Both Loss-of-Function and Gain-of-Function Mutations in *snf* Define a Role for snRNP Proteins in Regulating *Sex-lethal* Pre-mRNA Splicing in Drosophila Development

Helen K. Salz and Thomas W. Flickinger[1]

Department of Genetics, Case Western Reserve University, Cleveland, Ohio 44106-4955

Manuscript received January 22, 1996
Accepted for publication March 29, 1996

ABSTRACT

The Drosophila *snf* gene encodes a protein with functional homology to the mammalian U1A and U2B″ snRNP proteins. Studies, based on the analysis of three viable alleles, have suggested a role for *snf* in establishing the female-specific splicing pattern of the sex determination switch gene, *Sex-lethal*. Here, we show that the non-sex-specific lethal null allele is required for female sex determination, arguing against the formal possibility that the viable alleles disrupt a function unrelated to *snf's* wild-type function. Moreover, we find *snf* is required for normal cell growth and/or survival, as expected for a protein involved in a cell-vital process such as RNA splicing. We also show that of the three viable alleles only one, snf^{JA2}, is a partial loss-of-function mutation. The other two viable alleles, snf^{1621} and snf^{e8H}, encode antimorphic proteins. We find the antimorphic proteins are mislocalized and correlate their mislocalization with their molecular lesions and mutant phenotypes. Finally, we provide genetic evidence that the antimorphic alleles interfere with the autoregulatory splicing function of the Sex-lethal protein. Based on these studies we suggest a model in which the snRNP protein, Snf, functions with Sex-lethal to block recognition of the regulated male-specific exon.

IN higher eukaryotes, primary RNA transcripts (pre-mRNA) undergo a complex series of processing events in the nucleus before they are transported to the cytoplasm. In one of these processing events, known as RNA splicing, the introns are removed and the exons are ligated together (for review, see MANLEY 1993; MOORE *et al.* 1993). Selection and removal of introns takes place within the spliceosome, a large complex that includes both small nuclear ribonucleoprotein complexes (snRNPs) and non-snRNP proteins. Assembly of the spliceosome takes place in an ordered step-wise fashion, and it is during the early steps of spliceosome assembly that the identification of the intron/exon boundaries takes place. The initial selection of the 5′ splice sites is accomplished by the stable association of the U1 snRNP with the pre-mRNA. Similarly, the initial selection of the 3′ splice site is accomplished through a stable interaction of the U2 snRNP with the branch site. While the base-pairing interactions between the splice sites and the RNA component of the U1 and U2 snRNPs guide the snRNPs to the correct position on the pre-mRNA, additional protein-protein interactions are required for spliceosome assembly. For example, the U2 auxiliary factor (U2AF) binds to the polypyrimidine tract to facilitate the interaction of the U2 snRNP with the branch site (ZAMORE and GREEN 1989). Similarly, the SR protein, ASF/SF2, facilitates binding of the U1 snRNP to the 5′ splice site (KOHTZ *et al.* 1994). Another SR protein, SC35, is thought to bridge the interaction between the U1 and U2 snRNPs (WU and MANIATIS 1993). Interestingly, *in vitro* studies have shown that a large excess of SR proteins can initiate spliceosome complex formation without U1 snRNPs (CRISPINO *et al.* 1994; TARN and STEITZ 1994). The absence of U1 snRNPs, however, results in spliceosome complex formation that is not as accurate, suggesting that the role of the U1 snRNP is to guarantee that spliceosome formation occurs at authentic 5′ splice sites (TARN and STEITZ 1994).

Control of RNA splicing has proven to be a common means of tissue-, stage- and sex- specific gene regulation. Studies from a number of systems suggests that alternative splicing is controlled by differentially expressed proteins that modify the ability of the spliceosome to assemble at specific splice sites (for review, see HODGES and BERNSTEIN 1994). In Drosophila, the sex-specific splicing regulation of the binary switch gene *Sex-lethal (Sxl)* occurs via a negative regulatory mechanism where the recognition of the regulated male-exon is blocked in females (for review, see BAKER 1989; CLINE 1993; CRONMILLER and SALZ 1994). *Sxl* expression is limited to females because only the female-specific mRNAs have a long open reading frame (ORF). The male-specific mRNAs contain multiple stop codons within the regulated male-exon (BELL *et al.* 1988; SAM-

Corresponding author: Helen K. Salz, Department of Genetics, Case Western Reserve University School of Medicine, 10900 Euclid Ave., Cleveland, OH 44106-4955. E-mail: hks@po.cwru.edu

[1] *Present address:* Molecular Biology Department, NC2-121, The Cleveland Clinic Foundation, Cleveland, OH 44195.

UELS *et al.* 1991). Once established, the female-specific expression of *Sxl* is maintained throughout development (SALZ *et al.* 1989). Establishment of *Sxl* expression in female embryos, however, takes place at the level of transcription. During the first few hours of embryogenesis, somatic *Sxl* expression is initiated by transcriptional activation of the internal female-specific promoter (P_{Early}) in response to the appropriate dose (*XX*) of *X*-linked transcription factors (KEYES *et al.* 1992; ESTES *et al.* 1995). This initial burst of Sxl protein encoded by the P_{Early}-derived pre-mRNAs directs the splicing of the non-sex-specific pre-mRNAs that are generated from the constitutive promoter, P_{Late} (BELL *et al.* 1991; KEYES *et al.* 1992). By participating in its own splicing regulation, *Sxl* establishes an autoregulatory feedback loop that results in the irreversible commitment to the female sexual fate, including the vital process of *X*-chromosome dosage compensation. Consequently, loss-of-function mutations are lethal only to females and gain-of-function mutations are lethal only to males.

In addition to its autoregulatory splicing function, Sxl protein also directs the splicing pattern of at least one other pre-mRNA, encoded by the *transformer (tra)* gene (for review see BAKER 1989). Consistent with its role as a splicing regulator, Sxl binds to polypyrimidine sequences near the regulated splice sites on both the *Sxl* pre-mRNA and the *tra* pre-mRNA (BELL *et al.* 1988; SAMUELS *et al.* 1991; SAKAMOTO *et al.* 1992; SAKASHITA and SAKAMOTO 1994; WANG and BELL 1994; KANAAR *et al.* 1995). In each case, Sxl is thought to direct splice site selection by blocking spliceosome assembly at the regulated splice sites, however, the mechanism by which Sxl blocks spliceosome assembly appears to be different in each case. The best characterized mechanism is the regulated splicing of the *tra* pre-mRNA. Sxl directs the female-specific splicing of the *tra* pre-mRNA by promoting a shift in 3′ splice sites (SOSNOWSKI *et al.* 1989). In this case, Sxl appears to function by directly competing for the same binding site as a non-snRNP essential splicing factor, U2AF, thus preventing recognition of the regulated 3′ splice site (VALCARCEL *et al.* 1993).

Sxl directs the splicing of its own pre-mRNA by blocking recognition of the male-specific exon. *In vivo,* exon skipping is dependent on polypyrimidine sequences located downstream of the male exon 5′ splice site, thus suggesting that the key to *Sxl* autoregulation is blockage of the male exon 5′ splice site (SAKAMOTO *et al.* 1992; HORABIN and SCHEDL 1993a,b). However, because the critical sequences are located within the intron several hundred base pairs away from the regulated 5′ splice site, it is unlikely that Sxl directly prevents spliceosome assembly. Instead, it seems more plausible that Sxl's ability to block spliceosome assembly at a distance requires interactions with other proteins.

An excellent candidate for a protein that interacts with Sxl is encoded by the *snf* gene. *snf* was originally characterized as positive regulator of *Sxl* because in females, several different viable *snf* mutations disrupt *Sxl* autoregulation, resulting in the accumulation of *Sxl* mRNAs spliced in the male instead of the female mode (OLIVER *et al.* 1988, 1993; STEINMANN-ZWICKY 1988; SALZ 1992; ALBRECHT and SALZ 1993; BOPP *et al.* 1993). *snf*'s function, however, is not limited to sex determination. A complete loss of *snf* function results in lethality (FLICKINGER and SALZ 1994).

snf encodes a 28-kD nuclear protein that has extensive sequence similarity to two nearly identical vertebrate RNA binding proteins, U1A and U2B″ (FLICKINGER and SALZ 1994). Snf, U1A and U2B″ belong to a large family of RNA binding proteins that contain RNA recognition motifs (RRM; for review see, MATTAJ 1993; BURD and DREYFUSS 1994). In terms of size and structure, Snf is more similar to U2B″ than to U1A. In agreement with its extensive similarity to U2B″, Snf is recognized by a monoclonal antibody specific for the human U2B″ protein (FLICKINGER and SALZ 1994). In terms of function, however, Snf is the functional equivalent of both U1A and U2B″. Like U1A, Snf binds U1 snRNA and is incorporated into U1 snRNPs (HARPER *et al.* 1992; POLYCARPOU-SCHWARZ *et al.* 1996). Like U2B″, Snf binds U2 snRNA when bound to its partner the U2A′ protein and is incorporated into U2 snRNPs (POLYCARPOU-SCHWARZ *et al.* 1996). These results suggest that Snf is the only U1A/U2B″ protein in Drosophila, a hypothesis supported by the finding that antibodies specific for the human U1A protein fail to detect any other protein in Drosophila extracts (POLYCARPOU-SCHWARZ *et al.* 1996).

Although U1A and U2B″ are intensively studied as models of RNA-protein interactions, their function within the spliceosome is not known. In this paper we investigate the role that the Drosophila homologue *snf* plays in regulating pre-mRNA splicing by addressing the question of how a group of viable *snf* mutations specifically interferes with *Sxl* pre-mRNA splicing. In the first part of the paper, we establish, through our analysis of the *snf* null mutation (snf^{J210}), that a loss of *snf* function interferes with sex determination. This result argues against the formal possibility that the mutant *snf* proteins encoded by the viable alleles have a novel function unrelated to *snf*'s wild-type function. These data also establish that the loss of *snf* function results in cell growth and/or survival defects, a phenotype expected for a cell-vital process such as RNA splicing. In the second part of the paper, we establish, by using genetic dosage studies, that only one of the extant viable alleles (snf^{JA2}) is a partial loss-of-function (hypomorph). The other two alleles (snf^{1621} and snf^{e8H}) encode antimorphic proteins that exhibit dominant-negative properties under certain genetic conditions. We localize these antimorphic proteins and correlate their mislocalization with their molecular lesions and mutant phenotypes. Finally, we provide genetic evidence indicating that both loss-of-function and antimorphic al-

leles interfere with the autoregulatory function of the wild-type Sxl protein. Together, these results point to a model in which Sxl does not passively block spliceosome assembly, but instead recruits Snf, perhaps as a component of an snRNP, to block spliceosome assembly. This is the first suggestion that the role of U1A and/or U2B″ within the spliceosome is to interact with differentially expressed non-snRNP proteins to modulate splice site choice.

MATERIALS AND METHODS

Fly stocks and culture conditions: All mutations and balancer chromosomes are either described in the text or in LINDSLEY and ZIMM (1992). The *FM7c-lacZ* chromosome is a standard *FM7c* balancer chromosome with a *P*-element insertion that contains a β-galactosidase marker gene under control of the *fushi tarazu* promoter obtained from the Bloomington Stock Center. The *otu::Sxl* transgene was generated and generously provided to us by J. HAGAR and T. CLINE before publication. It should be noted that *snf* is described in LINDSLEY and ZIMM (1992) as *fs(1)A1621*. Elsewhere in the literature, *snf* is referred to as *sans-fille* (for which *snf* is an acronym), *liz* and *fs(1)1621*. Except where noted, all crosses were carried out at room temperature, which ranged from 22 to 25°, on a standard cornmeal, yeast, molasses, agar medium.

Lethal phase analysis: The lethal phase of snf^{J210} was determined by counting animals at the different stages of development according to standard procedures. In FLICKINGER and SALZ (1994) we reported that snf^{J210} was an embryonic lethal. Consistent with these findings we found that in embryos collected from snf^{J210}/*FM7-lacZ* mothers, the snf^{J210} hemizygotes (identified by the absence of β-galactosidase expression) are morphologically normal but only developed until about stage 14 (data not shown). Surprisingly, we find that the lethal phase varies with genetic background. Upon outcrossing the snf^{J210} chromosome to two different nonbalancer strains, snf^{J210} embryos hatch and die as first instar larvae (our unpublished observations and BEAT SUTER, personal communication). Whether the difference between late embryonic death and early larval lethality is of developmental significance remains to be determined.

Generation of somatic mosaics: A description of the methods and the stocks utilized to generate somatic clones can be found in XU and RUBIN (1993). Briefly, *y w snf*, P[ry^+;*hsp70::neo;FRT*]18A/*FM7* females were crossed to y^+ snf^+ P[ry^+;*hsp70::neo; FRT*]18A; *MRKS*, P[*hsp70::flp*]/*nkd* males, and the resulting larvae were subjected to a 1 hr heat shock in a 38° water bath during the first (24–48 hr), second (48–72 hr) or crawling third (hand-picked) instar. The resulting *y w snf*, P[ry^+;*hsp70::neo;FRT*]18A/snf^+, P[ry^+;*hsp70::neo;FRT*]18A; *MKRS*,P[*hsp70::flp*]/+ females were collected and screened for phenotypically *yellow* or *white* clones. Clones in the eye were identified by screening for white patches under a dissecting microscope. Clones in the wing and the foreleg were identified by screening mounted wings and forelegs for *yellow* bristles under a compound microscope. To control for variations in heat shock conditions as well as variations in the timing of heat shock, all mutant clones were induced at the same time as the control clones.

Immunolocalization in whole mounts: Embryos and salivary glands for antibody staining were collected and fixed according to standard methods. Snf protein was detected with mAb4G3 diluted 1:20 (HABETS *et al.* 1989; FLICKINGER and SALZ 1994). Detection was carried out either by staining with a biotinylated goat anti-mouse secondary antibody (Chemicon) diluted 1:5000 and visualized with the Vectastain ABC elite horseradish peroxidase (HRP) signal system and DAB staining according to manufacturer's instructions or with a Lissamine-Rhodamine conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories) diluted 1:200. β-galactosidase was detected by staining with a rabbit antibody against β-galactosidase (Cappel) diluted 1:200 and detected using an alkaline phosphatase conjugated goat anti-rabbit secondary antibody (BioRad) diluted 1:5000 and visualized using McGadey's reagent (BCIP and NBT). For simultaneous detection of both Snf and β-galactosidase, the primary antibodies were applied together overnight at 4°, then the secondary antibodies were applied together for 2 hr at room temperature. The color reactions for visualization were carried out in the following order: (1) embryos were treated with the Vectastain ABC elite HRP signal system, (2) stained with McGadey's reagent (BCIP and NBT) and (3) stained with DAB. Whole mount salivary glands were viewed using epifluorescence optics on a Zeiss Axiophot microscope and photographed under identical conditions. Embryos were viewed using Nomarski optics on a Leitz Diaplan microscope.

Immunolocalization to polytene chromosomes: Salivary glands from female larvae of the appropriate genotypes were dissected in phosphate-buffered saline (PBS) and prepared as described in ZINK and PARO (1989), except that the salivary glands were first fixed for 10 sec in 3.7% formaldehyde, 1% Triton X-100 in PBS and then fixed for 2 min in 3.7% formaldehyde, 50% acetic acid. The primary antibody, mAb4G3, was used at a concentration of 1:20 and incubated overnight at 4° in a humidified chamber. Detection was carried out with a Lissamine-Rhodamine conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories) diluted 1:200 and incubated for 2 hr at room temperature in a humidified chamber. Counterstaining of the chromosomes was done for several minutes with 0.5 mg/ml 4′,6-diamidino-2-phenylindole. Slides were mounted in 50% glycerol/PBS and viewed using epifluorescence optics on a Zeiss Axiophot microscope.

Sequencing of snf^{e8H}: Sequencing of snf^{e8H} and its parent chromosome was carried out by direct cycle sequencing of gel-purified PCR-amplified genomic DNA as described in FLICKINGER and SALZ (1994).

RESULTS

Zygotic *snf* function is required for sexual differentiation and a cell vital process during postembryonic development: As the first step in defining *snf*'s function, we asked whether zygotic function is necessary for normal sexual differentiation by characterizing the sexual phenotype of the *snf* null mutation, snf^{J210}. snf^{J210} is an unambiguous null mutation because it contains a small deletion of the entire ORF, starting in the 5′ untranslated region and extending into the adjacent nonvital gene, *deadhead (dhd)* (FLICKINGER and SALZ 1994; SALZ *et al.* 1994). Because snf^{J210} is a non-sex-specific lethal, we examined the phenotype of a small group of mutant cells in a genetic mosaic animal. Genetically marked clones homozygous for the *snf* null were generated by mitotic recombination, and those clones that fell within the sexually dimorphic region of the female foreleg were examined in detail (see MATERIALS AND METHODS). Our analysis of somatic clones induced during the second instar larval period supports a role for *snf* in sexual differentiation. All the clones we observed contained

TABLE 1

Sexual phenotype of somatic clones homozygous for a *snf* null allele

Time of clone induction	No. forelegs scored for clones	No. and sex of clones recovered in the foreleg
First instar	250	0
Second instar	140	5/5 sexual intermediates
Third instar	130	2/2 female

Frequency and sex of *y snf^{J210}/y snf^{J210}* clones in the sexually dimorphic region of the foreleg generated in a wild-type background by the flp-mediated recombination system during the first (24–48 hr), second (48–72 hr) or crawling third (handpicked) instar larvae (see MATERIALS AND METHODS for a detailed description).

bristles with a sexual phenotype that was intermediate between the expected male and female phenotype (Table 1; Figure 1A).

Surprisingly, clones induced later in development, during the third larval instar, failed to show any signs of sexual transformation: the bristles were phenotypically indistinguishable from normal female bristles (Figure 1B). Judging by the stability of the maternally provided Snf protein described below, we believe that the female sexual development of the clones induced late in larval development can be attributed to perdurance of the wild-type gene product through the few cell divisions remaining before differentiation (GARCIA-BELLIDO and MERRIAM 1971; RIPOLL 1977).

When clones were induced during the first instar larval period, we failed to recover any clones in the foreleg. Clones induced early in development are expected to be large, therefore the failure to recover clones may suggest that large patches of mutant cells result in the death of the organism. On the other hand, given the central roles that U1 and U2 snRNPs play in RNA processing, a defect in cell growth and/or cell survival would not be unexpected. To determine if the loss of *snf* function results in a cell growth and/or cell survival defect, we scored the number and size of *snf^{J210}* clones induced during the first, second and third instar larval periods in two other regions of the adult cuticle for which we had scorable markers: the eye, and the anterior portion of the wing blade in the row of bristles termed the "triple row." Surprisingly, we failed to observe any clones in the eye, irrespective of when in development clonal induction took place ($n > 500$ for each clonal induction time period).

In contrast to the situation in the eye, clones were recovered in the wing. Mutant clones induced during the first and second larval instars were rarely recovered (Table 2). The few clones we did recover were much smaller than control clones (Figure 2). Control clones ranged in size from one to 20 bristles, whereas *snf^{J210}* clones only consisted of a single bristle. The recovery

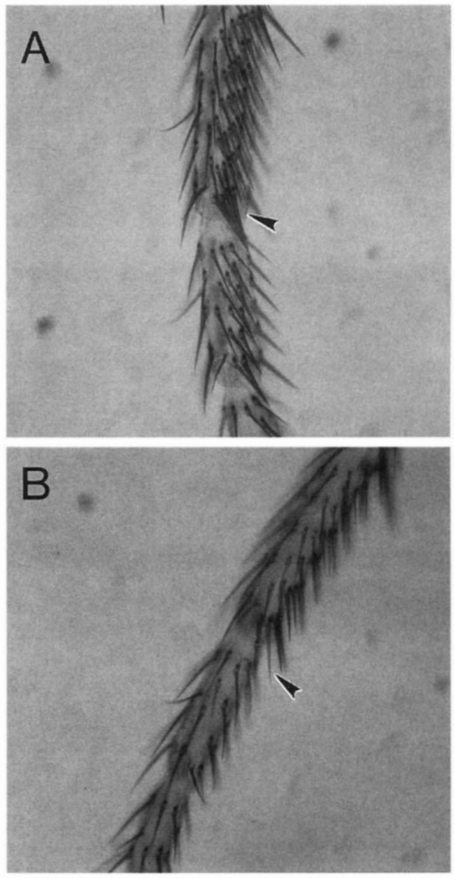


FIGURE 1.—Sexual phenotype of *snf^{null}* clones in the female foreleg. (A) Bristles within a *y snf^{J210}/y snf^{J210}* clone (indicated by arrowhead) induced during the second instar larvae are transformed into sexual intermediates: they are phenotypically neither male nor female. (B) A *y snf^{J210}/y snf^{J210}* clone that contains only a single bristle (indicated by the arrowhead) induced during the third instar larvae period fails to show any signs of sexual transformation: the bristle is phenotypically female.

of mutant clones increased dramatically when induced during the third larval instar. Although, again the mutant clones were smaller than control clones. Control clones ranged in size from one to 14 bristles, whereas *snf^{J210}* clones contained only one or two bristles. Thus, together these finding support the suggestion that *snf* is required for a cell-vital process necessary for cell growth and/or survival.

The maternally provided Snf protein is detectable throughout embryogenesis: Although *snf* is likely to encode a general splicing factor, we have found that male embryos hemizygous for the null allele complete embryonic development without any gross developmental abnormalities (see MATERIALS AND METHODS). Because we have previously shown that Snf protein is provided to the embryo by the mother (FLICKINGER and SALZ 1994), it is possible that the unexpected normal development of these *snf* null embryos is attributable to maternally provided protein lasting through embryogenesis. To examine this possibility directly, we collected embryos

TABLE 2

snf^{null} clones are recovered less frequently than control clones

	Clone frequency (no. of clones/no. of wings scored)[a]		
	First instar	Second instar	Third instar
snf^{J210}	0.008 (2/247)	<0.01 (0/100)	1.26 (88/70)
Control[b]	0.025 (6/236)	0.18 (18/98)	1.94 (128/66)

Comparison of the size and frequency of *y* snf^{J210}/*y* snf^{J210} and *y* control clones in the wing generated in a wild-type background by the flp-mediated recombination system during the first (24–48hr), second (48–72 hr) or crawling third (hand-picked) instar larvae (see MATERIALS AND METHODS for a detailed description).

[a] Mutant clones were identified by screening mounted wings for yellow bristles in the anterior portion of the wing blade termed the triple row under a compound microscope.

[b] Two sets of controls were run for each experiment. In the first set, snf^{+} clones were induced, and in the second set snf^{JA2} clones were induced. No differences in clone size or frequency were noted between the two control lines, therefore only the data from the snf^{JA2} controls are presented here. While snf^{JA2} is mutant for *snf*, it remains homozygous viable; thus as a derivative of the same parent chromosome as snf^{J210}, it provides the best "wild-type" control possible.

from snf^{J210}/*FM7-lacZ* mothers and stained them with antibodies against Snf and β-galactosidase. In this collection, the snf^{J210} hemizygous male embryos were identified by the absence of β-galactosidase expression. As expected for a maternally provided protein, we found that snf^{J210} embryos stained with antibodies against Snf early in embryogenesis. Interestingly, we also found that the maternally provided Snf protein continued to be detectable at near normal levels throughout embryogenesis (data not shown).

Gene dosage studies: Although *snf* encodes a vital gene, there is a class of viable alleles that have a pronounced effect on *Sxl* regulation. Of the three extant viable *snf* alleles only one, snf^{1621}, has a phenotype as a homozygote. snf^{1621} homozygous females are sterile because *Sxl* is not expressed in the germline (OLIVER *et al.* 1988, 1993; STEINMANN-ZWICKY 1988; SALZ 1992; BOPP *et al.* 1993). The other two alleles, snf^{JA2} and snf^{e8H}, are fertile as homozygotes; their only described phenotype is a female-lethal synergistic interaction with loss-of-function *Sxl* mutations (SALZ 1992; FLICKINGER and SALZ 1994). In all cases, *snf* exhibits a strong maternal effect with respect to this female-lethal synergistic interaction.

Here we present a series of gene dosage studies to determine the nature of the *snf* mutations. In these studies we compare the strength of the maternal-effect female-lethal synergistic interaction phenotypes of animals with different doses of mutant and wild-type *snf* alleles. As illustrated in Table 3, we have used two different assays to compare the strengths of the lethal synergistic interactions between *snf* and *Sxl*. In test no. 1, the viability of *snf*, +/+, *Sxl* daughters is compared to control animals from the same cross. This weak interaction between *Sxl* and *snf* provides a sensitive assay in which to compare the relative strengths of different mutant allele combinations. In some cases, however, this interaction proved to be too weak, therefore we have also included the results from a second stronger assay (test no. 2). In test no. 2 the lethal synergistic interaction was strengthened by the addition of *sis-a*, a mutation at another sex determination locus and the viability of *snf*, +/+, *Sxl*, *sis-a* females is compared to control animals (SALZ 1992). Together these assays provide a reliable measure of *snf* gene activity. Because the strength of these lethal synergistic interactions can be modified by different genetic backgrounds (data not shown), we have carefully controlled for genetic background and we have limited our comparisons to crosses with similar genetic backgrounds. It should be noted that, like snf^{1621} homozygous females, many of the mutant combinations described here result in female sterility due to their inability to express *Sxl* in the germline.

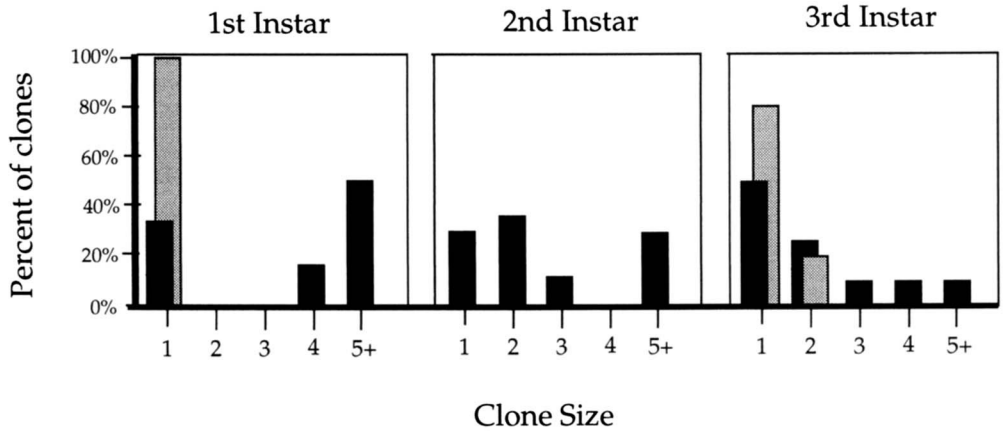


FIGURE 2.—snf^{null} clones are smaller than control clones in the wing. Comparison of the number of bristles in a *y* snf^{J210}/*y* snf^{J210} clone (▨) to that found in control clones (■) induced either during the first, second or third instar larval period (see legend under Table 2 and MATERIALS AND METHODS for details).

TABLE 3

***snf* gene dosage studies**

	Relevant maternal genotype[a]	Test no. 1[b]: lethal synergism with *Sxl* (% viability)	Test no. 2[c]: lethal synergism with *Sxl, sis-a* (% viability)
	$+/FM7$	ND[d]	100 (201)[e,f]
Group A	$snf^{J210}/FM7$	ND	49 (567)[e,f]
	$snf^{1621}/FM7$	ND	0.5 (575)[e,f]
	$snf^{e8H}/FM7$	ND	20 (202)[f,g]
	$snf^{JA2}/FM7$	ND	100 (380)[e,f]
Group B	snf^{JA2}/snf^{JA2}	84 (125)	10 (149)
	snf^{JA2}/snf^{J210}	5 (1168)	0.3 (1168)
Group C	snf^{1621}/snf^{1621}	<0.8 (151)	ND
	snf^{1621}/snf^{J210}	3 (760)	<0.1 (848)
Group D	snf^{e8H}/snf^{e8H}	4 (584)	<0.5 (196)[g]
	snf^{e8H}/snf^{J210}	3 (1378)	0.06 (1608)
Group E	snf^{1621}/snf^{JA2}	25 (826)	0.8 (904)
	snf^{e8H}/snf^{JA2}	38 (446)	6 (510)
	snf^{e8H}/snf^{1621}	0.9 (703)	0.5 (597)

Viability is assessed by comparing the number of *snf*, +/+, *Sxl, sis-a* or *snf*, +/+, *Sxl* females recovered to the number of reference males recovered from the same cross (expected). Number of expected animals shown in parentheses. If all the experimental animals are fully viable, we expect to recover the same number of males and females (100% viability). Due to the sensitivity of these assays, we find that there are small differences in the strength of the interactions due to differences in genetic background (data not shown), therefore in the assays presented here we have controlled for genetic background as much as possible by limiting our comparisons to crosses with similar genetic backgrounds designated groups A–E.

[a] The genotypes of the relevant chromosomes are as follows: snf^{J210} is *y w* snf^{J210}; snf^{JA2} is *y w* snf^{JA2}; snf^{e8H} is *w* snf^{e8H}; snf^{1621} is *w* snf^{1621}; snf^{+} is *w* dhd^{P8} (the parent chromosome for both snf^{J210} and snf^{JA2}; see FLICKINGER and SALZ 1994 for details). Both snf^{JA2} and snf^{J210} are also mutant for the nonvital gene *deadhead* (*dhd*), a thioredoxin homologue (FLICKINGER and SALZ 1994; SALZ *et al.* 1994). To cover the female-sterile phenotype associated with the loss-of-function *dhd* mutation, snf^{JA2}/snf^{JA2} and snf^{JA2}/snf^{J210} females carry a copy of the $P[w^{+}, dhd^{+}]$ transgene. To bypass *snf*'s female sterile phenotype, snf^{JA2}/snf^{J210}, snf^{1621}/snf^{1621}, snf^{1621}/snf^{J210}, snf^{e8H}/snf^{J210}, snf^{1621}/snf^{JA2} and snf^{1621}/snf^{e8H} females carry a copy of the $P[w^{+}, otu::Sxl^{+}]$ transgene (see text for details).

[b] In test no. 1, *Sxl* is *y cm* Sxl^{7B0}.

[c] In test no. 2 *Sxl sis-a* is *cm* Sxl^{fl}, *ct sis-a*.

[d] ND, not determined.

[e] Data from FLICKINGER and SALZ (1994) Table 1.

[f] Viability values in the group A crosses are based on the number of *FM7c* balancer males recovered. However, the number of expected males has been adjusted to reflect the fact that the *FM7c* balancer males are 40% as viable as nonbalancer males.

[g] Data from SALZ (1992) Table 2.

To overcome this sterility, we have taken advantage of an $otu::Sxl^{+}$ transgene kindly provided by JEFF HAGAR and TOM CLINE (personal communication). In this promoter-gene fusion construct, the female-specific *Sxl* cDNA (cF1) is expressed under control of the germline-specific *otu* promoter. We find that this $otu::Sxl^{+}$ transgene suppresses *snf*'s female-sterile phenotype but not its female lethal-synergistic interaction phenotype. The key results are summarized below.

snf is dosage sensitive: Whereas control animals do not display a lethal interaction with *Sxl*, we find that the null allele (snf^{J210}) does display a weak dominant female lethal-synergistic interaction (Table 3, test no. 2, group A). Only 49% of the expected snf^{J210},+/+, *Sxl, sis-a* females from snf^{J210}/+ mothers are recovered. This dosage-sensitive phenotype indicates that the level of Snf protein is critical in a sensitized genetic background.

snf^{JA2} is a hypomorph: snf^{JA2} is a small deletion that has the potential to encode a protein that is missing its C-terminal end (FLICKINGER and SALZ 1994). Because our Snf-specific antibody does not detect the snf^{JA2} mutant protein, we have been unable to confirm the size of this protein (FLICKINGER and SALZ 1994). The fact that homozygous animals are viable and fertile clearly demonstrates that this mutant allele produces a product that can accomplish most of its functions when in two copies. Nevertheless, we find that homozygous snf^{JA2}/snf^{JA2} females display an interaction phenotype. Furthermore, snf^{JA2} is classified as a loss-of-function mutation because we find that the interaction phenotype of $snf^{JA2}/null$ hemizygous females is stronger than homozygous females (Table 3, test no. 1 and test no. 2, group B). Whether the loss-of-function phenotype is due to the absence of a functional do-

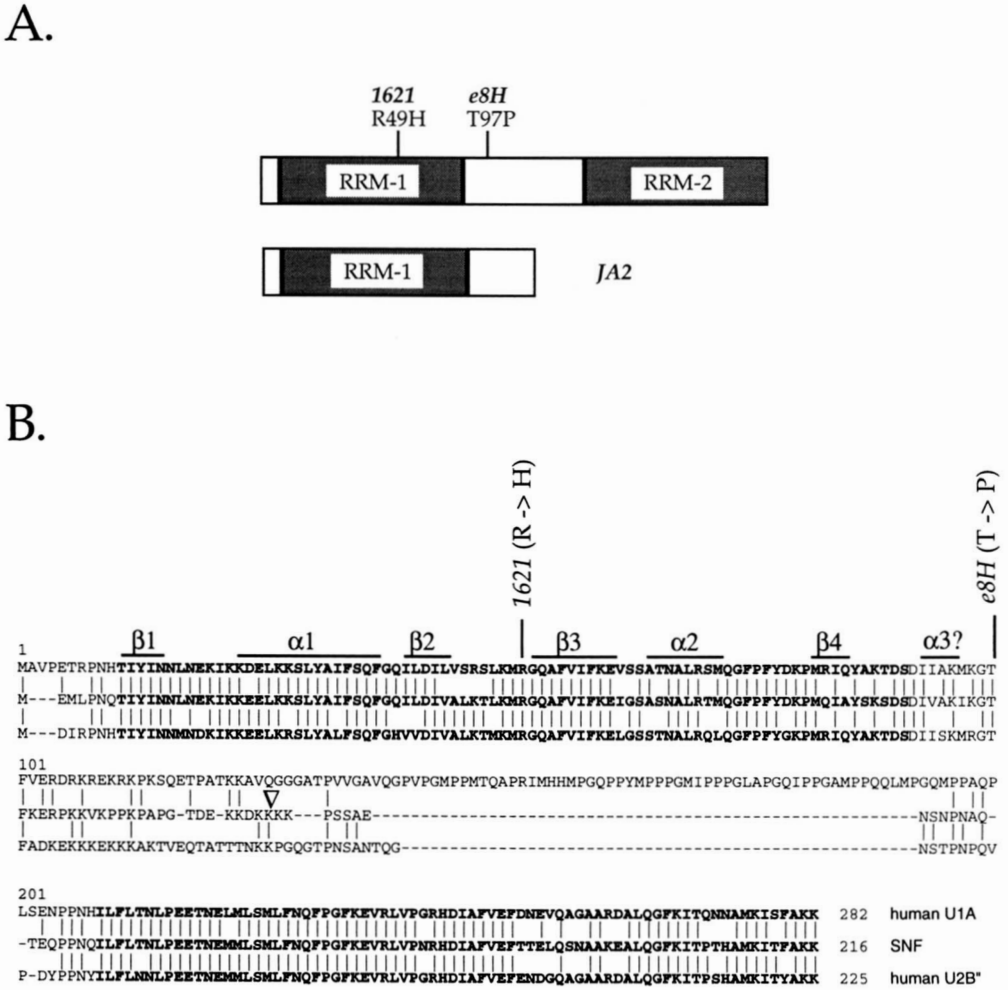
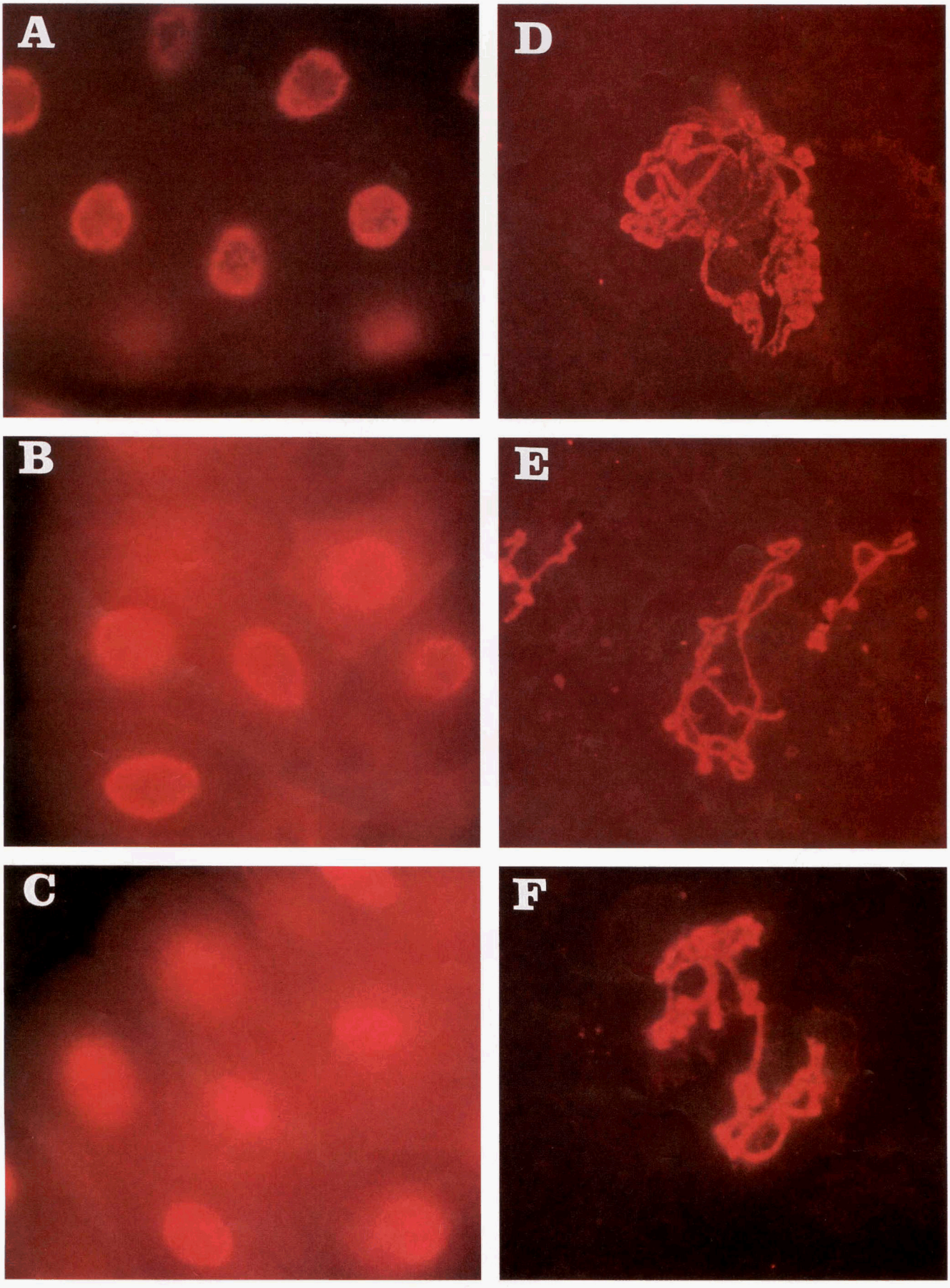


FIGURE 3.—Schematic of the Snf protein and location of the mutations in the viable *snf* alleles. (A) A schematic diagram of the 28-kD Snf protein. ▨, the location of the two RRM motifs. The EMS-induced mutation, snf^{1621}, is located within the N-terminal RRM and the EMS-induced snf^{e8H} mutation is located in the central region of the protein. Both of these mutations encode full length proteins that have dominant-negative (antimorphic) properties. snf^{JA2} is a small deletion that has the potential to encode a protein that is missing its C-terminal end, including the conserved C terminal RRM motif (RRM-2). Because our Snf-specific antibody does not detect the Snf^{JA2} mutant protein, we have been unable to determine whether the partial loss of function associated with snf^{JA2} is due to absence of a functional domain or whether the truncated Snf^{JA2} protein and/or RNA is less stable than the wild type. The null allele, snf^{J210}, is not shown as it deletes the entire gene starting in the 5′ untranslated region. (B) A comparison of the sequences of Snf with the human U1A and U2B″ proteins. Snf, U1A and U2B″ contain two highly conserved RRM motifs, marked in bold, separated by a central region that is less well conserved. The locations of the snf^{1621} and snf^{e8H} missense mutations are indicated. The breakpoint of the snf^{JA2} deletion mutation is within the only intron of the gene. △, the intron/exon boundary. The secondary structure of the U1A N-terminal RRM (RRM-1), both by itself and bound to its RNA target sequence, is known and illustrated above the sequence (HOWE *et al.* 1994; OUBRIDGE *et al.* 1994). RRM-1 folds in a characteristic $\beta\alpha\beta\beta\alpha\beta$ pattern in which the RNA interacts with the four-stranded β sheet. In addition, the C-terminal extension of RRM-1 contains a third α helix centered around residue 95 that is also required to position the RNA correctly. (Note, however that the extent of the α_3 helix remains to be determined.)

main of the protein or whether the truncated gene product is less stable than the wild-type product remains to be determined.

snf^{1621} is an antimorph: snf^{1621} is a missense mutation that encodes a full length protein (FLICKINGER and SALZ 1994). As illustrated in Table 3 (test no. 2, group A), snf^{1621} displays a stronger dominant lethal-synergistic interaction than the null allele (0.5 *vs.* 49%), indicating that this mutant protein has antimorphic function. Consistent with this classification, we find that the snf^{1621} interaction phenotype *is not* enhanced when hemizygous. The interaction phenotype of snf^{1621}/*null* hemizygous females is similar to the phenotype of snf^{1621}/snf^{1621} homozygous females (Table 3, test no. 1, group C). snf^{1621}/snf^{1621} homozygous and snf^{1621}/*null* hemizygous females rarely produce any daughters. Since *snf* is dosage-sensitive, the strength of the snf^{1621}/*null* interaction phenotype reflects the combined effects of both the loss-of-function and antimorphic gain-of-function phenotypes.

snf^{e8H} is an antimorph: Like snf^{1621}, snf^{e8H} is a missense mutation that encodes a full length protein (FLICK-


A
D
B
E
C
F

INGER and SALZ 1994; this report). As illustrated in Table 3 (test no. 2, group A), snf^{e8H} displays a stronger dominant lethal interaction than the null allele (20 *vs.* 49%), suggesting that this mutant protein has antimorphic function. Furthermore, as is the case for snf^{1621}, we find that interaction phenotype *is not* enhanced when hemizygous. The interaction phenotype of $snf^{e8H}/null$ hemizygous females is as strong as snf^{e8H}/snf^{e8H} homozygous females (4 *vs.* 3%, Table 3, test no. 1, group C). As mentioned above, the strength of the $snf^{e8H}/null$ interaction reflects the combined effects of both the loss-of-function and antimorphic phenotypes.

Interallelic complementation: The classification of the snf^{1621} and snf^{e8H} alleles as antimorphs is based on the observation that in dominant-lethal synergistic interaction assays both alleles have a more severe phenotype than the null. Surprisingly, when the snf^{e8H} and snf^{1621} alleles were tested in combination with the weak loss-of-function allele snf^{JA2}, this was not the case. Whereas $snf^{JA2}/null$ hemizygous females produced only 5% of the expected daughters (Table 3, test no. 1, group B), the trans-heterozygote females snf^{JA2}/snf^{1621} and snf^{JA2}/snf^{e8H} produced many more daughters (25 and 38%, respectively, Table 3, test no. 1, group E). These data indicate that snf^{JA2} partially complements the antimorphic alleles. In contrast, we find that snf^{e8H} and snf^{1621} do not complement each other. snf^{e8H}/snf^{1621} females rarely produce daughters (0.9%, Table 3, test no. 1, group E).

Molecular characterization of snf^{e8H}: To determine the lesion associated with the EMS induced mutation, snf^{e8H}, we amplified and sequenced the genomic DNA from the mutant chromosome and its parent chromosome. As illustrated in Figure 3, we found that the snf^{e8H} allele contains a single missense mutation that causes the substitution of a proline for a conserved threonine (T_{97}). This point mutation falls within the highly conserved central domain of the protein, just outside of the N-terminal RRM (RRM-1).

Localization of the mutant Snf^{1621} and Snf^{e8H} proteins: Previous studies have shown that the size and the amount of Snf^{1621} and Snf^{e8H} mutant protein detectable by Western blots does not differ from wild type (FLICKINGER and SALZ 1994). Consequently, it is unlikely that the mutant effect of snf^{1621} and snf^{e8H} is due to the amount of mutant protein produced. To further investigate how Snf^{1621} and Snf^{e8H} might interfere with the establishment of *Sxl* autoregulation, we examined the protein distribution of Snf^{e8H} and Snf^{1621} in whole mount salivary glands from homozygous female third instar larvae. Consistent with their homozygous viable phenotype, we find that the Snf^{e8H} and Snf^{1621} mutant proteins, like the wild-type Snf^{+} protein, are nuclear (Figure 4). Unlike the Snf protein, however, the Snf^{1621} and the Snf^{e8H} proteins do not appear to be strictly nuclear; staining is also visible in the cytoplasm (*cf.* cytoplasm in Figure 4A with that of Figure 4, B and C).

Although some of the Snf^{e8H} and Snf^{1621} mutant protein is mislocalized to the cytoplasm, staining is clearly visible within the nucleus. Are the mutant proteins correctly localized within the nucleus? The Snf^{+} protein, like that of other splicing factors, is associated with polytene chromosomes because pre-mRNAs remain closely aligned with the site of transcription as they are spliced (see Figure 4D; BEYER and OSHEIM 1988; AMERO *et al.* 1992). Given the viable phenotypes of both snf^{1621} and snf^{e8H}, we were not surprised to find that, like Snf^{+}, Snf^{1621} and Snf^{e8H} are correctly localized to polytene chromosomes (Figure 4, E and F).

In males, the viable alleles suppress ectopic *Sxl* activation: The strongest of the viable alleles, snf^{1621} is a temperature-sensitive suppressor of the male-specific lethal phenotype associated with the constitutive allele of *Sxl*, Sxl^{M1} (STEINMANN-ZWICKY 1988; SALZ 1992). Recent studies have shown that Sxl^{M1} is a transposon-induced constitutive *Sxl* allele whose behavior is dependent on the inappropriate expression of the wild-type protein (BERNSTEIN *et al.* 1995). This suggests that snf^{1621} may be interfering with the autoregulatory RNA-binding function of wild-type Sxl protein. On the other hand, these same studies suggested that the pre-mRNA generated from this transposon insertion allele differs from the wild-type pre-mRNA in its response to splicing-autoregulation (BERNSTEIN *et al.* 1995). If this is the case, then the observed Sxl^{M1}-snf^{1621} genetic interaction might be allele-specific with respect to the transposon containing pre-mRNA encoded by Sxl^{M1}.

To distinguish between these possibilities, we assayed whether snf^{1621} could suppress the male-specific lethality associated with the inappropriate expression of wild-type Sxl protein. To express the female-specific protein in the male, we took advantage of transgenic lines that express the product of a late *Sxl* cDNA (cF1) under control of a heat shock promoter (BELL *et al.* 1991). Because *Sxl* splicing is autoregulatory, the low levels of Sxl protein provided by the transgene initiate the female-specific splicing loop by splicing the *Sxl* pre-mRNAs from the endogenous Sxl^{+} allele in the female mode (BELL *et al.* 1991). As illustrated in Table 4, even under nonheat shock conditions, only 2% of the expected snf^{+}; *hsp70::SxlcF1/+* transgenic males were recovered. In

FIGURE 4.—Subcellular localization of wild-type and mutant Snf proteins in whole mount salivary glands and to polytene chromosomes. Staining with the Snf-specific antibody reveals that (A and D) Snf^{+} is nuclear in a salivary gland from a wild-type third instar larva and is associated with polytene chromosomes. (B and E) The Snf^{1621} protein is detectable in both the cytoplasm and the nucleus in snf^{1621}/snf^{1621} salivary glands and is associated with polytene chromosomes (C and F) The Snf^{e8H} protein is detectable in both the cytoplasm and the nucleus in snf^{e8H}/snf^{e8H} salivary glands and is associated with polytene chromosomes.

TABLE 4

Ectopic *Sxl* activation by the inappropriate expression of the Sxl protein is suppressed by *snf* mutations

Cross[b]	snf^X	Relative viability (%)[a] snf^X; *hsp70::Sxl/+*	No. of reference males (no transgene)[a] snf^X; +/+
A	snf^+	1.8 (2)	109
A	snf^{1621}	75 (148)	199
B	snf^{e8H}	67 (71)	106
C	snf^{JA2}	15 (15)	100

All crosses were carried out at 22°.

[a] Relative viability is assessed by comparing the number (shown in parentheses) of experimental males recovered to the number of reference males recovered from the same cross. If the experimental males were fully viable, we would expect to recover the same number of experimental and reference males.

[b] The full genotype of the mothers in each cross are as follows: (A) *y cho* snf^{1621}*/FM7; TM3* P[w^+, *hsp70::SxlcF1*] #19/+. (B) *w* snf^{e8H}*/FM7; TM3,* P[w^+,*hsp70::SxlcF1*]#19/+. (C) *y w* snf^{JA2}*/FM7; TM3* P[w^+,*hsp70::SxlcF1*]#19/+. The transgene P[W^+,*hsp70::SxlcF1*]#19 is located on the *TM3* balancer chromosome carrying the *Ser* dominant marker, therefore *Ser* is indicative of the presence or absence of the transgene. In cross A the *FM7* male progeny are utilized as the Snf^+ control flies.

contrast, 75% of the expected snf^{1621}*; hsp70::SxlcF1/+* transgenic males were recovered. These results demonstrate that snf^{1621} blocks the function of the wild-type Sxl protein.

To extend these observations, we examined whether this suppressor phenotype was unique to snf^{1621}. or whether the other two viable alleles could also suppress the male lethality associated with ectopic expression of *Sxl* cDNA cF1. The data presented in Table 4 (crosses B and C) indicate that antimorphic mutation snf^{e8H} is a strong suppressor (67% of the expected snf^{e8H}*; hsp70::SxlcF1/+* transgenic males were recovered) and the loss-of-function mutation snf^{JA2} is a weak suppressor (15% of the expected snf^{JA2}*, hsp70::SxlcF1/+* transgenic males were recovered).

DISCUSSION

The Snf protein has sequence and functional homology to both U1A and U2B″, and appears to be the only U1A/U2B″-like proteins in Drosophila (FLICKINGER and SALZ 1994; POLYCARPOU-SCHWARZ *et al.* 1996). Although these proteins have been extensively studied, their function within the spliceosome is not known. The analysis of the existing *snf* mutations, presented here, demonstrate that the Snf protein cooperates with the female-specific Sxl protein to block utilization of the male-specific exon of the *Sxl* pre-mRNA. These data provide the first suggestion that the role of U1A and/or U2B″ is to interact with differentially expressed proteins to modulate splice site choice.

***snf* is required for a cell-vital process, consistent with a role in general pre-mRNA splicing:** Our phenotypic analysis of the lethal null allele suggests that in Drosophila, Snf is an essential component of the RNA splicing machinery. By using genetic mosaics, we establish that *snf* is necessary for normal cell growth and/or cell survival during postembryonic development. This type of cell-vital function is consistent with a role in pre-mRNA splicing. The localization pattern of the Snf protein is also consistent with a role as a general splicing factor: Snf is nuclear and in salivary glands it is associated with nascent transcripts on polytene chromosomes. Snf is present in all cell types, at all stages of development and is maternally provided such that it is present in the embryo as zygotic transcription begins. We have also found that the protein supplied by the mother is stable, lasting through embryogenesis. Unfortunately, it is the stability of the maternally provided protein that prevents us from a direct analysis of *snf*'s role in RNA processing because there is no point at which we can find live embryos with no Snf protein. The alternative method of eliminating maternal Snf protein in embryos, by generating snf^{null} germline clones, is not possible because *snf* is required for female germline development.

***snf*'s role in regulating *Sxl* pre-mRNA splicing in the embryo:** *snf*'s role as a regulator of *Sxl* pre-mRNA splicing is based on the analysis of a group of viable alleles. Our phenotypic analysis of the lethal null allele strongly argues that *snf* is required for female sexual differentiation and, more importantly, argues against the formal possibility that the viable alleles disrupt a function unrelated to *snf*'s wild-type function. In genetic mosaics, homozygous mutant clones generated during the second instar larval period show abnormal differentiation with respect to the expected sexually dimorphic characteristics in the adult foreleg. Interestingly, these clones developed as sexual intermediates, suggesting that *Sxl* protein function has been reduced but not eliminated. A similar intersexual phenotype is observed in the foreleg region of females homozygous for Sxl^{2593}, a mutation that reduces but does not eliminate *Sxl* function (MARSHALL and WHITTLE 1978; CLINE 1984). In contrast, clones homozygous for a *Sxl* null allele are phenotypically male (SALZ *et al.* 1987). The similarity in sexual phenotype with Sxl^{2593} suggests that the intersexual phenotype of the *snf* clones is due to a reduction of protein encoding *Sxl* pre-mRNA splicing products. Since *snf* is also required for a cell-vital function, it is likely that the amount of Snf protein required to allow the cell to survive is also sufficient to allow some female-specific splicing to occur and hence feminization.

snf's role in activating *Sxl* in the embryo is readily apparent in a sensitized genetic background where the

dosage of Sxl^+ is reduced. Thus in the embryo, the *snf* mutations only prevent the establishment of *Sxl* expression under genetic conditions where Sxl protein levels are limiting. In contrast, when Sxl protein is not limiting, the *snf* mutations have no detectable effect on the establishment of *Sxl* expression. All the *snf* alleles display a strong maternal effect under these genetic conditions, consistent with our observation that a large amount of Snf protein is provided to the embryo by the mother and is stable throughout embryogenesis. Moreover, we find that the amount of Snf protein provided by the mother is critical under these sensitized genetic conditions, as demonstrated by the finding that the null allele, snf^{J210}, exhibits a dominant maternal-effect lethal synergistic interaction phenotype. Dosage-sensitive phenotypes are entirely consistent with biochemical studies that have shown that the relative levels of splicing factors influence splice site selection (EPERON *et al.* 1993; KOHTZ *et al.* 1994; STAKINS and REED 1994; ROMAC and KEENE 1995).

snf^{1621} and snf^{e8H} encode proteins that interfere with *Sxl* splicing autoregulation: Two of the viable alleles, snf^{1621} and snf^{e8H}, display a more severe dominant synergistic interaction phenotype than the lethal null allele. This exaggerated mutant phenotype indicates that the mutant proteins actively interfere with *Sxl* splicing regulation. Consistent with this, we find that snf^{1621} and snf^{e8H} are strong suppressors of the male-specific lethal phenotype elicited by the inappropriate expression of the wild-type Sxl protein. The mechanism by which the mutant Snf^{1621} and Snf^{e8H} proteins interfere with *Sxl* splicing remains to be determined. However by analogy with the information gathered from the human U1A protein, it is likely that the two mutant Snf proteins have reduced RNA binding activities and are therefore not efficiently incorporated into snRNPs.

The snf^{1621} mutation is associated with a missense mutation that causes the substitution of a histidine for an arginine (R_{49}) residue in the amino terminal RNA binding domain (FLICKINGER and SALZ 1994). *In vitro* structure/function studies with vertebrate U1A have established that this arginine is essential for its RNA binding activity (NAGAI *et al.* 1990; JESSEN *et al.* 1991). Furthermore, recent structural data have shown that the arginine located at this position not only contacts the U1 snRNA, but also forms intramolecular hydrogen bonds with neighboring amino acids within the N-terminal RRM domain (HOWE *et al.* 1994; OUBRIDGE *et al.* 1994). One can imagine that the arginine to histidine change present in snf^{1621} will disrupt these intramolecular hydrogen bonds and thereby reduce its binding for its target RNA. Similarly, snf^{e8H} encodes a full length protein that contains a threonine to proline change in a highly conserved basic region just outside of the N-terminal RRM. Although this basic region is not within the region of the protein shown to be necessary and sufficient for *in vitro* RNA binding, it is still within a region that contacts the RNA (NAGAI *et al.* 1990; JESSEN *et al.* 1991; HOWE *et al.* 1994). Consequently, it is likely that the substitution of a proline for a conserved threonine interferes with its RNA binding activity *in vivo*.

In vivo support for the suggestion that the mutant Snf^{1621} and Snf^{e8H} proteins have a lowered binding affinity for their target RNAs is provided by the mislocalization of the mutant proteins. We find that in contrast to the wild-type protein, which is strictly nuclear, the Snf^{1621} and Snf^{e8H} mutant proteins are located in both the cytoplasm and the nucleus. Studies examining the intracellular distribution of the vertebrate U1A protein have shown that U1A will shuttle back and forth between the cytoplasm and the nucleus unless actively retained by binding its target RNA (KAMBACH and MATTAJ 1992). By analogy, the presence of cytoplasmic protein suggests that there is a significant amount of mutant protein that does not bind to its target snRNA.

How might the free mutant protein interfere with *Sxl* splicing regulation? One possibility is that the free mutant Snf protein in the nucleus binds another splicing factor rendering it nonfunctional. An excellent candidate for a protein that interacts with Snf is Sxl because direct Snf^+/Sxl^+ protein-protein interactions have been detected *in vitro* (G. DESHPANDE, M. SAMUELS and P. SCHEDL, personal communication). This suggests a model in which the free mutant Snf protein directly interferes with Sxl's function by capturing it and preventing it from blocking recognition of the regulated male exon. Critical to our model is the prediction that the mutant Snf proteins retain the ability to interact with the wild-type Sxl protein. Indeed, DESHPANDE, SAMUELS and SCHEDL (personal communication) have shown a Snf^{1621}/Sxl^+ protein-protein interaction comparable to the Snf^+/Sxl^+ interaction.

Antagonistic functions for snRNPs in splice site selection—a model: Together, our analysis of the existing *snf* mutations demonstrates that Snf cooperates with Sxl to block utilization of the male-specific exon of the *Sxl* pre-mRNA and suggests a model in which the Sxl protein blocks spliceosome assembly by forming a nonproductive snRNP/Sxl complex. This hypothesis is supported by the finding that the Sxl and Snf proteins are found together in RNA-protein aggregates (SAMUELS *et al.* 1994). This model implies that Snf-containing snRNPs can have antagonistic roles in splicing. On the one hand, the primary function of U1 and U2 snRNPs is to promote spliceosome assembly. On the other hand, when complexed with Sxl the Snf-containing snRNPs block splice site utilization. Thus the availability of Sxl to the Snf-containing snRNP dictates whether it functions to promote or block splice site usage.

Whether *snf*'s role in splice site regulation is part of its U1 snRNP function and/or its U2 snRNP function is not known. However, because the critical step in *Sxl* autoregulation is blockage of the male-specific 5′ splice site (HORABIN and SCHEDL 1993a,b), we propose that

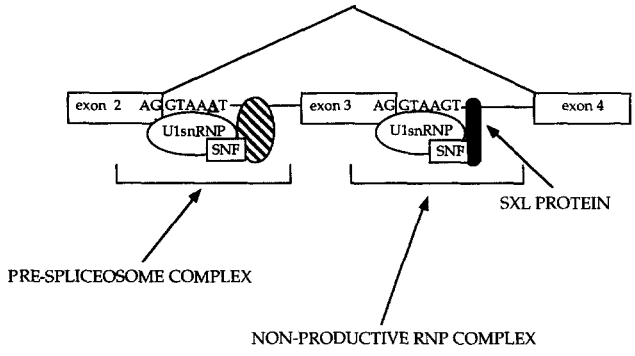


FIGURE 5.—*snf*'s role in *Sxl* splicing autoregulation: the Sxl-U1 snRNP interaction model. In females, the male-specific exon (exon 3) is excluded from the processed *Sxl* mRNA. The critical step in exon skipping is blockage of the male-specific 5′ splice site by the Sxl female-specific RNA binding protein (black oval) that is bound to poly (U) sites within the intron (see text for details and references). In this model we propose that Sxl blocks recognition of this 5′ splice site by forming a nonproductive (inactive) complex with the U1 snRNP via its interaction with Snf. At unregulated 5′ splice sites, the U1 snRNP is free to form a prespliceosome complex with other splicing factors (indicated by the striped oval). Interestingly, this regulated male 5′ splice site has a perfect match (AG/GTAAGT) for base pairing to U1 snRNA, whereas the competing 5′ splice site of exon 2 has a mismatch at the highly conserved +5 residue (AG/GTAAAT) (MOUNT *et al.* 1992; HORABIN and SCHEDL 1993a,b). As in other systems, regulation of splice site selection may depend on differences in the relative strengths of splice sites.

Sxl complexes with the U1 snRNP via its interaction with Snf to block spliceosome assembly. In our Sxl-U1 snRNP interaction model, illustrated in Figure 5, we propose that the Sxl protein binds to its target sequences on the *Sxl* pre-mRNA and blocks recognition of the regulated 5′ splice site by forming a nonproductive (inactive) complex with the U1 snRNP by interacting with the Snf protein. At unregulated 5′ splice sites, the U1 snRNP is free to form a prespliceosome complex with other splicing factors. Interestingly, recent studies in both *Saccharomyces cerevisiae* and human cells have failed to find a function for U1A in spliceosome complex formation (LIAO *et al.* 1993; JAMISON *et al.* 1995). U1 snRNPs that are missing their U1A protein component can still recognize and initiate spliceosome formation at 5′ splice sites. These results raise the possibility that *snf*'s function within the U1 snRNP is required only for modification of splice site selection.

Does Snf function with Sxl to regulate *tra* pre-mRNA splicing? In addition to its autoregulatory function, the Sxl protein also regulates the 3′ splice site selection of the *tra* pre-mRNA (SOSNOWSKI *et al.* 1989). Is *snf* also required for *tra* pre-mRNA splicing regulation? Since *snf* is necessary for *Sxl* autoregulation and *Sxl* regulates *tra*, genetic interaction assays do not allow us to distinguish between a direct interference with *tra* pre-mRNA splicing and an indirect effect caused by reducing the amount of available Sxl protein. Nonetheless, given that Snf is an snRNP protein, it is tempting to speculate that Snf is utilized by Sxl to regulate *tra* pre-mRNA splicing by blocking 3′ splice site usage. *In vitro* studies have suggested that Sxl competes with the U2AF protein for a stable association with the pre-mRNA (VALCARCEL *et al.* 1993). Since U2AF is normally present in the early prespliceosomal complexes with U1 snRNP (BENNETT *et al.* 1992; STAKINS and REED 1994), Sxl could actively block spliceosome assembly by replacing U2AF in this complex through an interaction with Snf.

We are grateful to J. HAGAR and T. CLINE for allowing us to use their *otu::Sxl* transgene before publication. We are also grateful to I. MATTAJ, B. SUTER, T. CLINE and P. SCHEDL for communicating results before publication. We thank M. SEDENSKY and P. MORGAN for comments on the manuscript and C. CRONMILLER, G. MATERA, S. MOUNT and J. WISE for their critical reading of early versions of this manuscript. We thank P. HUNT for unrestricted access to her Zeiss microscope and K. MATTHEWS of the Bloomington Stock Center, L. BELL and D. BOPP for fly stocks. T.W.F. was supported by a National Research Service Award (HD07104). This work was funded by a grant from the National Science Foundation (MCB-9305074) to H.K.S.

LITERATURE CITED

ALBRECHT, E. B., and H. K. SALZ, 1993 The Drosophila sex determination gene *snf* is utilized to establish the female-specific splicing pattern of *Sex-lethal*. Genetics **134:** 801–807.

AMERO, S. A., G. RAYCHAUDHURI, C. L. CASS, W. J. VAN VENROOIJ, W. J. HABETS *et al.*, 1992 Independent deposition of heterogeneous nuclear ribonucleoproteins andsmall nuclear ribonucleoprotein particles at sites of transcription. Proc. Natl. Acad. Sci. USA **89:** 8409–8413.

BAKER, B. S., 1989 Sex in flies: the splice of life. Nature **340:** 521–524.

BELL, L. R., E. M. MAINE, P. SCHEDL and T.W. CLINE, 1988 *Sex-lethal*, a Drosophila sex determination switch gene, exhibits sex-specific RNA splicing and sequence similarity to RNA binding proteins. Cell **55:** 1037–1046.

BELL, L. R., J. I. HORABIN, P. SCHEDL and T. W. CLINE, 1991 Positive autoregulation of *Sex-lethal* by alternative splicing maintains the female determined state in Drosophila. Cell **65:** 229–239.

BENNETT, M., S. MICHAUD, J. KINGSTON and R. REED, 1992 Protein components specifically associated with prespliceosome and spliceosome complexes. Genes Dev. **6:** 1986–2000.

BERNSTEIN, M., R. A. LERSCH, L. SUBRAHMANYAN and T. W. CLINE, 1995 Transposon insertions causing constitutive *Sex-lethal* activity in *Drosophila melanogaster* affect *Sxl* sex-specific transcript splicing. Genetics **139:** 631–648.

BEYER, A. L., and Y. N. OSHEIM, 1988 Splice site selection, rate of splicing and alternative splicing on nascent transcripts. Genes Dev. **2:** 754–762.

BOPP, D., J. I. HORABIN, R. A. LERSCH, T. W. CLINE and P. SCHEDL, 1993 Expression of the *Sex-lethal* gene is controlled at multiple levels during Drosophila oogenesis. Development **118:** 797–812.

BURD, C. G., and G. DREYFUSS, 1994 Conserved structures and diversity of functions of RNA-binding proteins. Science **265:** 615–621.

CLINE, T. W., 1984 Autoregulatory functioning of a Drosophila gene product that establishes and maintains the sexually determined state. Genetics **107:** 231–277.

CLINE, T. W., 1993 The Drosophila sex determination signal: how do flies count to two? Trends Genet. **9:** 385–390.

CRISPINO, J. D., B. J. BLENCOWE and P. A. SHARP, 1994 Complementation by SR proteins of pre-mRNA splicing reactions depleted of U1snRNP. Science **265:** 1866–1869.

CRONMILLER, C., and H. K. SALZ, 1994 The feminine mystique: the initiation of sex determination in Drosophila, pp. 171–204 in *Molecular Genetics of Sex Determination*, edited by S. S. WACHTEL. Academic Press, San Diego.

EPERON, I. C., D. C. IRELAND, R. A. SMITH, A. MAYEDA and A. R. KRAINER, 1993 Pathways for selection of 5′ splice sites by U1snRNPs and SF2/ASF. EMBO J. **12:** 3607–3617.

ESTES, P. A., L. N. KEYES and P. SCHEDL, 1995 Multiple response elements in the *Sex-lethal* early promoter ensure its female-specific expression pattern. Mol. Cell. Biol. **15:** 904–917.

FLICKINGER, T. W., and H. K. SALZ, 1994 The Drosophila sex determination gene *snf* encodes a nuclear protein with sequence and functional similarity to the mammalian U1A snRNP protein. Genes Dev. **8:** 914–925.

GARCIA-BELLIDO, A., and J. R. MERRIAM, 1971 Genetic analysis of cell heredity in imaginal discs of Drosophila melanogaster. Proc. Natl. Acad. Sci. USA **68:** 2222–2226.

HABETS, W. J., M. H. HOET, B. A. W. DE JONG, A. VAN DER KEMP and W. J. VAN VENROOIJ, 1989 Mapping of B cell epitopes on small nuclear ribonucleoproteins that react with human autoantibodies as well as with experimentally-induced mouse monoclonal antibodies. J. Immunol. **143:** 2560–2566.

HARPER, D. S., L. D. FRESCO and J. D. KEENE, 1992 RNA binding specificity of a Drosophila snRNP protein that shares sequence homology with mammalian U1-A and U2-B proteins. Nucleic Acids Res. **20:** 3645–3650.

HODGES, D., and S. I. BERNSTEIN, 1994 Genetic and biochemical analysis of alternative RNA splicing. Adv. Genet. **31:** 207–281.

HORABIN, J. I., and P. SCHEDL, 1993a Regulated splicing of the Drosophila *Sex-lethal* male exon involves a blockage mechanism. Mol. Cell Biol. **13:** 1408–1414.

HORABIN, J. I., and P. SCHEDL, 1993b *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.

HOWE, P. W. A., K. NAGAI, D. NEUHAUS and G. VARANI, 1994 NMR studies of U1snRNA recognition by the N-terminal RNP domain of the human U1A protein. EMBO J. **13:** 3873–3881.

JAMISON, S. F., Z. PASMAN, J. WANG, C. WILL, R. LUHRMANN *et al.*, 1995 U1 snRNP-ASF/SF2 interaction and 5′ splice site recognition: characterization of required elements. Nucleic Acids Res. **23:** 3260–3267.

JESSEN, T.-H., C. OUBRIDGE, C. H. TEO, C. PRITCHARD and K. NAGAI, 1991 Identification of molecular contacts between the U1A small nuclear ribonucleoprotein and U1 RNA. EMBO J. **10:** 3447–3456.

KANAAR, R., A. L. LEE, D. Z. RUDNER, D. E. WEMMER and D. C. RIO, 1995 Interaction of the Sex-lethal RNA binding domains with RNA. EMBO J. **14:** 4530–4539.

KEYES, L. N., T. W. CLINE and P. SCHEDL, 1992 The primary sex-determination signal of Drosophila acts at the level of transcription. Cell **68:** 933–943.

KOHTZ, J. D., S. F. JAMISON, C. L. WILL, P. ZUO, R. LUHRMANN *et al.*, 1994 Protein-protein interactions and 5′ splice-site recognition in mammalian mRNA precursors. Nature **368:** 119–124.

LIAO, X. C., J. TANG and M. ROSBASH, 1993 An enhancer screen identifies a gene that encodes the yeast U1 snRNP A protein: implications for snRNP protein function in pre-mRNA splicing. Genes Dev. **7:** 419–428.

LINDSLEY, D., and G. G. ZIMM, 1992 *The Genome of Drosophila melanogaster.* Academic Press, San Diego.

MANLEY, J., 1993 Question of commitment. Nature **365:** 14.

MARSHALL, T., and J. R. S. WHITTLE, 1978 Genetic analysis of the mutation *Female-lethal* in Drosophila melanogaster. Genet. Res. **32:** 103–111.

MATTAJ, I., 1993 RNA recognition: a family matter? Cell **73:** 837–840.

MOORE, J. M., C. C. QUERY and P. A. SHARP, 1993 Splicing of precursors to messenger RNAs by the spliceosome, pp. 303–358 in *The RNA World,* edited by R. F. GESTELAND and J. F. ATKINS. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

MOUNT, S. M., C. BURKS, G. HERTZ, G. D. STORMO, O. WHITE *et al.*, 1992 Splicing signals in Drosophila: intron size, information content and consensus sequences. Nucleic Acids Res. **20:** 4255–4262.

NAGAI, K., C. OUBRIDGE, T. H. JESSEN, J. LI and P. R. EVANS, 1990 Crystal structure of the RNA-binding domain of the U1 small nuclear ribonucleoprotein A. Nature **348:** 515–520.

OLIVER, B., N. PERRIMON and A. P. MAHOWALD, 1988 Genetic evidence that the *sans fille* locus is involved in Drosophila sex determination. Genetics **120:** 159–171.

OLIVER, B., Y.-J. KIM and B. S. BAKER, 1993 *Sex-lethal,* master and slave: a hierarchy of germline sex determination in Drosophila. Development **119:** 897–908.

OUBRIDGE, C., N. ITO, P. R. EVANS, C.-H. TEO and K. NAGAI, 1994 Crystal structure at the 1.92Å resolution of the RNA binding domain of the U1A spliceosomal protein complexed with an RNA hairpin. Nature **372:** 432–438.

POLYCARPOU-SCHWARZ, M., S. I. GUNDERSON, S. KANDELS-LEWIS, B. SERAPHIN and I. W. MATTAJ, 1996 Drosophila SNF/D25 combines the functions of the two snRNP proteins U1A and U2B″ that are encoded separately in human, potato and yeast. RNA **2:** 11–23.

RIPOLL, P., 1977 Behavior of somatic cells homozgyous for zygotic lethals in *Drosophila melanogaster.* Genetics **86:** 357–376.

ROMAC, J. M.-J., and J. D. KEENE, 1995 Overepxression of the arginine-rich carboxy-terminal region of U1 snRNP 70 K inhibits both splicing and nucleocytoplasmic transport of mRNA. Genes Dev. **9:** 1400–1410.

SAKAMOTO, H., K. INOUE, I. HIGUCHI, Y. ONO and Y. SHIMURA, 1992 Control of Drosophila *Sex-lethal* pre-mRNA splicing by its own female-specific product. Nucleic Acids Res. **20:** 5533–5540.

SAKASHITA, E., and H. SAKAMOTO, 1994 Characterization of RNA binding specificity of the Drosophila Sex-lethal protein by *in vitro* ligand selection. Nucleic Acids Res. **22:** 4082–4086.

SALZ, H. K., 1992 The genetic analysis of *snf:* a Drosophila sex determination gene required for activation of *Sex-lethal* in both the germline and the soma. Genetics **130:** 547–554.

SALZ, H. K., T. W. CLINE and P. SCHEDL, 1987 Functional changes associated with structural alterations induced by mobilization of a *P* element inserted in the *Sex-lethal* gene of Drosophila. Genetics **117:** 221–231.

SALZ, H. K., E. M. MAINE, L. N. KEYES, M. E. SAMUELS, T. W. CLINE *et al.*, 1989 The Drosophila female-specific sex determination gene, *Sex-lethal,* has stage-, tissue-, and sex-specific RNAs suggesting multiple modes of regulation. Genes Dev. **3:** 708–719.

SALZ, H. K., T. W. FLICKINGER, E. MITTENDORF, A. PELLICENA-PALLE, J. P. PETSCHEK *et al.*, 1994 The Drosophila maternal effect locus *deadhead* encodes a thioredoxin homolog required for female meiosis and early embryonic development. Genetics **136:** 1075–1086.

SAMUELS, M. E., P. SCHEDL and T. W. CLINE, 1991 The complex set of late transcripts from the Drosophila sex determination gene *Sex-lethal* encodes multiple related polypeptides. Mol. Cell. Biol. **11:** 3584–3602.

SAMUELS, M. E., D. BOPP, R. A. COLVIN, R. F. ROSCIGNO, M. A. GARCIA-BLANCO *et al.*, 1994 RNA binding by Sxl proteins *in vitro* and *in vivo.* Mol. Cell. Biol. **14:** 4975–4990.

SOSNOWSKI, B. A., J. M. BELOTE and M. MCKEOWN, 1989 Sex specific aternative splicing of RNA from the *transformer* gene results from sequence-dependent splice site blockage. Cell **3:** 449–459.

STAKINS, D., and R. REED, 1994 SR proteins promote the first specific recognition of pre-mRNA and are present together with the U1 small nuclear ribonucleoprotein particle in general splicing enhancer complex. Mol. Cell. Biol. **14:** 7670–7682.

STEINMANN-ZWICKY, M., 1988 Sex determination in Drosophila: the *X*-chromosomal gene *liz* is required for *Sxl* activity. EMBO J. **7:** 3889–3898.

TARN, W.-Y., and J. A. STEITZ, 1994 SR proteins can compensate for the loss of U1snRNP functions *in vitro.* Genes Dev. **8:** 2704–2717.

VALCARCEL, J., J. SINGH, P. D. ZAMORE and M. R. GREEN, 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 L. R. BELL, 1994 The Sex-lethal amino terminus mediates cooperative interactions in RNA binding and is essential for splicing regulation. Genes Dev. **8:** 2072–2085.

WU, J. Y., and T. MANIATIS, 1993 Specific interactions between proteins implicated in splice site selection and regulated alternative splicing. Cell **75:** 1061–1070.

XU, T., and G. M. RUBIN, 1993 Analysis of genetic mosaics in developing and adult Drosophila tissues. Development **117:** 1223–1237.

ZAMORE, P. D., and M. R. GREEN, 1989 Identification, purification and biochemical characterization of U2 small nuclear ribonucleoprotein auxiliary factor. Proc. Natl. Acad. Sci. USA **86:** 9243–9247.

ZINK, B., and R. PARO, 1989 In vivo binding pattern of a trans-regulator of the homeotic genes in *Drosophila melanogaster*. Nature **337:** 468–471.

Communicating editor: T. SCHÜPBACH