The N-terminal domain of Sxl protein disrupts Sxl autoregulation in females and promotes female-specific splicing of tra in males

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SUMMARY

Sex determination in Drosophila depends upon the post-transcriptional regulatory activities of the Sex-lethal (Sxl) gene. Sxl maintains the female determined state and activates female differentiation pathways by directing the female-specific splicing of Sxl and tra pre-mRNAs. While there is compelling evidence that Sxl proteins regulate splicing by directly binding to target RNAs, previous studies indicate that the two Sxl RNA-binding domains are not in themselves sufficient for biological activity and that an intact N-terminal domain is also critical for splicing function. To further investigate the functions of the Sxl N terminus, we ectopically expressed a chimeric protein consisting of the N-terminal 99 amino acids fused to β-galactosidase. The Nβ-gal fusion protein behaves like a dominant negative, interfering with the Sxl autoregulatory feedback loop and killing females. This dominant negative activity can be attributed to the recruitment of the fusion protein into the large Sxl:Snf splicing complexes that are found in vivo and the consequent disruption of these complexes. In addition to the dominant negative activity, the Nβ-gal fusion protein has a novel gain-of-function activity in males: it promotes the female-specific processing of tra pre-mRNAs. This novel activity is discussed in light of the blockage model for the tra splicing regulation.

Key words: Sex-lethal, Alternative splicing, Drosophila, Sex determination, Protein:protein interaction

INTRODUCTION

Somatic sex determination in D. melanogaster is controlled by the binary switch gene, Sex-lethal (Sxl). The Sxl gene is activated in females by the primary sex determination signal, the X chromosome to autosome ratio (X/A), while gene remains off in males (Keyes et al., 1992). The X/A signaling system functions only transiently in the early embryo and, during the remainder of development, the Sxl gene is responsible for both, ensuring that the determined state is remembered, and for orchestrating sexual differentiation. These two facets of sexual development are controlled by post-transcriptional regulatory mechanisms, which operate at the level of splicing and translation (Baker, 1989; Hodgkin, 1989; Cline and Meyer, 1996; Kelley and Kuroda, 1995).

The determined state in females is maintained by a Sxl autoregulatory feedback loop in which Sxl proteins promote their own synthesis by directing the female-specific splicing of Sxl pre-mRNAs. Exon 2 of the Sxl pre-mRNA is joined directly to exon 4, skipping the male-specific exon, exon 3. The resulting female mRNAs encode proteins of ~350 aa, which have two RRM-type RNA-binding domains. The male state is maintained by the default splicing of Sxl pre-mRNAs. The 3rd exon is incorporated into the processed mRNAs and it has stop codons that truncate the Sxl open reading frame which begins in exon 2 (Cline, 1984; Bell et al., 1988, 1991).

Sxl directs differentiation by regulating several gene cascades that control different aspects of somatic sexual development. These include the transformer (tra)→doublesex (dsx) and tra→fruitless (fru) sexual differentiation pathways (Butler et al., 1986; Boggs et al., 1987; Nagoshi et al., 1988; Burtis et al., 1989; Ryner et al., 1996) and the male specific lethal-2 (msl-2) dosage compensation system (Bashaw and Baker, 1995; Kelley et al., 1995; Zhou et al., 1995). Sxl controls the dsx and fru pathways by regulating tra splicing. In females, Sxl directs the splicing machinery to skip the default 3′ splice site of the 2nd tra exon and uses instead a downstream 3′ site. The Tra protein produced from the female spliced mRNA (Sosnowski et al., 1989; Inoue et al., 1990; Sosnowski et al., 1994) directs the female-specific expression of dsx and fru. In males, tra mRNA spliced in the default pattern encodes only a short non-functional polypeptide and, in the absence of Tra protein, the male versions of dsx and fru are expressed. Finally, Sxl turns off the dosage compensation system in females by blocking the translation of msl-2 mRNAs (Bashaw and Baker, 1997; Kelley et al., 1997; Gebauer et al., 1998). It may also function in an msl-2-independent dosage compensation system by directly downregulating the translation of X-linked genes whose transcripts contain Sxl protein binding sites in their 3′ UTRs (Kelley et al., 1997; Yanowitz et al., 1999). Because the activity state of the dsx and fru sexual differentiation and the dosage compensation pathways are linked through Sxl, loss-
and gain-of-function mutations in Sxl are both ‘sex-transforming’ and lethal (Cline, 1978; Gergen, 1987; Bernstein and Cline, 1994; Bernstein et al., 1995).

The regulatory activities of Sxl protein depend upon a direct interaction with target RNAs (Sakamoto et al., 1992; Sakashita and Sakamoto, 1994; Kanaar et al., 1995; Samuels et al., 1994; Wang and Bell, 1994). The simplest case is that of tra, which has two closely spaced Sxl-binding sites in the polypyrimidine tract of the default 3′ splice site (Inoue et al., 1990). In vitro splicing experiments suggest that Sxl blocks the utilization of this splice site by competing with the general splicing factor U2AF for binding to the polypyrimidine tract. As a consequence, U2AF interacts with the weaker polypyrimidine tract of the downstream 3′ splice site and activates this female 3′ site by recruiting U2 snRNP (Valcarcel et al., 1993). This simple competition model cannot account for Sxl autoregulation because the key target sites for Sxl protein in Sxl pre-mRNAs are located 200 or more nucleotides away from the 3′ and 5′ splice sites of the male exon (Sakamoto et al., 1992; Horabin and Schedl, 1993). Hence, if Sxl prevents the assembly of functional complexes on the male exon splice sites, it must do so by an indirect mechanism, and not by competing with general splicing factors for overlapping binding sites. One possibility is that Sxl proteins bound to target sites in the flanking introns interact with snRNP complexes assembled on each male exon splice site, inactivating these complexes (Salz, 1992; Deshpande et al., 1996; Salz and Flickinger, 1996). Several findings are consistent with this possibility. First, Sxl proteins are assembled into large, rapidly sedimenting complexes in vivo. These complexes contain not only Sxl proteins, but also the U1 and U2 snRNPs and Sxl pre-mRNAs (Samuels et al., 1994; Deshpande et al., 1996). Second, mutations in the snf gene disrupt autoregulation and exacerbate the female lethal effects of Sxl mutations (Salz, 1992; Albrecht and Salz, 1993; Flickinger and Salz, 1994; Salz and Flickinger, 1996). snf encodes the fly homolog of two mammalian snRNP proteins, the U1A protein of the U1 snRNP, and the U2B* protein of the U2 snRNP, and is a component of both the U1 and U2 snRNPs of Drosophila (Flickinger and Salz, 1994; Harper et al., 1992; Polycarpou-Schwartz et al., 1997). Third, Sxl:Snf complexes can be detected in vivo, and these two proteins are found to interact with each other in vitro (Deshpande et al., 1996; Samuels et al., 1998).

In vitro studies have shown that the two 90 aa RRM domains, R1 and R2, are responsible for RNA binding and for the two known Sxl protein:protein interactions (Samuels et al., 1994, 1998; Sakashita and Sakamoto, 1994, 1996; Wang et al., 1997). R1 and R2 interact with themselves and with each other and R1 but not R2 interacts with Snf. Although the two RRM domains are clearly important for the regulatory activities of the Sxl protein, they are not in themselves sufficient to carry out its regulatory functions. This was first suggested by the discovery of truncated Sxl proteins in the head of adult D. melanogaster males, which appeared to be generated by initiation at an AUG codon in exon 4 downstream of the male exon instead of at the usual AUG in exon 2 (Bopp et al., 1991). Since these proteins lack only about 40 aa from the normal N terminus, but still contain both RRM domains, they should bind to the appropriate target RNAs (cf Kanaar et al., 1995; Samuels et al., 1998). However, none of the expected female-specific splice products are detected in adult males.

While it was initially thought that the truncated proteins lacked regulatory activity only because they were present at very low levels, this explanation was called into question by ectopic expression experiments of Wang and Bell (1994) in Drosophila tissue culture cells. They showed that a Sxl protein lacking the first ~40 aa is defective in its autoregulatory activity. These findings have been extended by more recent experiments, which assayed the regulatory activities of the same N-terminal truncation in transgenic animals (Yanowitz et al., 1999). Remarkably, the N-terminal amino acids are not essential for dosage compensation, but they play a critical role in splicing as the autoregulatory activity of the truncation is severely impaired and it is unable to activate the tra-dependent female differentiation pathways. The failure to regulate tra is contrary to the expectations of the simple blockage model. Since only the RRM domains are required for specific, high-affinity binding to target RNAs in vitro, this model predicts that the N-terminal truncation should be as active as the full-length protein in preventing U2AF from binding to the default 3′ splice site.

One plausible explanation for the impaired activity of the truncation is that splicing regulation in vivo requires interactions between N-terminal sequences of Sxl and other proteins (which are presumably components of the Drosophila splicing machinery). These interactions could function to stabilize the binding of Sxl to the default 3′ splice site of tra pre-mRNAs and to the introns of Sxl pre-mRNAs. Alternatively, these interactions might play a more significant and direct role in mediating the splicing choice. In either case, it might be possible to interfere with the somatic splicing functions of the Sxl gene in females by ectopically expressing this putative N-terminal interaction domain. In the studies reported here, we have tested this hypothesis by generating transgenic animals carrying transgenes, which constitutively express a Sxl N-terminal:β-galactosidase fusion protein.

**MATERIALS AND METHODS**

**Fly stocks**

All fly stocks, unless otherwise noted in the text, are referenced in Lindesly and Zimm (1992). Flies were grown on standard Drosophila medium and maintained at room temperature (22°C), unless otherwise specified.

**Plasmid construction and Drosophila transformation**

β-gal fusion construct was made as follows. (1) PCR was used to generate a Sxl fragment, N, extending from the translation start site to amino acid 99 (Samuels et al., 1991). The 5′ oligonucleotide introduces a XhoI restriction site just upstream of the ATG, while the 3′ oligonucleotide introduces a BamHI site. After restriction digestion, the PCR fragment was cloned into Blue Script (BS) and sequenced. (2) An EcoRI fragment containing the lacZ-SV40 sequences from the C4-β-gal vector of Thummel et al. (1988) was inserted into the HindIII site of Bluescript to generate C4-β-gal-BS. (3) The N fragment was inserted into the Xho-K BamHI sites of C4-β-gal-BS to give N-C4-β-gal. (4) The final fusion of the first 99 aa of Sxl to β-gal was inserted as an XhoI-NcoI into the hsp83 mini-white vector (Horabin and Schedl, 1993). Germline transformations (Spradling and Rubin, 1982) were done by injecting plasmid DNA and helper vector, pTurbo into w1 embryos.

**Embryo staining**

Anti-Sxl monoclonals m114 and m5 are described in Bopp et al.
RT-PCR analysis and Southern blotting

RNA from 5-10 flies was prepared as described in Bell et al. (1991). Reverse transcription was performed according to the procedure of Frohman et al. (1990). To make Sxl cDNAs, primer MES21 was used in the RT reaction. PCR was then performed with primers T41-3 and BellA1 (primers described in Bell et al., 1991) spanning the sex-specific Sxl splice junctions. 1/10th of the Sxl RT reaction was amplified for 30 cycles of the following conditions: 95°C for 1 minute, 55°C for 45 seconds, 72°C for 30 seconds. 1% of the reaction volume was amplified for 30 cycles of the following conditions: 95°C for 1 minute, 99°C for 1 minute, 15 cycles of 95°C for 1.5 minutes, 55°C for 1 minute, 55°C for 1 minute, 15 cycles of 95°C for 1.5 minutes, 55°C for 1 minute, 72°C for 1 minute, 10% of the reaction volume was loaded on 2% agarose gels and Southern blotted onto nitrocellulose. Blots were hybridized with randomly primed Sxl MS3 cDNA.

To make tra cDNAs, reverse transcription was performed with the tra-3′ primer: Approximately 1/10th of the tra RT reaction was amplified with tra-5′ and tra-2 primers. The sequence of the three tra primers is as follows: tra-3′ GA TCTGGAGCGAGTGCGTCTG tra-5′ GGTCAACACTGAGAAATGC tra-2 CT TCTTACCCGATCTTGTTTCTC.

RESULTS

Generation of transgenic lines expressing a Sxl N-terminal β-gal fusion protein

To learn more about how the N-terminal domain contributes to the regulatory activities of the Sxl protein, we generated a chimeric gene consisting of the coding sequences for the first 99 amino acids of the Sxl protein fused in frame to β-galactosidase-coding sequences. This fusion gene was placed under control of the constitutive but heat-inducible hsp83 promoter and introduced into flies (see Fig. 1A). Four independent transgenic lines carrying the hsp83:Nβ-gal transgene were generated: N-21, N-41, N-141 and N-172. As expected, western blots probed with β-gal antibody showed that embryos carrying the Nβ-gal transgene express a protein that migrates more slowly than full-length β-gal (not shown). Similarly, a uniform β-gal antibody staining pattern is observed in 0-12 hour embryos from homozygous transgene stocks, and fusion protein appears to be distributed in both the nucleus and cytoplasm (not shown).

Expression of the amino terminal fragment of Sex lethal results in dose-dependent female-specific lethality

If the N-terminal domain participates in interactions that are critical for the regulatory activities of the Sxl protein, then the Nβ-gal fusion protein might interfere with the functioning of endogenous Sxl and have female lethal consequences. To test this possibility, we compared the relative viability of males and females homozygous for the hsp83:Nβ-gal transgene. Presented in Table 1A is the relative viability of homozygous transgenic males and females for three autosomal insertions, N-21, N-41 and N-172. At 25°C, the viability of females from all three lines is reduced by about 25% compared to their male sibs. These female lethal effects can be enhanced by raising the flies at 29°C, (see lines N-41 and N-172 in Table 1A) a temperature where the hsp83 promoter is expected to be more active, while they can be suppressed by raising the flies at 18°C (data not shown), where the promoter should be less active.

The correlation between temperature and viability suggests that the female lethal effects of the transgene arise from expression of the fusion protein. If this is correct, then female lethality should also depend upon transgene copy number. As shown in Table 1B for the N-41 and N-172 lines, the viability of males carrying either one or two copies of the hsp83:Nβ-gal transgene is at most only slightly less than that of males lacking the transgene. In contrast, there is a dose-dependent reduction in female viability. For females carrying one copy of the transgene, viability is reduced about 15%, while it is reduced to nearly 25% for females carrying two copies. It could be argued that the female lethal effects observed in animals containing two transgenes arise from a lesion caused by insertion of the transposable element, and not from ectopic expression of the Nβ-gal fusion protein. To show that this is not the case, we crossed N-41 heterozygous females to N-172 heterozygous males. As can be seen in Table 1C, the viability of males trans-heterozygous for the N-41 and N-172 inserts is equivalent to that of males carrying only a single copy of the transgene. In contrast, the viability of females trans-
heterozygous for the transgene inserts is only about 70% of that of females carrying a single copy of either transgene. Although the hsp83:Nβ-gal transgene reduces the viability of females but not males, it should be noted that (in these and subsequent experiments) the surviving females are usually phenotypically wild type. However, we infrequently observe weak transformations.

**The hsp83:Nβ-gal transgene behaves like a dominant negative mutation**

A plausible explanation for the female-specific lethality of the hsp83:Nβ-gal transgene is that the ectopically expressed fusion protein interferes with the regulatory activities of the Sxl gene. If the transgene acts like a dominant negative, then reducing the number of copies of the wild-type Sxl gene should exacerbate its phenotypic effects. To determine if this is the case, we varied the dose of Sxl while keeping the dose of the transgene constant. As shown in Table 2A, the viability of transgenic females heterozygous for the Sxl deletion, Sxl7B0, is reduced compared to sibs who have two wild-type Sxl genes.

We next varied the dose of the transgene in females that have only a single wild-type copy of Sxl. For this purpose, females heterozygous for the N-41 insertion were crossed to Sxl7B0 males that were also heterozygous for N-41. As shown in Table 2B, introducing a single copy of the transgene into Sxl7B0/+ females reduced viability by about 20%, while two copies of the transgene reduced viability by nearly 50%. In contrast, the transgene had only a modest effect on male viability (see Table 2B).

We conclude from the findings presented in Table 2A, B that the hsp83:Nβ-gal transgene behaves like a classical dominant negative mutation. To confirm this conclusion, we tested for interactions between the hsp83:Nβ-gal transgene and the Snf mutant allele, Snf1621. While females heterozygous for Snf1621 are fully viable, females trans-heterozygous for Snf1621 and a Sxl loss-of-function mutation have reduced viability due to a failure in establishing/maintaining the Sxl autoregulatory feed back loop (Salz, 1992). If the hsp83:Nβ-gal transgene behaves like a dominant negative Sxl mutation, it would be expected to show a similar synergistic interaction with Snf1621.

In the experiment shown in Table 2C, we varied the dose of the hsp83:Nβ-gal transgene in females heterozygous for Snf1621. As can be seen in Table 2, female viability drops as the number of copies of the transgene is increased and only about half of the Snf1621/+ females survive when there are two copies of the transgene.

**The hsp83:Nβ-gal transgene interferes with Sxl protein expression**

The fact that the phenotypic effects of the hsp83:Nβ-gal transgene are sensitive to the relative dose of the endogenous Sxl gene argues that the β-gal fusion protein acts like a classical dominant negative mutation, interfering with some critical regulatory activity of the Sxl protein. In principle, two different (but not mutually exclusive) mechanisms could account for the female lethality. The Nβ-gal fusion protein could prevent the endogenous Sxl protein from properly downregulating the dosage compensation system. Alternatively, the fusion protein could turn off the endogenous Sxl gene by disrupting Sxl autoregulation.

To determine if the Nβ-gal fusion protein interferes with autoregulation, we compared Sxl protein expression in

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**Table 1A. Female lethal effects induced by the Nβ-gal transgene**

<table>
<thead>
<tr>
<th>Line no.</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C</td>
<td>N-172</td>
<td>1585</td>
</tr>
<tr>
<td></td>
<td>N-41</td>
<td>1265</td>
</tr>
<tr>
<td></td>
<td>N-21</td>
<td>712</td>
</tr>
<tr>
<td>29°C</td>
<td>N-172</td>
<td>1342</td>
</tr>
<tr>
<td></td>
<td>N-41</td>
<td>1239</td>
</tr>
</tbody>
</table>

Crosses were set up between males and females carrying two copies of the indicated transgene insert and the number of offspring were counted. % viability measurements were calculated as follows, total number of females/total number of males × 100.

The genotype of the flies: w/w; P[w+, Nβ-gal]

No sex-specific lethality was observed either at 25°C or 29°C in the w/+ stock.

**Table 1B. Female-specific lethality induced by the Nβ-gal transgene is dose dependent**

<table>
<thead>
<tr>
<th>Cross</th>
<th>N-41/+ × Male (N-41/+)</th>
<th>N-41/+ × Male (N-172/+)</th>
<th>N-172/+ × Male (N-172/+)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(+) females</td>
<td>92% (512)</td>
<td>79% (876/2)</td>
</tr>
<tr>
<td></td>
<td>(+) males</td>
<td>100% (552)</td>
<td>94% (1042/2)</td>
</tr>
</tbody>
</table>

The viability measurements were conducted at 25°C.

The genotype of the flies used is w/w; P[w+, Nβ-gal]/+. The males carrying one copy of the transgene were mated with females carrying one copy of the same transgene. The number of progeny belonging to the different classes was counted. The number obtained for the w/w; P[w+, Nβ-gal]/+ (single copy) class was normalized by dividing by 2 and subsequently used for comparison. The flies were distinguished based on the intensity of the eye colour.

% viability measurements were calculated as follows, total number of flies belonging to a given genotype/ total number of males belonging to w/w class × 100.

**Table 1C. The female-specific lethality induced by the Nβ-gal transgene is independent of insertion site**

<table>
<thead>
<tr>
<th>Cross</th>
<th>N-41/TM3 × Males (N-41/TM3)</th>
<th>N-41/TM3 × Males (N-41/TM3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>females</td>
<td>88% (960/2)</td>
</tr>
<tr>
<td></td>
<td>males</td>
<td>100% (100%)</td>
</tr>
</tbody>
</table>

The viability measurements were conducted at 25°C.

The complete genotype of the flies used is w/w; P[w+, Nβ-gal]/+ TM sb. The two different inserts are indicated as N-172/TM3sb and N-41/TM3sb. N-172/TM3sb males were mated with N-41/TM3 females. Numbers of progeny belonging to different classes were counted. The number obtained for the N-172/TM3sb (single copy) class was normalized by dividing by 2 and subsequently used for comparison.

% viability measurements were calculated as follows, total number of flies belonging to a given genotype/ total number of males belonging to N-172/TM3sb class × 100.
collections of wild-type and transgenic 0-12 hour embryos. Wild-type embryo population could be divided into two roughly equal classes based on the pattern of Sxl protein expression. Sxl protein is not expressed in male embryos, while female embryos express Sxl protein and are uniformly and darkly stained with Sxl antibody. A different result was obtained for the transgene embryos. First, instead of two classes, the transgene embryos could be divided into three classes: embryos that do not express Sxl protein and are, like wild-type males (Fig. 2A), unstained; embryos that express Sxl protein and, like wild-type females (Fig. 2B), are uniformly stained, and finally, embryos that express reduced levels of Sxl protein and show patchy antibody staining (Fig. 2C). Second, as indicated in Table 3, more than 60% of the transgenic embryos, instead of the expected 50% fall in the unstained class while only about 30% of the embryos show the normal female staining pattern. These results indicate that Nβ-gal fusion protein interferes with autoregulation.

We next examined the expression of Msl-2 protein in wild-type and transgenic embryos. As expected, approximately 50% of the wild-type embryos express Msl-2 protein, while the other half do not. By contrast, 70% of the hsp83:Nβ-gal

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Table 2. The Nβ-gal transgene behaves like a dominant negative

<table>
<thead>
<tr>
<th>Cross</th>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sxl^{BO}+/+; N-41/N-41</td>
<td>Sx^{BO}+/+; N-41/N-41</td>
</tr>
<tr>
<td></td>
<td>Sxl^{BO}+/+; N-41/N-41</td>
<td>Sx^{BO}+/+; N-41/N-41</td>
</tr>
<tr>
<td>Females (Sxl^{BO}/Bin; N-41/N-41) × Males (N-41/N-41)</td>
<td>66%</td>
<td>80%</td>
</tr>
<tr>
<td></td>
<td>(676)</td>
<td>(822)</td>
</tr>
<tr>
<td>Males (Sxl^{BO}/N-41/N-41</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>(1021)</td>
<td>(5)</td>
</tr>
</tbody>
</table>

Females of the genotype ywSxl^{BO}/Bin, P[w+, Nβ-gal] were mated with males carrying two copies of the transgene i.e. P[w+, Nβ-gal]. The progeny belonging to the different classes were counted and compared.

ywSxl^{BO}/Bin is a deficiency chromosome that removes the Sxl locus.

% viability measurements were calculated as follows: total number of flies belonging to a given genotype / total number of progeny belonging to Sxl^{BO}/Y; N-41/N-41 class × 100.

**Table 2. The Nβ-gal transgene behaves like a dominant negative**

**A**

<table>
<thead>
<tr>
<th>Cross</th>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sxl^{BO}+/+</td>
<td>Sxl^{BO}+/+; tg/+</td>
</tr>
<tr>
<td></td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>Females (tg+/+) × Males (Sxl^{BO}/Y; tg+/)</td>
<td>100%</td>
<td>82%</td>
</tr>
<tr>
<td></td>
<td>(346)</td>
<td>(572/2=286)</td>
</tr>
<tr>
<td>Males (+/Y; +/+</td>
<td>100%</td>
<td>96%</td>
</tr>
<tr>
<td></td>
<td>(546)</td>
<td>(1056/2=528)</td>
</tr>
</tbody>
</table>

Males of the genotype ywSxl^{BO}; tg+/ were mated with females carrying a single copy of the transgene. The number of progeny belonging to each class was counted, normalized and compared. The number obtained for the tg+/ (single copy) class was normalized by dividing by 2 and subsequently used for comparison.

For females, the total number of flies belonging to a given genotype / total number of progeny belonging to ywSxl^{BO}/+; +/+ class × 100.

For males, the total number of flies belonging to a given genotype / total number of progeny belonging to +/Y; +/+ class × 100.

**B**

<table>
<thead>
<tr>
<th>Cross</th>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sxl^{BO}+/+</td>
<td>Sxl^{BO}+/+; tg/+</td>
</tr>
<tr>
<td></td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>Females (tg+/+) × Males (Sxl^{BO}/Y; tg+/)</td>
<td>100%</td>
<td>81%</td>
</tr>
<tr>
<td></td>
<td>(346)</td>
<td>(572/2=286)</td>
</tr>
<tr>
<td>Males (+/Y; +/+</td>
<td>100%</td>
<td>90%</td>
</tr>
<tr>
<td></td>
<td>(546)</td>
<td>(1056/2=528)</td>
</tr>
</tbody>
</table>

Males of the genotype ywSnf^{621}/+; tg+/ were mated with females carrying a single copy of the transgene. The number of progeny belonging to each class was counted, normalized and compared. The number obtained for the tg+/ (single copy) class was normalized by dividing by 2 and subsequently used for comparison.

For females, the total number of flies belonging to a given genotype / total number of progeny belonging to ywSnf^{621}/+; +/+ class × 100.

For males, the total number of flies belonging to a given genotype / total number of progeny belonging to +/Y; +/+ class × 100.
transgenic embryos are stained with the Msl-2 antibody, while only 30% are not. We presume that this latter group corresponds to transgenic female embryos that have wild-type Sxl protein expression. (Recall that 30% of the transgenic embryos had wild-type Sxl protein expression.) Conversely, the former group should be composed of male embryos and of female embryos that either had no Sxl protein expression or had an abnormal pattern of Sxl protein expression. Taken together, these findings argue that the female lethal effects of the Nβ-gal transgene arise, for the most part, indirectly through a disruption of Sxl autoregulation. Of course, we cannot exclude the possibility that the fusion protein also antagonizes Sxl regulation of Msl-2 protein expression.

The hsp83:Nβ-gal transgene alters Sxl RNA splicing

To confirm that the Nβ-gal transgene interferes with autoregulation, we examined the splicing of Sxl mRNAs. Male Sxl mRNAs differ from female because they contain an additional ~190 bp male exon (exon 3). The splicing pattern of Sxl mRNAs can be examined by RT-PCR with primers derived from sequences upstream and downstream of exon 3. In wild-type males, we observe RT-PCR amplification products corresponding to mRNAs spliced exons 2-3-4, but do not observe products corresponding to the exons 2-4 splice (see Fig. 3B). Similarly, in wild-type females, the male 2-3-4 splice Sxl mRNA is not detected, and instead we observe a band corresponding to the female mRNA (exons 2-4: Fig. 3B). While the splicing pattern of Sxl mRNAs in transgenic males resembles that in wild-type males, the splicing pattern in the surviving Nβ-gal transgenic females differs from that of wild-type females. Although the transgenic animals have female Sxl mRNAs (exons 2-4), autoregulation is incomplete and we also observed mRNAs spliced in the male pattern (exons 2-3-4: Fig. 3B).

Nβ-gal fusion protein is in a complex that contains Sxl

We sought to understand how the Nβ-gal fusion protein interferes with the regulation of Sxl pre-mRNA splicing. Previous studies have shown that Sxl protein, components of the splicing machinery and pre-mRNAs are assembled into large rapidly sedimenting complexes in vivo (Samuels et al., 1994; Deshpande et al., 1996). Hence, one possibility is that the Nβ-gal fusion protein is recruited into these same splicing complexes. It could then poison the complexes because of its large bulk, or it could prevent a key step in the regulated splice by competing with Sxl protein for interactions with some constituent of the complex.

If the Nβ-gal fusion protein is assembled into complexes with Sxl, it should be possible to immunoprecipitate the fusion protein with Sxl antibodies and, conversely, Sxl protein with β-galactosidase antibodies. In the experiment shown in Fig. 4A, we immunoprecipitated embryo extracts with Sxl antibody and then probed western blots of the immunoprecipitated proteins with β-galactosidase antibodies. As expected, the Nβ-gal fusion protein is not detected in Sxl immunoprecipitates of wild-type embryos, or in control immunoprecipitates of transgenic embryos using GST antibodies. The Nβ-gal fusion protein is, however, present in Sxl immunoprecipitates of extracts prepared from transgenic embryos. In the experiment shown in Fig. 4B, we immunoprecipitated wild-type and transgenic embryo extracts with β-galactosidase antibody, and then probed the western blots of the immunoprecipitated proteins with Sxl antibodies. Sxl protein is not detected when β-galactosidase antibodies are used to immunoprecipitate extracts from wild-type embryos, or, more importantly, from transgenic embryos expressing a "normal" β-galactosidase protein that lacks the Sxl protein sequences at the N terminus (see hsp83-β-gal in Fig. 4B). We do, however, detect Sxl protein in β-galactosidase antibody immunoprecipitates from the transgenic lines (N-41 and N-172), which express the Nβ-gal fusion protein. These results indicate that the Nβ-gal fusion protein can assemble into a complex with Sxl protein in vivo. Since Sxl:Sxl interactions in vitro are mediated by the RRM domains and not the N terminus (Sakashita and Sakamoto, 1996; Samuels et al., 1998), we presume that the Nβ-gal fusion protein interacts with Sxl in vivo through an intermediate.

The Nβ-gal fusion protein associates with Snf in vivo

In wild-type embryos, the Sxl splicing complexes contain Snf protein, U1 and U2 snRNPs, other unidentified proteins and pre-mRNAs. Of these constituents, the association between Sxl and Snf is relatively stable and survives immunoprecipitation without cross-linking. If the Nβ-gal fusion protein is recruited into these same Sxl splicing complexes, it may be possible to

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**Table 3**

<table>
<thead>
<tr>
<th>(A) Anti-Sxl antibody staining</th>
<th>Dark (%)</th>
<th>Partial (%)</th>
<th>Unstained (%)</th>
<th>n</th>
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<tr>
<td>Wt</td>
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<td>54</td>
<td>150</td>
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<tr>
<td>N'</td>
<td>30</td>
<td>5</td>
<td>65</td>
<td>300</td>
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</table>

<table>
<thead>
<tr>
<th>(B) Anti-Msl-2 antibody staining</th>
<th>Stained (%)</th>
<th>Unstained (%)</th>
<th>n</th>
</tr>
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<td>49</td>
<td>100</td>
</tr>
<tr>
<td>N'</td>
<td>69</td>
<td>30</td>
<td>200</td>
</tr>
</tbody>
</table>

*n* = total number of embryos.

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![Fig. 3. The hsp83:Nβ-gal transgene interferes with Sxl autoregulation. Sxl RT-PCR analysis of transgenic flies: Sxl RT-PCR was performed with primers upstream and downstream of the male exon 3. The RT primer is in exon 8, while the PCR primers are from exons 2 and 7. Results were visualized either by staining the gels with ethidium bromide (see photo) or by probing Southern blots with radioactively labeled Sxl cDNA (not shown). Sxl splicing was assayed in females and males from three different transgenic lines. The experiment presented here shows the results from the N172 line. *w* females and males serve as controls. Flies were raised at 25°C. Lanes from left to right: (1) wild-type males; (2) wild-type females; (3) Tg (172/172) males; (4) Tg (172/172) females.**
detect a similar association between the fusion protein and Snf. To determine if this is the case, we immunoprecipitated extracts from control and transgenic embryos with β-galactosidase antibodies. The immunoprecipitates were then probed with anti-Snf antibody. As can be seen in Fig. 4C, Snf protein is observed in the β-galactosidase immunoprecipitates of transgenic embryos but not in wild-type immunoprecipitates.

In vitro studies with purified proteins demonstrate that Sxl can interact directly with Snf via the R1 RRM domain and that this interaction does not require RNA (Samuels et al., 1998). Surprisingly, the in vivo Sxl:Snf complexes differ from the complexes formed in vitro with purified protein in that they can be disrupted by RNase digestion (Deshpande et al., 1996). From our previous studies, the target for RNase digestion appears to be the pre-mRNA and not the snRNA. While it is not understood why the in vivo Sxl:Snf complexes are disrupted by RNase digestion, this sensitivity likely reflects some feature(s) of the Sxl complexes (which appear to contain many other components) or of Sxl:Snf interactions in vivo which are not faithfully recapitulated in in vitro experiments with purified proteins. Nevertheless, if our hypothesis concerning the dominant negative activity of the Nβ-gal fusion protein is correct, then its association with Snf in embryonic extracts should also be RNase sensitive. To test this, we treated transgenic embryo extracts with RNase prior to immunoprecipitation with β-galactosidase antibody. As can be seen in Fig. 4C, the Nβ-gal fusion protein:Snf complexes are also disrupted by RNase treatment.

**Sedimentation behavior of the Sxl:Nβ-gal and the Sxl:Snf complexes**

When nuclear extracts from wild-type embryos are fractionated on sucrose gradients, Sxl is found predominantly in rapidly sedimenting complexes near the bottom of the gradient. A different sedimentation profile is observed for Sxl:Snf complexes. The more rapidly sedimenting Snf in the more rapidly migrating fractions near the bottom of the gradient. A different sedimentation profile is observed for Sxl:Snf complexes. The more rapidly sedimenting Snf in the more rapidly migrating fractions near the bottom of the gradient. A different sedimentation profile is observed for Sxl:Snf complexes. The more rapidly sedimenting Snf in the more rapidly migrating fractions near the bottom of the gradient. A different sedimentation profile is observed for Sxl:Snf complexes. The more rapidly sedimenting Snf in the more rapidly migrating fractions near the bottom of the gradient. A different sedimentation profile is observed for Sxl:Snf complexes. The more rapidly sedimenting Snf in the more rapidly migrating fractions near the bottom of the gradient. A different sedimentation profile is observed for Sxl:Snf complexes.
sedimenting fractions. In contrast, only very little Snf can be co-immunoprecipitated with Sxl antibody from fractions at the top of the gradient even though these fractions contain the bulk of the Snf protein (Deshpande et al., 1996).

Since the Nb-gal fusion protein associates with both Sxl and Snf in co-immunoprecipitation experiments, it was of interest to determine whether it is also assembled into large, rapidly sedimenting complexes. We fractionated nuclear extracts from Nb-gal transgene and wild-type embryos on sucrose gradients and examined the distribution of Sxl, the fusion protein and Snf by western blotting fractions from the gradient. The results of these experiments are shown in Fig. 5. As observed in wild-type extracts, the Snf protein in extracts from transgenic embryos sediments slowly and is found predominantly in factions at the top of the gradient (see Fig. 5A). While the Sxl protein in extracts from transgenic embryos migrates more rapidly than bulk Snf (see transgene Sxl in Fig. 5A), its distribution differs markedly from that observed in wild-type extracts. In wild-type extracts, most of the Sxl protein is found in the bottom third to one half of the gradient and there is only very little protein trailing into the upper portions of the gradient (see WT in Fig. 5A; see also Samuels et al., 1994 and Deshpande et al., 1996). Although Sxl protein from transgenic embryo extracts is detected in the very rapidly sedimenting fractions near the bottom of the gradient, substantial amounts of protein are also found in fractions from the middle and top half of the gradient (see Fig. 5A). Like Sxl, the Nb-gal fusion sediments more rapidly than bulk Snf; however, unlike Sxl, little fusion protein is found in the bottom half of the gradient (Fig. 5A).

These findings demonstrate the sedimentation profile of Sxl protein is substantially altered in extracts from Nb-gal transgene embryos. We wondered whether the more slowly sedimenting Sxl protein is associated with either Snf or the Nb-gal fusion protein. To answer this question, we immunoprecipitated each fraction with Sxl antibody and then tested whether the immunoprecipitable complexes contain either Snf or the Nb-gal fusion protein. In wild-type gradients, the immunoprecipitable Snf protein co-fractionates with Sxl. Like Sxl, most of the immunoprecipitable Snf protein is in fractions in the bottom one third to one half of the gradient; however, immunoprecipitable Snf protein can also be found in more slowly migrating factions (Fig. 5B; see also Deshpande et al., 1996). The profile of immunoprecipitable Snf protein in gradients prepared from Nb-gal transgene extracts is similar to that seen in wild-type extracts, except that there appears to be slightly more immunoprecipitable Snf protein in the more slowly migrating fractions. Of most interest, the immunoprecipitable Nb-gal fusion protein is not found in the rapidly sedimenting Sxl complexes in the bottom 1/3rd of the gradient (Fig. 5B). Rather, it is in complexes that sediment in the top 2/3rds of the gradient. These results suggest that the Nb-gal fusion protein does not assemble into the normal rapidly sedimenting Sxl:Snf splicing complexes, but instead is associated with more slowly sedimenting and presumably abnormal or incomplete complexes.
Nβ-gal fusion protein affects male development

The data presented in the previous sections indicate that the female lethal effects of the hsp83:Nβ-gal transgene are most likely a direct consequence of a disruption in Sxl autoregulation. Since the Sxl gene can be completely deleted in males without any detectable effects on viability, morphology or behavior, the Nβ-gal transgene would not, in principle, be expected to have any effect on male-specific developmental pathways. However, contrary to this expectation, approximately 10% of the males in all of the lines exhibit morphological alterations characteristic of sex transformations. These transformations include reduced or patchy abdominal pigmentation, extra sternite hairs, rotated genitalia (see Fig. 6) and alterations in the number or shape of the sex combs.

In females, the phenotypic effects of the hsp83:Nβ-gal transgene can be enhanced by raising the temperature and suppressed by lowering the temperature. To test whether this is also true for male feminization, we crossed wild-type females to males heterozygous for the N-41 transgenic line, and examined the viability and morphology of the resulting transgenic animals at either 25°C or 18°C. The lethal effects of a single copy of the transgene on female progeny from this cross decreases from about 16% at the higher temperature to about 6% at the lower temperature (not shown). On the contrary, decreasing the temperature does not reduce the frequency of feminization; the number of partially feminized males increases from 7% to about 21% as the temperature is dropped from 25°C to 18°C (not shown).

Expression of yolk protein mRNA in hsp83:Nβ-gal transgene males

The morphological alterations observed in transgenic males closely resemble those caused by the inappropriate expression of the female form of the Dsx transcription factor, DsxF, in males (Burtis and Baker, 1989). While the targets for DsxF in cuticular development are unknown, the expression of the yolk protein yp1 mRNA in the adult fat body is under the direct control of DsxF (An and Wensink, 1995; Erdman et al., 1996; Cho and Wensink, 1997). If the cuticular transformations in males carrying the hsp83:Nβ-gal transgene arise from the inappropriate expression DsxF, we would expect to find yp1 mRNA in these animals. As shown in the northern blot in Fig. 7, a low but readily detectable level of yp1 mRNA is present in transgenic N-172 and N-21 males, but not in the control wild-type males.

Expression of yp1 mRNA in transgenic males does not require the Sxl gene

In females Tra, together with Tra-2, directs the female-specific splicing of dsx mRNA. It is unlikely that the hsp83:Nβ-gal transgene promotes the synthesis of DsxF in males by directly altering the splicing pattern of dsx mRNAs. Rather, the transgene would be expected to act upstream of dsx processing, functioning either to turn on the Sxl autoregulatory feedback loop or to direct the female-specific processing of tra mRNAs. Arguing against the activation of Sxl is the fact that we failed to detect female spliced Sxl transcripts in males carrying the hsp83:Nβ-gal transgene (see Fig. 3). However, the hsp83:Nβ-gal transgene could induce the expression of Sxl proteins by some novel splicing mechanism. To test this possibility we introduced the hsp83:Nβ-gal transgene into males carrying the Sxl deletion, SxlΔ. We found that the frequency of feminized 7BO males carrying two copies of the transgene was close to 10%, essentially the same as that observed in a SxlΔ background. In addition, as shown in Fig. 7 the 7BO transgenic males express yp1 mRNA. These findings indicate that the Nβ-gal fusion protein must induce the expression of DsxF by a mechanism that is independent of Sxl.

Feminization of the hsp83:Nβ-gal males is mediated by tra

We used two different approaches to determine whether tra is the regulatory target for the hsp83:Nβ-gal transgene. In the first, we examined the splicing of tra pre-mRNAs in transgenic males using PCR primers corresponding to sites upstream and downstream of the regulated tra splice. Two amplification products are seen in wild-type males; one corresponds to unprocessed tra RNA (or amplified DNA) while the other to downstream female-specific 3′ splice site of exon 2 (Fig. 8A). Females have both of these products as well as a third more rapidly migrating product that corresponds to tra RNAs in which the first exon 1-2 splice is to the downstream female-specific 3′ site rather than to the upstream...
specific primers, PCR conditions and probe). (A) Lanes from left to right. (1) Wild-type male; (2) wild-type female; (3) wild-type copy of the Sxl gene instead of the Sxl+ gene, we considered below. In the second experiment, to investigate whether the effects of the hsp83:Nβ-gal transgene on cuticle morphology and expression ypl mRNA depend upon a wild-type tra gene, we introduced, the hsp83:Nβ-gal transgene into tra mutant males. Unlike tra+ transgenic males, none of the tra− transgenic males showed evidence of feminization nor were we able to detect ypl mRNA (not shown). With the important caveat that this a negative result, it suggests that tra is required for feminization.

**tra splicing in individual transgenic animals**

In the analysis of tra pre-mRNA splicing shown in Fig. 8A, female spliced products were detected in males from the N-172 line but not the N-41 line. One plausible explanation for this result is the low penetrance of the sex transformations caused by the hsp83:Nβ-gal transgene. Only about 10% of the transgenic males in each line show evidence of feminization, while the remaining males are phenotypically wild type. If significant levels of female spliced tra mRNAs are only present in a similar percentage of the adult male flies, we might not have detected female amplification products in the N-41 line in this experiment because the RNA was prepared from only 10 animals. Consistent with this possibility, female spliced products were observed in other preparations of RNA from N-41 males. To investigate this question further, we examined the tra splicing in individual transgenic males. The experiment in Fig. 8B shows the tra splicing pattern in individual N-21 and N-41 transgenic males that are either Sxl+/Sxl− or Sxl+. Some transgenic males have female spliced tra mRNAs, while others do not. Out of fifty Sxl+/Sxl− transgenic N-21 and N-41 males, seven had amplification products corresponding to the female spliced tra mRNA, while the remainder did not. We also examined ypl mRNA expression in individual N-41 Sxl+/Sxl− males. Seven of sixty males had detectable levels of ypl mRNA.

**DISCUSSION**

Nβ-gal fusion protein interferes with Sxl function in females

The Sxl gene controls somatic sexual development by post-transcriptional regulatory mechanisms that operate at the level of RNA splicing and mRNA translation. There is compelling evidence that these regulatory activities depend upon direct interactions between Sxl protein and target RNAs, and that these interactions are mediated by the two RRM domains (Kanaar et al., 1995; Wang et al., 1997; Sakashita and Sakamoto, 1996; Samuels et al., 1998). In addition to RNA binding, other interactions (e.g. protein:protein) are likely to be important for the diverse regulatory activities of the Sxl protein in vivo. For example, there is evidence that the N-terminal domain may participate in interactions that are important for the splicing functions of the Sxl protein. Experiments in tissue culture cells and transgenic animals have shown that a Sxl protein lacking the N-terminal 40 aa is severely compromised in its ability to activate the female splicing of Sxl and tra, but has nearly full function in the regulation of Msl-2 protein expression (Wang and Bell, 1994; Yanowitz et al., 1999). Although the specific regulatory defects of this N-terminal truncation are mostly explained by an inability to participate in some protein:protein interaction that is important for splicing, other mechanisms such as a weakened RNA binding can not be entirely excluded. For this reason, we sought a different approach for investigating the role of the N-terminal domain in the regulatory functions of the Sxl protein. The strategy that we used is to ask whether the N-terminal domain has any detectable biological activities in vivo in the absence of the two RRM domains and the C terminus. For this purpose, we generated a transgene that expresses a chimeric protein consisting of the Sxl N terminus fused to β-galactosidase. If the N-terminal domain participates in critical protein:protein interactions, this chimeric Nβ-gal fusion protein might compete with the endogenous Sxl protein and behave like a classical dominant negative mutation in females.

Our genetic analysis of hsp83:Nβ-gal transgenic lines is completely consistent with this prediction. The hsp83:Nβ-gal transgene reduces female viability, but has little effect on males. As expected for a dominant negative, the severity of the lethal effects depends upon the dose of the transgene relative to Sxl+. The conclusions drawn from these genetic experiments are directly supported by the finding that the transgene disrupts the Sxl autoregulatory circuit. Thus, these results provide

![Fig. 8](https://example.com/fig8.png)
independent evidence that the N terminus of the Sxl protein plays a critical role in its regulatory functions.

How does the Nβ-gal fusion protein interfere with Sxl autoregulation? Previous studies have shown that Sxl protein from nuclear extracts is assembled into large multicomponent complexes, which contain snRNPs and Sxl pre-mRNAs (Samuels et al., 1994; Deshpande et al., 1996). The most plausible hypothesis is that the fusion protein is recruited into these Sxl splicing complexes, interfering with their proper assembly and/or function. A number of lines of evidence support this model. First, the Nβ-gal fusion protein can be co-immunoprecipitated from nuclear extracts with antibodies directed against Sxl. Second, both Sxl and the U1/U2 snRNP protein Snf can be co-immunoprecipitated with β-galactosidase antibodies. Thus the Nβ-gal fusion protein must assemble into complexes in vivo that contain either Sxl or Snf or both proteins. Third, sucrose gradient sedimentation of nuclear extracts prepared from transgenic embryos reveals that the distribution of Sxl protein complexes is markedly altered from that observed in wild-type extracts. In wild-type extracts, the Sxl (Snf) complexes sediment rapidly through the sucrose gradient and are found predominantly in fractions in the bottom one half to one third of the gradient. While rapidly sedimenting Sxl (Snf) splicing complexes are present in extracts from transgenic embryos, a significant fraction of the Sxl protein is associated with more slowly sedimenting complexes that migrate in the top half of the gradient. Fourth, immunoprecipitation of the sucrose gradient fractions with Sxl antibody shows that the Nβ-gal fusion protein is incorporated into Sxl protein complexes. These sedimentation experiments also reveal an important difference between the Sxl complexes containing the Nβ-gal fusion protein and those that do not. The complexes containing the Nβ-gal fusion protein correspond to the abnormal, slowly sedimenting Sxl complexes, while the complexes that lack the fusion protein sediment rapidly like the wild-type complexes.

We infer from the reduced sedimentation rate that the Sxl Nβ-gal fusion protein complexes lack constituents found in wild-type complexes and/or have an abnormal conformation. The fusion protein could potentially prevent the assembly of the wild-type complexes by blocking the recruitment of some key component(s). Alternatively, the fusion protein could destabilize the splicing complexes so that they partially disassemble during sedimentation analysis. By either model, the splicing complexes formed with the fusion protein would be non-productive, and this could account for the dominant negative effects of the hsp83:Nβ-gal transgene on Sxl autoregulation.

A key step in this process is the recruitment of the fusion protein into the Sxl splicing complexes. Since the fusion protein lacks the RNA-binding domains of Sxl, we presume that it must be recruited into the complexes via protein:protein interactions rather than by recognition of pre-mRNAs. Only two Sxl protein:protein interactions have thus far been documented, Sxl:Sxl and Sxl:Snf. In vitro studies with purified proteins and two hybrid experiments indicate that the RRM domains and not the N terminus mediate both of these interactions. This would suggest that the fusion protein is recruited by as yet unidentified components of the splicing machinery and not by interactions with Sxl or Snf. While it is not possible to generate embryonic extracts that have the fusion protein but lack Sxl (or Snf), the gain-of-function phenotypes of the hsp83:Nβ-gal transgene in males (see below) suggests that the fusion protein can be incorporated into splicing complexes in the complete absence of Sxl protein. Perhaps it will be possible to identify which components of the splicing apparatus interact with the Sxl-N terminus and/or are important for autoregulation by screening for mutations that either enhance or suppress the dominant negative activity of the hsp83:Nβ-gal transgene.

Nβ-gal fusion protein feminizes males

While we expected the hsp83:Nβ-gal transgene would have an antimorphic activity in females, an entirely unanticipated finding is that it also behaves like a gain-of-function ‘mutation’ in males. Though both the expressivity and penetrance of this gain-of-function activity are low, the transgene appears to activate the female dxf differentiation pathway and partially feminizes males. Feminization does not involve nor does it require the ectopic activation of the Sxl gene in transgenic males; the Sxl gene is not turned on in transgenic males and feminization is observed in SxlΔBO males. This implies that the fusion protein targets a regulatory step(s) downstream of Sxl. Here the obvious candidate is tra. Two lines of evidence are consistent with this possibility. First, we detect female spliced tra mRNAs in transgenic males. Second, tra mutations appear to suppress feminization. (For the purposes of the discussion that follows, we presume that the Nβ-gal fusion protein promotes tra splicing by a mechanism that is related to that used by Sxl itself. While unlikely, we can not exclude the possibility that the mechanism is entirely novel.)

The gain-of-function activity of the Nβ-gal fusion protein is difficult to reconcile with the prevailing blockage model for the regulation of tra splicing (Sosnowski et al., 1989; Valcarcel et al., 1993). In this model, Sxl protein prevents the generic splicing factor U2AF from binding to the polypyrimidine tract of the default 3' splice site, forcing it instead to interact with the weaker downstream female-specific 3' splice site. A strong prediction of this model is that Sxl must be able to bind to the default polypyrimidine tract in order to block the binding of U2AF. Since specific binding to target RNAs requires the two Sxl RRM domains, the Nβ-gal fusion protein should have absolutely no tra regulatory activity. This prediction is not fulfilled. A second, weaker prediction is that the two Sxl RRM RNA-binding domains should be sufficient to regulate tra splicing. Consistent with this prediction, in vitro splicing experiments in Hela extracts with an adenovirus-tra hybrid pre-mRNA have shown that a Sxl protein without the N-terminal domain can promote the use of the female 3’ splice site (Granadino et al., 1997). However, while the N terminus does not seem to be essential in Hela extracts, it is important in vivo in flies since Sxl protein with a 40 aa N-terminal truncation is severely impaired in tra splicing regulation (Yanowitz et al., 1999). Taken together these findings call into question the simple blockage model and suggest that regulation of tra splicing may be different from that previously envisioned.

Both the impaired tra splicing activity of the Sxl N-terminal truncation and the ability of the Nβ-gal fusion protein to induce the female tra splice argue that the N-terminal domain plays a key role in the regulatory mechanism. To account for the regulatory activity of the Nβ-gal fusion protein, we must first suppose that it can associate with tra pre-mRNAs without actually binding directly to the RNA. The most-likely
mechanism is through interactions with generic RNA-binding proteins (or other components of the splicing machinery) that associate with tra pre-mRNAs. In this respect, it is interesting to note that, when tra and tra-2 bind to the splicing enhancer in the female exon of dsx pre-mRNAs, they form multicomponent complexes with generic SR-RRM proteins. The binding of these different factors to the dsx splicing enhancer seems to be facilitated by protein:protein interactions that modulate sequence specificity (Lynch and Maniatis, 1996).

A good candidate for a factor that could recruit the fusion protein to tra (and Sxl) pre-mRNAs would be the product of fl(2)d. Granadino and co-workers have shown that the fl(2)d gene is essential not only for Sxl autoregulation but also tra pre-mRNA splicing (Granadino et al., 1996, 1997). In the second step, the Sxl N-terminal domain in the fusion protein must promote the utilization of the female splice site. It has long been thought that this is accomplished by preventing the assembly of a functional U2 snRNP complex on the default 3’ splice site (Valcarcel et al., 1993). The fact that the Sxl N-terminal truncation fails to promote the utilization of the female splice site in vivo (Yanowitz et al., 1999) suggests that regulation does not pivot upon a competition between Sxl and U2AF for binding to the default pyrimidine tract. Likewise it is difficult to envision how the Nβ-gal fusion protein would be able to compete with U2AF for binding to tra pre-mRNA.

These results argue that blockage requires not only the binding of Sxl protein to the default splice site, but also depends upon some interaction between the N terminus of the Sxl protein and the splicing machinery. (This interaction could be quite distinct from the one that initially recruits the Nβ-gal fusion protein to tra pre-mRNAs.) For example, the N terminus (in Sxl or in the fusion protein) could contact some component of the U2 snRNP associated with the default branch point. This interaction could prevent this U2 snRNP from participating in lariat formation. An alternative possibility suggested by the weak gain-of-function activity of the Nβ-gal fusion protein is that the Sxl protein promotes female splicing by activating the weak female 3’ splice site. Though the experiments of Sosnowski et al. (1989) are consistent with the expectations of a simple blockage model, they do not conclusively exclude an activation model (or a combination of blockage and activation). In this case, Sxl proteins bound to the default 3’ splice site would interact with proteins associated with the downstream female 3’ splice site to activate the weaker splice site. Discriminating between these possibilities will require the identification and analysis of the factors that enable the Nβ-gal fusion protein to promote the female splicing of tra mRNAs.

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