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flex, an X-linked female-lethal mutation in *Drosophila melanogaster* controls the expression of *Sex-lethal*

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SUMMARY

The *Sex-lethal* (*Sxl*) gene is required in Drosophila females for sexual differentiation of the soma, for gem cell differentiation and dosage compensation. We have isolated three new alleles of *female-lethal-on-X (flex)*, an X-linked female-lethal mutation and have characterized its function in sex determination. SXL protein is missing in *flex/flex* embryos, however transcription from both Sxl_{Pe}, the early *Sxl* promoter and Sxl_{Pm}, the late maintenance promoter, is normal in *flex* homozygotes. In *flex/flex* embryos, *Sxl* mRNA is spliced in the male mode. Analysis of *flex* germline clones shows that it also functions in oogenesis, but in contrast to *Sxl* mutants that show an early arrest tumorous phenotype, *flex* mutant egg chambers develop to stage 10. In *flex* ovarian clones, *Sxl* RNA is also spliced in the male form. Hence, *flex* is a sex-specific regulator of *Sxl* functioning in both the soma and the germline. Genetic interaction studies show that *flex* does not enhance female lethality of *Sxl* loss-of-function alleles but it rescues the male-specific lethality of both of the gain-of-function *Sxl* mutations, Sxl^{M1} and Sxl^{M4}. In contrast to mutations in splicing regulators of *Sxl*, the female lethality of *flex* is not rescued by either Sxl^{M1} or Sxl^{M4}. Based on these observations, we propose that *flex* regulates *Sxl* at a post-splicing stage and regulates either its translation or the stability of the SXL protein.

Key words: Sex determination, Dosage compensation, *Sex-lethal*, Splicing, Translational control, *flex*

INTRODUCTION

In *Drosophila melanogaster*, the sex determination process is initiated by the ratio of the number of X chromosomes (X) to the number of sets of autosomes (A). This ratio is assessed by a counting process involving the products of X-linked numerator and autosomal denominator elements (Bridges, 1925; reviewed in Cline, 1993; Cline and Meyer, 1996). This signal is transmitted to *Sex-lethal* (*Sxl*), the master switch gene located on the X chromosome which then is activated sex specifically. *Sxl* regulates all aspects of sexual differentiation by either activating or repressing downstream genes (reviewed by Cline and Meyer, 1996). *Sxl* is regulated at two levels: at the level of transcription (as described above) and subsequently at the level of splicing. When the X:A ratio is 1.0, as in XX embryos, an embryo-specific *Sxl* promoter (*Pe*) is activated during nuclear cycles 12-14 (Erickson and Cline, 1993; Keyes et al., 1992). In XY embryos, whose X/A ratio is 0.5, *Sxl* remains 'off' and SXL protein is not synthesized. After the blastoderm stage, *Pe* becomes inactive and expression is regulated by a maintenance promoter, *Pm*, which is active in both sexes. However, the accumulation of Sex-lethal protein (SXL) is restricted to females through a positive autoregulatory RNA splicing mechanism (Bell et al., 1991; Cline, 1984). Alternately spliced transcripts are generated by the splicing process and this prevents the inclusion of a translation-terminating exon in Sxl_{Pm}-derived mRNA in females (Bell et al., 1988; Bopp et al., 1991). In addition to *Sxl* itself, three genes are known to be involved in the female-specific splicing of *Sxl* pre-mRNA: *snf* (Albrecht and Salz, 1993; Salz, 1992), *fl(2)d* (Granadino et al., 1990, 1992), *vir* (Hilfiker and Nothiger, 1991; Hilfiker et al., 1995).

In addition to its function in regulating female somatic development, *Sxl* also imposes the female mode of X-chromosome dosage compensation (reviewed in Baker et al., 1994; Kelley and Kuroda, 1995; Lucchesi, 1983, 1997). Misregulation of *Sxl* upsets dosage compensation resulting in sex-specific lethality because of inappropriate levels of X-linked gene expression (Cline, 1978; Gorman et al., 1993; Lucchesi and Skripsky, 1981; Gergen 1987). The products of five genes, *msl-1*, *msl-2*, *msl-3* and *mle* (collectively referred to as the *msl*s) and *mof* are required for the proper regulation of dosage compensation (Belote and Lucchesi 1980a,b; Hilfiker et al., 1997; for reviews see Baker et al., 1994; Gorman and Baker, 1994; Lucchesi and Manning, 1987; Kuroda et al., 1993).

The genes regulating germline sex determination are substantially different from those governing somatic sex

determination. They include cell-autonomous (germ cell intrinsic components) and cell non-autonomous factors (somatic signals, Horabin et al., 1995; Nöthiger et al., 1989; Staab and Steinmann-Zwicky, 1996; Steinmann-Zwicky et al., 1989; reviewed by Pauli and Mahowald, 1990; Steinmann-Zwicky, 1992). Proper formation of the female germline requires *Sxl* (Bopp et al., 1993; Granadino et al., 1993; Nöthiger et al., 1989; Oliver et al., 1993; Schupbach, 1985; Steinmann-Zwicky et al., 1989). Analysis of germline-specific mutants of *Sxl* show that *Sxl* is required for proper differentiation of female germ cells and its activity is maintained by autoregulation (Bopp et al., 1993; Hager and Cline, 1997; Schüpbach, 1985; Steinmann-Zwicky et al., 1989). Mutations in *snf*, *fl(2)d*, *ovo* and *otu*, show phenotypes similar to those observed in *Sxl* mutants and are required for the proper expression of *Sxl* in XX germ cells (Granadino et al., 1992; Oliver et al., 1993; Pauli et al., 1993).

From an EMS screen designed to identify additional X-linked genes affecting sex determination, one allele of a mutation named *flex* (*female-lethal on X*) was isolated (Anand, 1993). We isolated three additional alleles that, like the original allele, showed female-specific lethality. To gain insight into what causes this phenotype, we studied the expression of *Sxl* in *flex* homozygous embryos. We find that Sex-lethal protein (SXL) is not present in embryos homozygous for *flex*, which is presumably the cause of the lethality. Monitoring the transcription of *Sxl* shows that both the *Pe* and *Pm* promoters are activated normally. However, in *flex* homozygous females, *Sxl* transcripts are spliced in the male mode. *flex* is also required for the development of the female germline and functions in the regulation of *Sxl* in this tissue.

Genetic experiments show that *flex* rescues the male lethality of gain-of-function mutations of *Sxl* (Sxl^M). Unexpectedly, the lethality of homozygous *flex* females is not rescued by Sxl^M mutations. *flex* function is exclusively sex specific and we propose that it functions at the post-splicing level in either the translation of the SXL protein or its maintenance.

MATERIALS AND METHODS

Flies were maintained on standard cornmeal-yeast-sugar-agar medium and the embryos were collected on glucose agar plates.

Fly stocks

Unless otherwise indicated all mutations and chromosomes are described in Lindsley and Zimm (1992). $flex^1$(Anand, 1993) was the original *flex* allele used in this study. $flex^2$ was the EMS allele and $flex^3$ and $flex^4$ were the γ-irradiated alleles of *flex* isolated subsequently. Sxl^{Ml} and Sxl^{M4} were used to study interaction with *flex*. Sxl^{fP7BO} served as a negative control for the staining reactions. The stock carrying the Sxl_{Pe} promoter construct fused to *lacZ* (Keyes et al., 1992) on the second chromosome was used to look for activation of Sxl_{Pe}. *y w* $FRT^{9\text{-}2}$ */ y w* $FRT^{9\text{-}2}$ and ovo^{D2} v^{24} $FRT^{9\text{-}2}$/C(1)DX, *y f*/Y; FLP^{38}/FLP^{38} (Chou and Perrimon, 1992) were used to generate germline clones of *flex*.

X-Gal staining of embryos

Embryos carrying chromosomes with *lac-Z* promoter-fusion constructs were dechorionated and fixed in glutaraldehyde buffer. Following fixation, they were rehydrated in PBS containing 0.3% TX100. The embryos were transferred to a cavity block and incubated at 37°C in an X-Gal-staining solution (3.1 mM $K_4Fe(CN)_6$, 3.1 mM $K_3Fe(CN)_6$, 0.3%(v/v) TX100 and 0.2% X-Gal in PBS) until the blue stripes appeared (Montell et al., 1992). The reaction was stopped by rinsing the embryos in PBS-TX.

Anti-SXL staining of embryos

Embryos of interest were collected, dechorionated, fixed in formaldehyde and stained with the SXL ascites. The procedure followed was essentially that described by Bopp et al. (1991). Horseradish-peroxidase-conjugated detection reagents from the Vectastain ABC kit (Vector Laboratories, Burlingame, California) were used. The reaction was visualized with DAB (3,3′-diaminobenzidine) and stopped by rinsing in PBS when a dark-brown color developed.

For double labeling, embryos were first stained with X-Gal and subsequently with anti-SXL antibody. Embryos of the appropriate genotype were collected, stained with X-Gal, and the stained and the unstained embryos were separated. They were then devitellinized by shaking vigorously in 5 ml of heptane and 10 ml of methanol, rehydrated and stained with anti-SXL antibody as described before.

In situ hybridization

Hybridizations to *Sxl* transcripts were done using digoxigenin-labeled *Sxl* probes of sequences corresponding to the male-specific exon (g2) and an exon present in mature transcripts of both sexes (h1) (Samuels et al., 1991). Double-stranded probes (used for hybridization to whole mounts of embryos) were prepared by the random-primed labeling reaction as described in the manufacturer's protocol (Boehringer and Mannheim). Embryos of interest were collected, dechorionated, fixed and processed for in situ hybridization essentially as described by Tautz and Pfeifle (1989). Hybridization was carried out overnight at 48°C in formamide. Sense and antisense RNA probes (used for hybridization to whole mounts of ovaries) were prepared by labeling with the RNA polymerases (T3, T7 or SP6) according to the standard procedure.

For double labeling, embryos were collected, dechorionated, fixed and stained with X-Gal as described before (Montell et al., 1992). The unstained embryos were separated and devitellinized and processed for in situ hybridization (Cohen and Cohen, 1992) with *Sxl* exons as probes.

Staining embryos for anti-H4Ac16

Embryos of interest were collected, dechorionated, fixed in formaldehyde and stained with the polyclonal antibodies R14 and R41 to detect different isoforms of histone H4. The staining procedure used was that of Turner and Fellows (1989). For detection, the Vectastain ABC kit was used.

Germline clones

Clones homozygous for *flex* were generated by the FLP-DFS technique (Chou and Perrimon, 1992). Females of the genotype *y cho cv v f* $flex^2$ *FRT* (18E)/FM7 were crossed to ovo^{D2} *FRT* (18E)/Y; FLP^{38}/FLP^{38} males and the progeny were heat shocked for 2 hours at 37°C between L2 and L3. *y cho cv v f* $flex^2$ *FRT*/ovo^{D2} *FRT* females were then tested for their fertility.

RESULTS

flex is a female-specific lethal mutation

A mutation showing female-specific lethality, *flex*, was isolated in a screen for additional X-linked genes affecting sex determination (Anand, 1993). To identify more alleles, approximately 15,000 EMS mutagenised chromosomes and 8,000 γ- irradiated chromosomes were screened for non-complementation of the female lethal phenotype of $flex^1$. The screen was designed without selection bias, to identify male or female lethal alleles (Fig. 1). One EMS-induced allele, $flex^2$, and two γ-radiation alleles, $flex^3$ and $flex^4$, were obtained. As

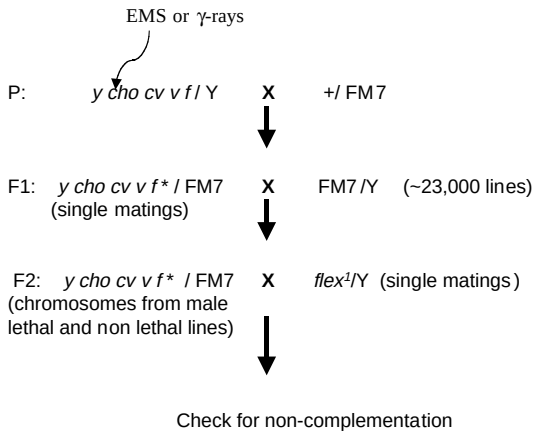

Fig. 1. Mutagenesis screen for the isolation of additional *flex* alleles.

observed for *flex*^{1}, hemizygous males of all three new alleles show the same viability as their balancer brothers and are fertile (Table 1). The lethal period was determined for all four alleles by counting the number of embryos that hatched. In all four alleles, about 25% of the eggs did not hatch and there were also a few dead larvae (< 1%) arrested at first instar stage. All four mutations are strong loss-of-function alleles, as they show the same lethal phenotype as homozygotes, as heteroallelic combinations and over a deficiency uncovering the locus.

SXL protein is not present in *flex/flex* female embryos

The sex-specific lethality associated with *flex* suggests that *Sxl* is misregulated in embryos homozygous for *flex*. Therefore, we stained embryos (2-13 hour) from wild type and a cross of *flex*/Bal females and *flex* males. While 50% of wild-type embryos, presumably the females, stain with anti-Sxl antibody (Fig. 2B, Bopp et al., 1991), only approximately 25% of embryos from the *flex* cross were positive (data not shown). This result is consistent with *flex/flex* females not expressing SXL.

To verify this hypothesis, we repeated the experiment using a FM7 chromosome carrying a *ftz*-promoter-*lac-Z* transgene that allowed us to identify the homozygous and hemizygous *flex* embryos. As a negative control for SXL staining, we did the same experiment with flies carrying a male viable deletion uncovering *Sxl* (*Sxl*^{7BO}). After X-Gal staining, embryos with the balancer chromosomes (Fig. 2A) were counted and amounted to 50% in the *flex* and *Sxl* experiments. The lacZ-positive embryos were manually separated and both the balancer and non-balancer embryos were stained with anti-Sxl antibody. About 50% of the embryos positive for β-gal stained with the anti-Sxl antibody (result not shown). But, neither the *Sxl*^{7BO}/*Sxl*^{7BO} females and *Sxl*^{7BO}/Y male embryos (0 of 166, Fig. 2C) nor the *flex* embryos stained with the antibody: *flex*^{1} (0/265), for *flex*^{2} (0/134, Fig. 2D) and for *flex*^{4} (0/179). These results show that *flex* plays a role in regulating the expression of SXL.

Transcription of *Sxl* is normal in *flex/flex* embryos

The expression of the *Sxl* early mRNAs depends on the maternal and zygotic genes, the numerator and the denominator elements, that control the activation of the early embryonic promoter *Pe* (Keyes et al., 1992). After the cellular blastoderm stage *Pe* is turned off and *Sxl* expression is maintained by the maintenance promoter *Pm*.

To examine whether *flex* controls the choice of sexual identity by regulating the function of *Sxl*$_{Pe}$ in the early embryo, a *Pe-lac-Z* reporter construct (Keyes et al., 1992) was crossed into *flex*^{2} and *flex*^{4} backgrounds. Embryos from the *flex* stocks and a *flex*$^{+}$ stock, all homozygous for the *lac-Z* reporter, were stained with X-Gal. The result was the same for all three genotypes, about 50% of the embryos were positive, indicating that *flex* does not control the transcriptional activation of *Sxl*$_{Pe}$ (results not shown).

In situ hybridization was performed to investigate if *flex* controls the transcription of *Sxl* from *Pm*. The probe used was a 1.0 kb *Sxl* exon (h1) present in the transcripts of both male and female post-blastoderm stage embryos. Virtually 100% of (3-3.5 hour) embryos from the wild-type and *flex*^{1} and *flex*^{2} populations showed positive staining and the distribution of the RNA was identical in all embryos (Fig. 2). Thus, *Sxl* transcription from both promoters is normal in *flex/flex* embryos and the distribution of the *Sxl* mRNA is not affected.

Sxl mRNA is spliced in the male-specific form in *flex/flex* female embryos

Since *Sxl* expression is controlled post-transcriptionally at the level of splicing, we investigated whether *flex* affects *Sxl* splicing. To this end, we carried out in situ hybridization with the male-specific *Sxl* exon (#3). Embryos from homozygous and hemizygous *flex*^{2}, *flex*^{4} as well as *Sxl*^{7B0} deletion embryos (3-3.5 hour old) were first identified by their lack of the characteristic *ftz* blue stripes, associated with the marked FM7

Table 1. All *flex* alleles are female lethal

	Paternal chromosome					
Maternal chromosome	*flex*^{4}		*flex*^{3}		*flex*^{2}	
	Sons	Daughters	Sons	Daughters	Sons	Daughters
flex^{3}	1064*	0	729	0	640	0
Balancer	1034	1104	748	794	611	693
flex^{2}	1104	0	843	0	986	0
Balancer	1120	1151	800	789	1007	1014
flex^{4}	1509	0	937	0	782	0
Balancer	1503	1482	907	963	756	731
Df(1)JA27	767	0	733	0	767	0
FM*7*	1002	989	977	1000	988	1012

Complete genotypes of *flex* alleles=*w cv f flex*; FM7, *y*^{31d} *sc*^{8} *w*^{a} *sn*^{X2} *v*^{of} *g*^{4} *B*.
*Numbers are pooled from three independent experiments.

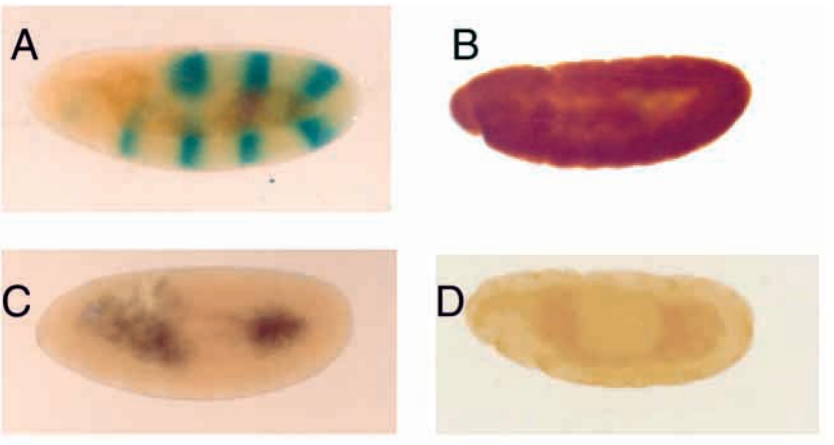

Fig. 2. Detection of SXL in mutant embryos. (A) Heterozygous (*flex*/+) embryos carrying a balancer chromosome with a *ftz-lac-Z* marker were stained for β-gal staining. (B) *flex*$^{+}$ (wild type) embryo staining with mSxl18, a monoclonal anti-SXL antibody; SXL staining is uniform throughout the embryo. (C) In *Sxl*^{7BO} and (D) *flex*^{2} embryos, SXL is not present. Anterior is to the left and dorsal to the top.

chromosome. In situ hybridization experiments using exon #3 was carried out on the unstained embryos. While, as expected, none of the *Sxl*^{7B0} embryos stained, almost 100% of the *flex* embryos expressed the male-specific exon (similar to what is shown in Fig. 3; Table 2), confirming that the female-specific splicing is disrupted in homozygous *flex* embryos.

In *flex/flex* female embryos histone H4 is acetylated at position 16, a characteristic of male dosage compensation

The enrichment of a particular isoform of acetylated histone H4 (H4Ac16) on the male X chromosome is taken as evidence of dosage compensation (Turner et al., 1992). Using an antibody raised against H4Ac16 (R14), increased staining can be detected in stage 9 male embryos (Franke et al., 1996; Rastelli et al., 1995). Since SXL is not present in *flex*/*flex* embryos, we investigated whether the acetylated H4 isoform is augmented in chromosomally female *flex* embryos.

Stage 14 (11-13 hours old) embryos collected from wild-type, *Sxl*^{7BO} and *flex* (*flex*^{2} and *flex*^{4}) stocks were stained with anti-H4Ac16, R14 antibody (Fig. 4). As shown in Table 3, 47% of the wild-type embryos show weak, background staining with the R14 antibody, while the other 50%, presumably the males, show intense staining in the anterior end. In contrast, only 27% of the embryos from the *Sxl*^{7BO} cross show weak background staining with H4Ac16, which are presumably *Sxl*^{7BO} /Bal females. Similarly, only about 25% of embryos from the *flex* crosses did not stain strongly, presumably representing the

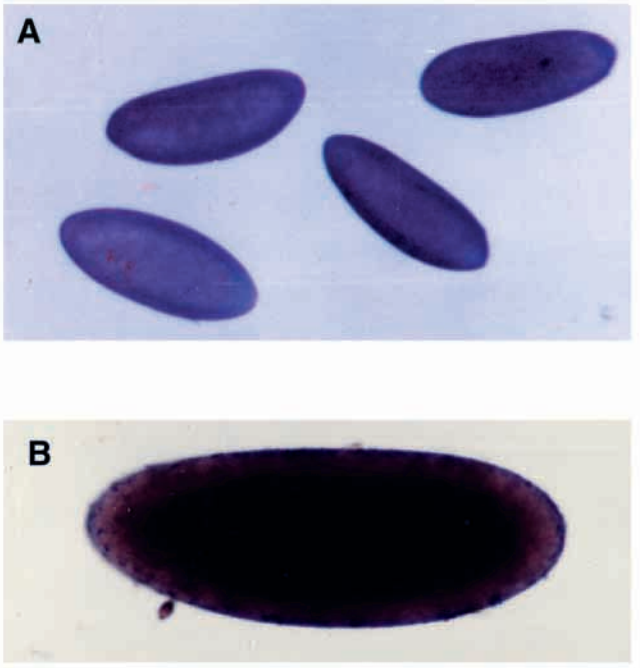

Fig. 3. *Sxl* expression in wild-type and mutant embryos. (A) All wild-type embryos show hybridization with exon number h1 present in both male and female transcripts. (B) All *flex* embryos also hybridize with the h1 probe. One representative embryos is shown. The negative control (embryos that were treated the same way but no probe was added) did not show any staining (figure not shown). The distribution of *Sxl* transcripts is uniform and identical to wild type in these embryos.

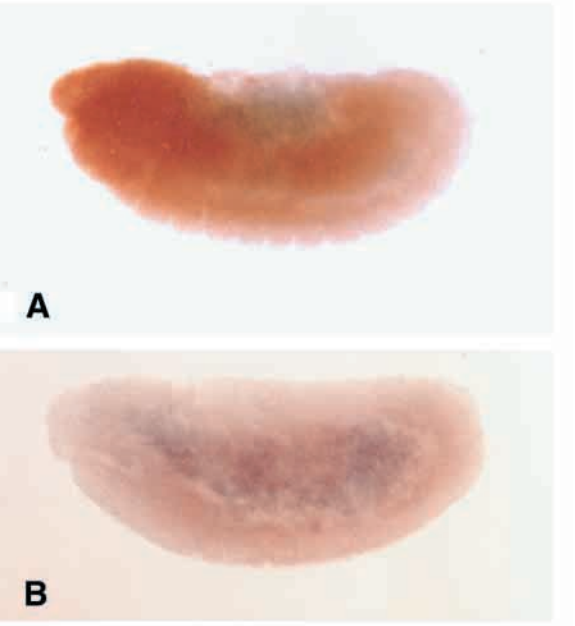

Fig. 4. Distribution of the histone H4Ac16 isoform in embryos carrying *flex*. The presence of H4Ac16 was detected with the polyclonal antibody R14. (A) An embryo showing the male characteristic distribution of H4Ac16. (B) A presumably female embryo showing only background staining. 75% of the *flex* embryos (Table 2) show the pattern illustrated in A indicating that they behave like males and are dosage compensated.

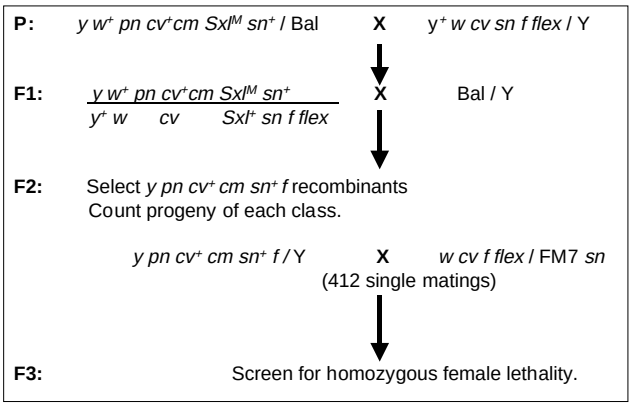

Fig. 5. Interaction between Sxl^{M} and *flex*.

flex/Bal females. This shows that the *flex* homozygous female embryos stain at the same level as males with the R14 antibody. R41 is an anti-histone H4 antibody that does not show any sex-specific difference in its distribution in embryos and was used as a control. As expected, virtually 100% of embryos from all crosses stained with this antibody, irrespective of their sex and genotype. These results confirm that dosage compensation in *flex/flex* female embryos is in the male mode, as would be expected since these embryos lack SXL.

flex suppresses male lethality of Sxl^{M1} and Sxl^{M4}

Sxl^{M1} and Sxl^{M4} are two gain-of-function mutations of *Sxl*, which result in the presence of SXL in chromosomally male animals, leading to inappropriate regulation of dosage compensation and subsequent male lethality (Bernstein et al., 1995; Cline, 1978). Mutations in *snf*, *fl(2)d* and *vir*, the splicing regulators of *Sxl*, can rescue Sxl^{M}-induced male lethality to varying degrees (Salz, 1992; Granadino et al., 1992; Hilfiker et al., 1995). We were therefore interested in finding out whether *flex* is able to rescue males carrying Sxl^{M1} or Sxl^{M4}. To this end, we recombined Sxl^{M} and *flex* onto the same chromosome, and scored for the survival of males carrying the two mutations (Fig. 5, Table 4). The presence of Sxl^{M} was monitored with the help of the two closely linked markers *carmine* (*cm*; 0.01 cM distal from *Sxl*) and *singed* (*sn*; 0.1 cM proximal from *Sxl*). The presence of *flex* was monitored by the presence of *forked* (*f*, 4.5 cM distal from *flex*).

The map distance between *Sxl* and *flex* is about 40 cM. Therefore, from a total of 971 males obtained from a recombination experiment involving Sxl^{M4} and *flex*, 388 are expected to be recombinants between *Sxl* and *flex*. Half of these should be the recombinant double-mutant males, while the other half should be wild type for both genes. The 81 double-mutant (Sxl^{M4} *flex)* males that were found to survive

Table 2. RNA in situ hybridization experiment with the male-specific *Sxl* exon

Genotype of embryos	Stained	Unstained	Total	Percentage stained
Sxl^{7BO} /$Sxl^{7BO;}$ Sxl^{7BO}/Y	0	210	210	0
$flex^{2}$ / $flex^{2}$, $flex^{2}$/Y	314	61	375	84
$flex^{4}$/ $flex^{4}$, $flex^{4}$/Y	263	24	287	92

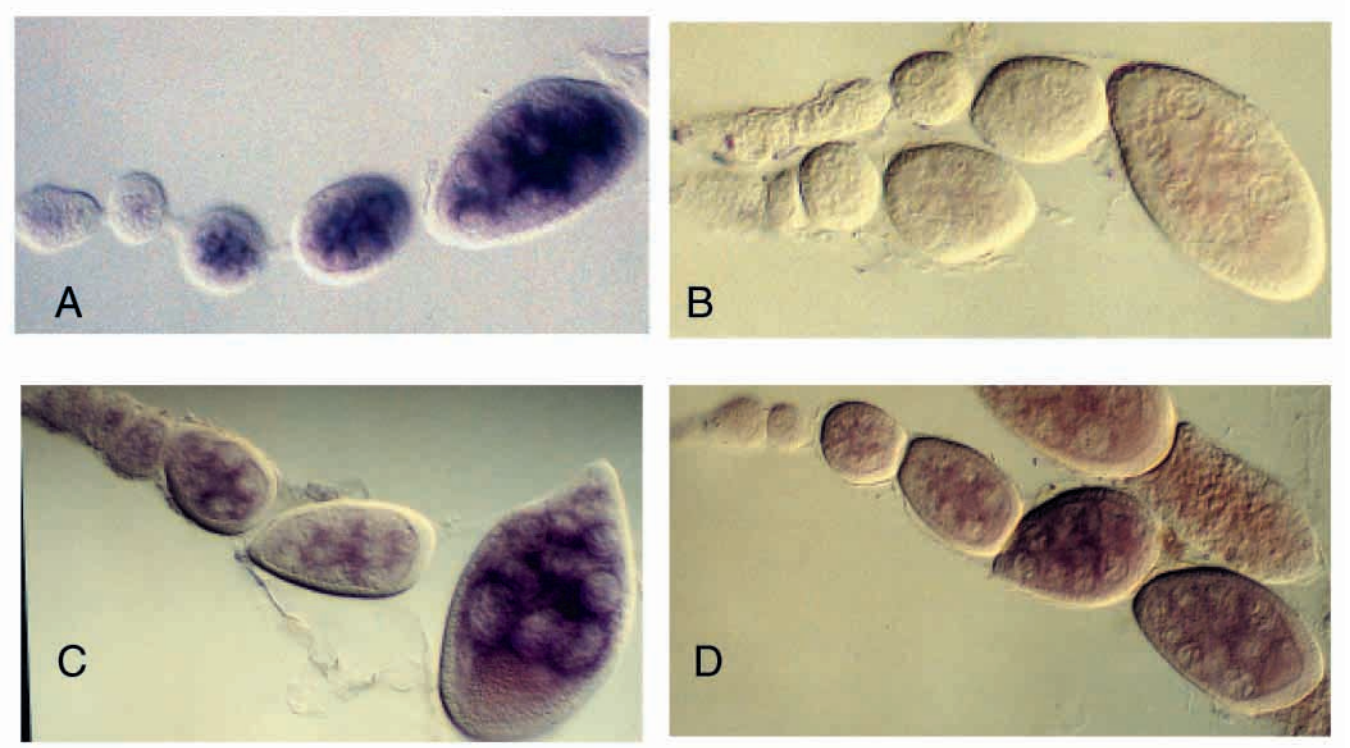

Fig. 6. Detection of *Sxl* expression in germline clones of *flex*. (A) Wild-type ovariole hybridized with the h1 probe, which detects mature *Sxl* transcripts in females. (B) Wild-type ovariole after hybridization with the male-specific exon #3. Only background staining is observed. (C) *flex/flex* germline clone hybridized with the h1 probe shows the same distribution of *Sxl* transcripts as in wild type. (D) A *flex/flex* germline clone hybridized with the male-specific exon #3 showing strong cytoplasmic staining (compare B and D). *Sxl* transcripts retain the male-specific exon in *flex/flex* clones indicating abnormal splicing.

Table 3. Detection of H4 isoforms upon staining with antibodies R14 and R41

	R14			R41		
Parental genotype	Stained	Unstained	Percentage unstained	Stained	Unstained	Percentage unstained
OR	147	131	47.12	232	8	3.3
Sxl^{7BO}/Bal×Sxl^{7BO}/Y	191	72	27.3	213	26	10.8
$flex^{2}$/ Bal×$flex^{2}$ /Y	195	61	23.6	184	20	10.2
$flex^{4}$ /Bal×$flex^{4}$/Y	291	93	24.2	435	31	6.6

represent 41.7% of expected recombinants. The result for Sxl^{M1} *flex* was similar; 59.6% of the expected recombinant males survived (Table 4). None of the surviving males were Sxl^{M} and $flex^{+}$ ruling out any unanticipated interaction. We conclude from this experiment that $flex^{2}$ rescues both $Sxl^{M\,1}$ and Sxl^{M4}. The rescue is slightly temperature sensitive, fewer males survive at 29°C than at 25°C. This rescue does not appear to be allele specific because $flex^{1}$ also rescues both Sxl^{M1} and Sxl^{M4}.

The presence of *Sxl* on the chromosomes was not further tested, since the flanking markers *cm* and *sn* are very closely linked to *Sxl*. To further verify the presence of *flex* in the rescued males, we tested for the homozygous female lethal phenotype of *flex* by crossing the 412 Sxl^{M} *flex* males singly to *flex*/FM7 *sn* females. In all the crosses, only FM7 chromosome bearing males and females and Sxl^{M} *flex* males survived (approximately 30 offspring of each genotype counted from each cross). Therefore, the presence of Sxl^{M} does not rescue the female lethality of *flex* homozygotes. The males that carry both Sxl^{M} and *flex* had no obvious evidence of sex transformation and were fertile. The genetic interactions between Sxl^{M} and *flex* confirms that the lethality of *flex*/*flex* homozygotes is due to a post-transcriptional perturbation in *Sxl*.

Sxl is spliced in the male form in *flex/flex* ovarian germline clones

Since most of the regulators of *Sxl* also function in the germline to maintain the expression of *Sxl*, we investigated whether in *flex/flex* germ cells *Sxl* regulation is disrupted. We found that *flex* function is essential for normal oogenesis. *flex/flex* germline clones induced in *flex*/ovo^{D2} females using the FLP-DFS system (Chou and Perrimon, 1992) did not produce fertile eggs. Inspection of ovarian *flex/flex* clones showed that oogenesis initiated normally, but the egg chambers did not develop past stage 10 (Bhattacharya et al., 1999).

In such germline clones, the splicing of *Sxl* was monitored by in situ hybridization using the antisense *Sxl* exons as probes (Fig. 6). The clones were generated in a background of ovo^{D2} which has been shown to arrest before stage 6 of oogenesis (Pauli et al., 1995). Any egg chamber of a later stage was considered to represent a *flex/flex* clone. Large amounts of *Sxl* transcripts were detected in all germ cells when the *Sxl* exon present in the transcripts of both sexes was used as a probe (Fig. 6C). The pattern of expression is identical to that observed in wild-type ovarioles (Fig. 6A; Bopp et al., 1993). When whole-mount ovaries were hybridized with a probe that detects only the male exon sequences, intense cytoplasmic staining was observed only in *flex* clones, especially at stages 8-10 (Fig. 6D), and only very faint staining in $flex^{+}$ egg chambers (Fig. 6B). No staining was observed when the control sense probes were used (data not shown). This indicates that *flex* is essential for *Sxl* splicing and, in its absence, transcripts containing the male-specific exon accumulate. Hence, *flex* regulates *Sxl* not only in somatic cells, but also in the female germline.

Table 4. Rescue of Sxl^{M} by *flex*

		Number of males with			
Maternal genotype	Temperature	$Sxl^{M}flex$	$Sxl^{M}flex^{+}$	Sxl^{+} *flex;* Sxl^{+} $flex^{+}$	Percentage rescue
$Sxl^{M4}/flex$	25°C	81	0	891	41.7
	29°C	52	0	804	30.6
$Sxl^{M1}/flex$	25°C	171	0	1267	59.6
	29°C	108	0	988	49.3

Percentage rescue represents the actual number of Sxl^{M} *flex* males/expected number of double-mutant males. The expected number of double-mutant males is calculated from the map distance between *Sxl* and *flex*.

Cross 1: *y pn cm* Sxl^{M4} sn^{+}/Bal females×*w cv sn f* $flex^{2}$/Y males. In the F_2, *y pn* cv^{+} *cm* sn^{+} *f*/Y males were scored as carrying both Sxl^{M4} and $flex^{2}$. Males with only Sxl^{M4} (*y pn* cv^{+} *cm* sn^{+} f^{+}/Y) were not recovered. Cross 2*: y pn cm* Sxl^{M1} sn^{+}/Bal females×*w cv sn f* $flex^{2}$/Y males. The experiment was the same as described for Sxl^{M4}.

DISCUSSION

flex functions as a positive regulator of *Sxl*

We report on the characterization of *flex*, a female-specific lethal mutation on the X chromosome. In *Drosophila*, such sex-specific lethality is usually indicative of a gene that functions as part of the sex-determination pathway. We found that SXL is absent in *flex/flex* female embryos throughout embryogenesis. We further found that, while *Sxl* transcription from both the early (*Pe)* and the late constitutive promoter (*Pm*) is normal, male-specific splicing is observed in *flex* female embryos. The absence of *flex* results in dosage compensation in chromosomally female embryos, as seen by the presence of H4Ac16 in these animals. Male-specific splicing is also observed in ovarian germline clones homozygous for *flex*. But, homozygous mutant cells survive in clones generated in the thorax and abdomen. Clones on the female forelegs did not show any appearance of sex combs (data not shown). Hence, it is unlikely that *flex* has a general, non-sex-specific function, but rather *flex* is a positive regulator of *Sxl*, which is essential for female-specific splicing, and is required for the expression of the SXL.

flex is not essential for *Sxl* transcription

The sex-specific regulation of expression of *Sxl* in the early embryo is regulated both at the level of transcription and splicing. The products of the counting elements *da*, *sisA*, *sisB*,

her and others, regulate the transcription from the *Pe* promoter in response to the X/A ratio. Splicing is controlled by *snf, fl(2)d* and *vir* (reviewed in Cline and Meyer, 1996).

Our experiments show that, in embryos homozygous for *flex*, there is no apparent defect in the activation of both the *Pe* and *Pm* promoters of *Sxl*. We also investigated genetically whether *flex* affects transcription of *Sxl*. We determined if *flex* shows any dose-dependent synergistic interactions with *da, sisA, sisB* and Sxl^{f}. *Trans*-heterozygous combinations of these genes and *flex* showed no effect on the sex ratio (data not shown). In contrast, all X/A counting elements show dose-dependent synergistic interactions with one another and with Sxl^{f1} (Cline, 1988; Torres and Sanchez, 1989). Hence, it is unlikely that *flex* is a counting element like *da*, *sisA* or *sisB*, and suggests that *flex* is not a part of the transcriptional machinery controling *Sxl* activation.

Rescue of Sxl^{M} by *flex*

The Sxl^{M} mutations are caused by insertions into the *Sxl*-coding region that result in the constitutive expression of the gene from Sxl_{Pm} irrespective of the X/A ratio (Bernstein et al., 1995). These *Sxl* gain-of-function mutations can rescue the female-lethality caused by mutations in *da, sisA, sisB* and *her* (Cline, 1978, 1988; Pultz and Baker, 1995). On the contrary, the splicing regulators *snf, vir* and *fl(2)d* rescue the male lethality caused by Sxl^{M} (Salz, 1992; Hilfiker et al., 1995; Granadino et al., 1992). Hence, interaction with Sxl^{M} provides a useful tool to investigate at which level genes in the pathway function. We found that both alleles of *flex* tested ($flex^{1}$ and $flex^{2}$) rescued the Sxl^{M} male lethality.

The observed interaction between *flex* and Sxl^{M} is similar to that seen between *snf, vir* and *fl(2)d* (Salz, 1992; Hilfiker et al., 1995; Granadino et al., 1992) and, since these mutations affect *Sxl* regulation at the post-transcriptional level, *flex* is also likely to function post-transcriptionally. Indeed, in our in situ hybridization experiments, we found that splicing of the *Sxl* transcript in the female form is affected in *flex* embryos resulting in the default male-specific splice. In other aspects also, *flex* behaves similar to *vir*. It suppresses Sxl^{M1} and surviving males are fertile showing no sex transformation (Hilfiker et al., 1995), unlike *fl(2)d1*, where the *trans*-heterozygous males are sex transformed (Granadino et al., 1992).

A clear difference in function between the splicing regulators and *flex* is evident from the observation that both Sxl^{M1} and Sxl^{M4} can rescue the female lethality of *vir* and *fl(2)d*, but they fail to rescue the female lethality of *flex* homozygotes. Also, none of the regulators of *Sxl* splicing rescue the male lethality of Sxl^{M4}. *flex* is the only mutation, that shows rescue of this phenotype. This difference in rescue could possibly be due to the fact that *snf* and *fl(2)d*1 are partial loss-of-function alleles.

Regulation and function of *Sxl* and *flex* in the female germline

Most of the genes involved in the somatic sex determination cascade are dispensable within the germ cells (Granadino et al., 1993; Marsh and Wieschaus, 1978; Schupbach, 1982; Steinmann-Zwicky, 1993, 1994; Horabin et al., 1995). *Sxl*, although necessary for oogenesis, does not have a master regulatory function for sex determination in the germline (Horabin et al., 1995; Steinmann-Zwicky, 1994). The expression of *Sxl* in the germline depends both on an inductive signal from the soma and on an autonomous signal given by the X:A ratio, which is measured by elements different from those used in assessing the X:A ratio in the soma (Granadino et al., 1993; Steinmann-Zwicky, 1993). *snf, fl(2)d* and *vir* which control the maintenance of *Sxl* activity in the soma are also required in the germline (Granadino et al., 1992; Hilfiker et al., 1995; Salz, 1992).

We found that *flex*, which is required for the expression of SXL in the soma, also regulates *Sxl* in the germline, but the loss-of-function *flex* phenotype can only be detected in mid-oogenesis. The germline phenotypes of *flex* and *Sxl* are different. *flex/flex* germline clones proceed normally through oogenesis till about stage 10 when the egg chambers disintegrate. These clones, when hybridized with the male-specific exon of *Sxl* show intense cytoplasmic staining especially after stage 6 of oogenesis (Fig. 6D), indicating the *Sxl* splicing is affected. The different germline phenotypes of *Sxl* and *flex* could be due to one of the following reasons. A perdurance effect of FLEX protein synthesized in heterozygous stem cells prior to mitotic recombination could initiate the production of SXL protein in *flex* clones thereby allowing them to survive the early stage of SXL requirement. This possibility is unlikely because *flex/flex* clones show no defect in the early stages of oogenesis even 2 weeks after induction of mitotic recombination and after several rounds of stem cell divisions.

A second possibility would be that the first few hours of development in heterozygous condition before the induction of mitotic recombination determine the female fate of germ cells irreversibly. The induction of Sxl^{-} germline clones by mitotic recombination rule out this possibility (Schutt et al., 1998). The phenotype observed in Sxl^{f4} germline clones is similar to that observed in pole cell transplantation experiments (Schupbach, 1985). The phenotypic differences observed in *Sxl* and *flex* mutant egg chambers suggest that only the later germline functions of *Sxl* require *flex*.

The mechanism controlling the initiation of *Sxl* expression in the germline is unknown. Initially, SXL is necessary in gonial cells at the tip of the germarium of the adult ovary for female-specific development of the germ cells. Later in oogenesis, the germ cells may become independent of the primary signals from the soma, and the X/A ratio may control SXL levels by autoregulation (Hager and Cline, 1997). Bopp et al. (1993) have shown that, concomitant with this change in regulation, SXL gets redistributed during oogenesis. In stem cells and early cystoblasts the protein is predominantly cytoplasmic. Once the cluster of 16-cell cysts is formed the protein is concentrated in the nuclei of the cystocytes.

The genes required for regulation of *Sxl* at early stages, such as *otu* and ovo^{D}, as well as the splice regulators *snf* and *fl(2)d*, display similar phenotypes to *Sxl*; oogenesis is arrested in the germarium and multicellular cysts are formed. Only one other gene, *vir*, displays a similar phenotype to *flex*, and it has been suggested that it functions in the splicing of *Sxl* at later stages of oogenesis, after the transition in regulation (Schutt et al., 1998).

flex functions in the control of protein expression

Our results demonstrate that, in *flex* homozygous embryos, splicing of *Sxl* is disrupted and SXL protein is absent. Two basic hypotheses can be put forward to explain these

observations. (i) *flex* could regulate sex-specific splicing of *Sxl* either directly or indirectly. (ii) *flex* could regulate *Sxl* expression at a post-splicing level, controlling either the translation or stability of the protein.

Two results argue against *flex* functioning at the splicing level. First, vir^{2f}, which has been found to behave like a null allele in females (Hilfiker et al., 1995), is capable of suppressing Sxl^{M1} but fails to suppress Sxl^{M4}. In contrast, two alleles of *flex*, also null alleles (based on genetic criteria) suppress both Sxl^{M1} and Sxl^{M4}. It is therefore unlikely that the difference in Sxl^{M4} rescue could be due to residual levels of *flex* function. Sxl^{M4} has been found to be completely constitutive, it functions independently of the all transcriptional and splicing regulators (Bernstein et al., 1995), but it does require *flex* for its function.

Second, in contrast to mutants in all splice regulators, *flex* homozygous females are not rescued by either Sxl^{M1} or Sxl^{M4}. In particular, Sxl^{M4}, the constitutive allele, would be expected to rescue *flex* females if *flex* is essential for *Sxl* splicing. Either Sxl^{M4} is not a constitutive allele, and requires *flex* for its splicing, or *flex* does not function as a splice factor. We therefore suggest, that *flex* abolishes the presence of SXL and interrupts the autoregulatory feedback loop in Sxl^{M1} and Sxl^{M4} males. The fact that the lethal periods of *Sxl* and *flex* overlap, late embryonic (Albrecht and Salz, 1993) and the lack of SXL in *flex* homozygous embryos throughout embryogenesis is consistent with this hypothesis; *flex* may perturb the positive autoregulation by eliminating SXL, leading to the accumulation of transcripts containing the male exon. This explanation is likely to apply to the female germline as well. Thus, we propose that *flex* may function at the post-splicing level, controlling either the translation or the stability of SXL. No matter at what aspect of *Sxl* expression *flex* regulates, among all post-transcriptional regulators of *Sxl* known, it is unique in its sex specificity.

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