p}\textit{[w+}, \textit{dhd}+, \textit{p}]; [D] \textit{w} \textit{snf}\textit{210}/FM7\textit{c}; [E] \textit{w} \textit{dhdP}8/FM7\textit{c}.

U1A and U2B$^*$. These two proteins, although very similar, bind different target RNAs (for review, see Kenan et al. 1991). U1A binds the U1 small nuclear RNA (snRNA) and is incorporated into the U1 snRNP complex, whereas U2B$^*$ [in conjunction with U2A$'$] binds the U2 snRNA and is incorporated into the U2 snRNP complex. In many respects, SNF resembles U2B$^*$ more closely than U1A. Not only does SNF have a similar overall structure and molecular weight to U2B$^*$ but it also contains a 5-amino-acid motif within the amino-terminal RRM domain [marked 5-MER in Fig. 4B] that is identical to that found in U2B$^*$. In the human protein, this penta-amer region has been shown to be necessary for the U1A protein to discriminate between U1 and U2 RNA (Scherly et al. 1990a,b; Bentley and Keene 1991). Functional studies, on the other hand, demonstrate that SNF is a U1 snRNP protein. An epitope-tagged SNF protein immunoprecipitates from both \textit{Drosophila} and human cell extracts, U1 snRNA but not U2 snRNA (Harper et al. 1992). Moreover, R. Mancebo and S. Mount have recently determined that the SNF protein is present in purified \textit{Drosophila} U1 snRNP complexes (pers. comm.). Clearly, \textit{snf} encodes a U1 snRNP protein. Whether SNF is the only U1A-like protein in \textit{Drosophila} remains to be determined.

\textbf{Role of \textit{snf} in establishing the female-specific splicing pattern of \textit{Sxl}}

Although the fact that a \textit{snf} null allele is a non-sex-specific lethal establishes that \textit{snf} is required for developmental processes unrelated to sex determination, its role in regulating \textit{Sxl} can provide us with valuable insights into its biochemical function. As illustrated in Figure 7, the female-specific SXL protein promotes exon skipping of its own pre-mRNA by binding directly to intron sequences surrounding the male-specific exon, thereby preventing the general splicing machinery from recognizing the male-specific 5' splice site (Sakamoto et al. 1992, Horabin and Schedl 1993a,b). Because it is also required to establish the female-specific splice site choice, \textit{snf} is likely to function in conjunction with \textit{Sxl} to bias the general splicing machinery against the recognition of the male 5' splice site. These studies suggest that as a component of a U1 snRNP, the SNF protein modulates 5' splice site recognition. Because U1 snRNPs function early in the splicing pathway, many studies have already implied that differential binding of the U1 snRNP to the 5' splice site plays a role in the regulation of splice site choice (Rosbash and S6raphin 1991; Hoff- man and Grabowski 1992; Siebel et al. 1992; Eperon et al. 1992, 1993; Nandabalan et al. 1993). Our studies, however, provide the first demonstration in a multicellular organism that mutations in a U1 snRNP protein alter splicing in vivo.

As a generic component of the U1 snRNP, SNF might be required for the recognition of all 5' splice sites. Our genetic data are certainly consistent with such a conclusion: The \textit{snf} null phenotype is a non-sex-specific lethal. Given this scenario, how do we explain the observation that the viable loss-of-function and gain-of-function mutations appear to affect the regulation of \textit{Sxl} preferentially? In the mammalian system, relative levels of splicing factors have been shown to interact in a concentration-dependent manner to influence splice site selection (Ge and Manley 1990; Krainer et al. 1990, Mayeda and Cold Spring Harbor Laboratory Press on July 20, 2018 - Published by genesdev.cshlp.org Downloaded from
the SXL protein is predominately cytoplasmic (Bopp et al. 1991, 1993). Thus, in the germ line, it would appear that only a limited amount of SXL protein is transported to the nucleus where it functions and, therefore, it is always rate limiting (Bopp et al. 1993). Consequently, even small reductions in the level of SNF protein would cause an imbalance in the germ line. In the soma, on the other hand, all of the SXL protein is nuclear localized, suggesting that it is not rate-limiting (Bopp et al. 1991).

Even as a component of a U1 snRNP, SNF might not be required for all splicing events. The fact that snf has greater sequence homology to U2B' than to U1A suggests that the function of snf may not precisely parallel that of the mammalian U1A protein. Perhaps snf is similar to the yeast U1A protein, which is not essential for the recognition of most 5' splice sites (Liao et al. 1993). In yeast, the absence of U1A has no effect on the assembly or function of U1 snRNPs, either in vitro or in vivo. By analogy, snf function would not be required for the function of most U1 snRNPs, but only for regulated splice sites, such as the Sxl male 5' splice site. If correct, this model predicts that a null mutation would not eliminate splicing but would instead only disrupt specific regulated splicing events. This possibility can now be tested.

Materials and methods

Note on nomenclature

A considerable amount of confusion surrounds the naming of the snf locus. snf was initially identified in a screen for female-sterile mutations and named fs(1)1621 (Gans et al. 1975). Once identified as a sex determination gene it was renamed sans-fille (snf) [Oliver et al. 1988] and liz [Steinmann-Zwicky 1988]. Although we have constantly used the acronym snf, pronounced sniff, others still identify the locus as fs(1)1621 and liz in the literature. In light of the fact that snf is an snRNP protein whose null phenotype is a non-sex-specific lethal, none of the names assigned to the snf locus are consistent with its phenotype. We therefore propose to retain the acronym snf and rename the locus splicing necessary factor.

Fly stocks and culture conditions

snf1621 is described in Lindsley and Zimm [1992] as fs(1)A1621. snf1621, Df(1)DEB4D, Dp(1;2)4FRDup, are described in Salz [1992], and dhd+ and the dhd+ transgene P(w+, dhd+) are described in Salz et al. (1994). The remaining mutations and balancer chromosomes are either described in the text or in Lindsley and Zimm [1992]. All crosses were carried out at room temperature, which ranged from 22°C-25°C, on a standard cornmeal, yeast, molasses, agar medium.

Isolation of P-element-induced snf mutations

To isolate P-element-induced snf mutations, a P[lacW] enhancer trap element [Bier et al. 1989] within the dhd gene was mobilized and screened for mutations that did not complement snf1621. To mobilize the P-element insertion, w dhd+/FM7c virgins were mated to males containing a source of transposase, Δ2-3 [Robertson et al. 1988], of genotype w; Sb Δ2-3/TM6. The F1 males [w dhd+/Sb Δ2-3/+ ] were mated to C(1)DX y w f...
virgin males. It is in the germ line of these F1 males that the P element can transpose. Single male progeny of this cross carried a new insertion event (recognized by dark eye color than dhd76 males, which have light yellow eyes) were crossed to Df(1)DEB4D/FM7c virgins. Fertility tests of the females with the deficiency from each vial were scored for an eggless female-sterile phenotype. One event, PR110, had this phenotype and was found not to complement snf1621.

Derivatives of snfPR110 were isolated as follows: snf1621 was isolated in a similar screen as described above except that it was selected for the loss of the P-element insertion as judged by the loss of the w+ eye color marker. snf1210 was isolated in a screen that allowed the recovery of lethals. The P element in snfPR110 was mobilized by crossing w snfPR110/FM7c virgins to wy Df/TMS, D2-3 males. The F1 males of genotype, w snfPR110 +/TMS, D2-3 were mated to wy Df(1)ovoG6/FM7c virgins. Df(1)ovoG6 is an X chromosome deficiency which does not uncover the snf locus [Olive et al. 1987]. Females of the genotype w snfPR110 y w Df(1)ovoG6 in which the P element had remobilized [recognized because the females have white eyes] were individually mated to FM7c males and stocks established in the next generation. Males from 335 derivative lines were viable and were therefore screened by PCR for the loss of the first snf exon (P2-P3 amplification product, Fig. 1) without the concomitant loss of the second dhd exon (P6-P7 amplification product, Fig. 1). None of these lines were deleted for the snf gene. Twelve lines contained homozygous lethal derivatives and therefore could not be analyzed by PCR. Consequently, the breakpoints were characterized by genomic Southern blots. One line, snf1210 was identified as a small deletion that only extended through the first exon of the snf gene.

Molecular analysis

Unless otherwise noted, standard molecular techniques followed the procedures described in Ausubel et al. [1987]. Five cDNAs corresponding to snf were obtained by screening a Drosophila head cDNA library in Agt11 [Itoh et al. 1986] using either a ~2.5-kb BamHI-KpnI or a ~2.8-kb KpnI--EcoRI genomic fragment as probes (Fig. 1). One cDNA clone, AKE-14, was characterized further and found to contain a 788-bp insert that hybridized exclusively to the altered transcript in snfPR110 [Fig. 2]. This cDNA was then subcloned into pBluescript (Stratagene) for sequence analysis. Unidirectional deletions of pBKE-14 were made using exonuclease III and mung bean nuclease [New England Biolabs] as described [Henikoff 1984]. These inserts were sequenced by the dyeoxy chain termination method [Sanger et al. 1977] using Sequenase v2.0 [U.S. Biochemical]. The genomic subclones, 2.5-kb BamHI-KpnI and 2.8-kb KpnI--EcoRI were also sequenced in a similar manner. Data base searches and alignments were generated by the BLAST programs at NCBI using the BLAST network service [Altschul et al. 1990].

RNA from adult flies was isolated as described in Chomczynski and Sacchi [1987]. Northern transfers and RNase T2 protection assays were carried out as described previously [Flickinger et al. 1992]. Sequencing of the mutant alleles was carried out by direct cycle sequencing of gel-purified PCR-amplified genomic DNA fragments with the CircumVent kit according to the manufacturer's directions [New England Biolabs]. In short, 1 μg of genomic DNA from wild-type or mutant snf alleles was amplified in a MJ Research PCT-100 thermocycler using 0.25 μM of each primer, 0.2 mM each dNTP [Promega], 2.5 units of Taq DNA polymerase [Boehringer Mannheim] in 100 μl of a standard buffer containing 3 mM MgCl2 [Ausubel et al. 1987]. The genomic DNA from snf1621 homozygotes and its parent chromosome were PCR amplified with the P2-P4 primer pair, whereas DNA from snf1210 heterozygotes was amplified using the P2-P7 and P1-P5 primer pairs, respectively [Fig. 1]. Forty cycles of the following parameters were used: 30 sec at 94°C, 45 sec at 49°C, 90 sec at 72°C, followed by a final 5-min extension step at 72°C. The following primers were used: P1, 5'-CGTCCACCAAGC-CATGATA-3'; P2, 5'-CTCAAATACAGACGAAAT-3'; P3, 5'-GAATCGGATTGGAAGTACG-3'; P4, 5'-AGATCTCACA-CATACACAAATAA-3'; P5, 5'-GATTGCCTTTGAGCCCTT-3'; P6, 5'-GTTCCCGTATAGTATCGAGA-3'; and P7, 5'-ATT-GTTCTTCTAGTCGCC-3'.

Protein analysis

Immunoblot analysis. Adult flies, embryos, and larvae were collected and frozen on dry ice and then homogenized in ~150 μl of 2X SDS loading buffer. Samples were boiled for ~5 min and then centrifuged for 1 min at 4000g to pellet the debris. Samples [4-30μl] were loaded on a 12.5% polyacrylamide gel and electrophoresed. Proteins were electrottered to a PVDF membrane [Tropix] as described [Towbin et al. 1979]. The membrane was blocked and then incubated with a 1:10 dilution of culture supernatant containing mAb 4G3 [Habets et al. 1989]. The primary antibody was visualized using chemiluminescence with the Western-Light kit according to the manufacturer's directions [Tropix]. In the immunoblots shown in Figure 5, the only antigen detected by mAb 4G3 is a 28-kD protein. However, we and others have observed that a larger cross-reacting protein is sometimes detected by this antibody in both Drosophila and HeLa cell extracts [Amuro et al. 1992; Tazi et al. 1993; Flickinger, unpubl.]. Because the larger protein is similar in size to U1A and is detected in purified U1 snRNPs, it was identified as U1A [Amuro et al. 1992; Tazi et al. 1993]. Interestingly, we do not detect this 37-kD protein in extracts from either snf1621 or snfPR110 animals under conditions where we do detect it in extracts from wild-type animals. Although the origin of this cross-reacting protein has yet to be determined, its absence in snf mutant extracts suggests the possibility that it is encoded by the snf gene. However, because we have no evidence of any alternative splicing within the snf gene, it is more likely that the expression of this 37-kD antigen represents a protein complex containing the SNF protein.

Immunolocalization in whole mounts. The fixation and antibody staining to embryos and salivary glands were performed as described in Salz et al. [1994]. The mAb 4G3 was diluted 1:10, stained with a biotinylated goat anti-mouse secondary antibody [Chemicon], diluted 1:5000 and visualized with the Vectastain ABC elite HRP signal detection system according to the manufacturer's instructions.

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