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The *Drosophila melanogaster fl(2)d* gene is needed for the female-specific splicing of *Sex-lethal* RNA

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In *Drosophila melanogaster*, sex determination and dosage compensation are under the control of the *Sex-lethal* (*Sxl*) gene. We have identified a gene, *female-lethal-2-d* (*fl(2)d*), located in the second chromosome, that interacts with *Sxl*. *fl(2)d* homozygous clones, induced during the larval stage of *fl(2)d*/+ females, develop male structures instead of female ones. *fl(2)d* homozygous females hyper-transcribe their two X chromosomes, as measured by comparing the level of the X-linked *sgs-4* transcript, which is dosage compensated, with that of the autosomal *sgs-3* transcript. Thus, with respect to the processes of sex determination and dosage compensation, loss-of-function mutations at the *fl(2)d* and at the *Sxl* genes are equivalent. Moreover, *fl(2)d* homozygous female larvae express the *Sxl* transcripts characteristic of males. These results indicate that the *fl(2)d* gene is needed for the sex-specific splicing pattern of the *Sxl* RNA that occurs in females, thus suggesting the involvement of the *fl(2)d* gene in the positive autoregulatory pathway of *Sxl*.

Key words: dosage compensation/*Drosophila*/sex determination/*Sxl* splicing

Introduction

In *Drosophila melanogaster*, sex determination and dosage compensation (hypertranscription of the male X chromosome) are under the control of the *Sex-lethal* (*Sxl*) gene. The functional state of the *Sxl* gene is determined by the X:A ratio signal: in 2X;2A flies *Sxl* will be ON, while in X;2A flies *Sxl* will be OFF (Cline, 1978). Two X elements of this X:A signal have been identified: *sisterless-a* (*sis-a*) (Cline, 1986) and a region of the *achaete-scute* complex that has been named *sis-b* (Cline, 1988) and which corresponds to the *scute(T4)* gene (Torres and Sánchez, 1989). Once the state of activity of *Sxl* is defined, an event that occurs at blastoderm stage, the X:A signal is no longer used and the activity state of *Sxl* remains fixed (Sánchez and Nöthiger, 1983). The capacity of the *Sxl* gene to function as a stable 'switch' is thought to be due to a positive autoregulatory function of the *Sxl* gene product (Cline, 1984). The *Sxl* gene controls the expression of two independent sets of regulatory genes: the sex determination genes and the genes involved in controlling dosage compensation (Lucchesi and Skripsky, 1981).

The *Sxl* gene produces two temporally separate sets of transcripts. The early set is composed of three transcripts found only around blastoderm stage. The late set is formed by three other transcripts, present in both females and males, which appear slightly later in embryogenesis and persist throughout the remainder of development and in adult life. The three female late transcripts overlap extensively and share most exons, but differ at their 3′ ends. The three male late transcripts are similar to their female counterparts, except for the presence of an additional internal exon (exon 3), which contains a translation stop codon; consequently the male late transcripts give rise to presumably inactive truncated proteins (Bell *et al.*, 1988). Another *Sxl* transcript appears in adult females associated specifically with the development of the germline (Salz *et al.*, 1989).

Our previous work has identified a gene, *female-lethal-2-d* (*fl(2)d*), located in the second chromosome, that interacts with *Sxl* (San Juán, Granadino and Sánchez, in preparation). We have characterized two recessive EMS-induced *fl(2)d* mutations. The $fl(2)d^1$ mutation is lethal in females and semilethal in males. The $fl(2)d^2$ mutation is lethal in both sexes. The $fl(2)d^1/fl(2)d^2$ combination is lethal in females and semilethal in males. The genetic and developmental analysis of the *fl(2)d* mutations, as well as their interactions with *Sxl* mutations, suggest that *fl(2)d* is needed for the maintenance regulatory pathway of the *Sxl* gene throughout the development of female flies.

In this work we present the effect of the *fl(2)d* mutations on the two processes controlled by *Sxl*: sex determination and dosage compensation. We show that loss-of-function mutations at the *fl(2)d* and at the *Sxl* genes are equivalent, with respect to both processes. We further demonstrate that $fl(2)d^+$ activity is required for the sex-specific splicing pattern of the primary *Sxl* transcript which occurs in females, thus supporting our proposal about the involvement of the *fl(2)d* gene in the maintenance regulatory pathway of *Sxl*.

Results

Loss-of-function fl(2)d mutations affect sex determination

We have investigated the effect of *fl(2)d* on sex determination by clonal analysis. The genotypes of the irradiated females are given in the legend to Figure 1. Mitotic recombination in the experimental females gives rise to *yellow-Minute*$^+$ clones that will be either homozygous (recombination I) or heterozygous (recombination II) for $fl(2)d^2$ (see Figure 1). If the $fl(2)d^2$ mutation is affecting sex determination, *yellow* clones should develop either male ($fl(2)d^2/fl(2)d^2$) or female ($fl(2)d^2/+$) structures. In control females, all *yellow* clones will develop female structures. Because *Sxl* function is required throughout development for the cells to follow the female developmental programme (Cline, 1979; Sánchez and Nothiger, 1982), $fl(2)d^2$ homozygous clones were induced during larval development (48−72 h after ovoposition).

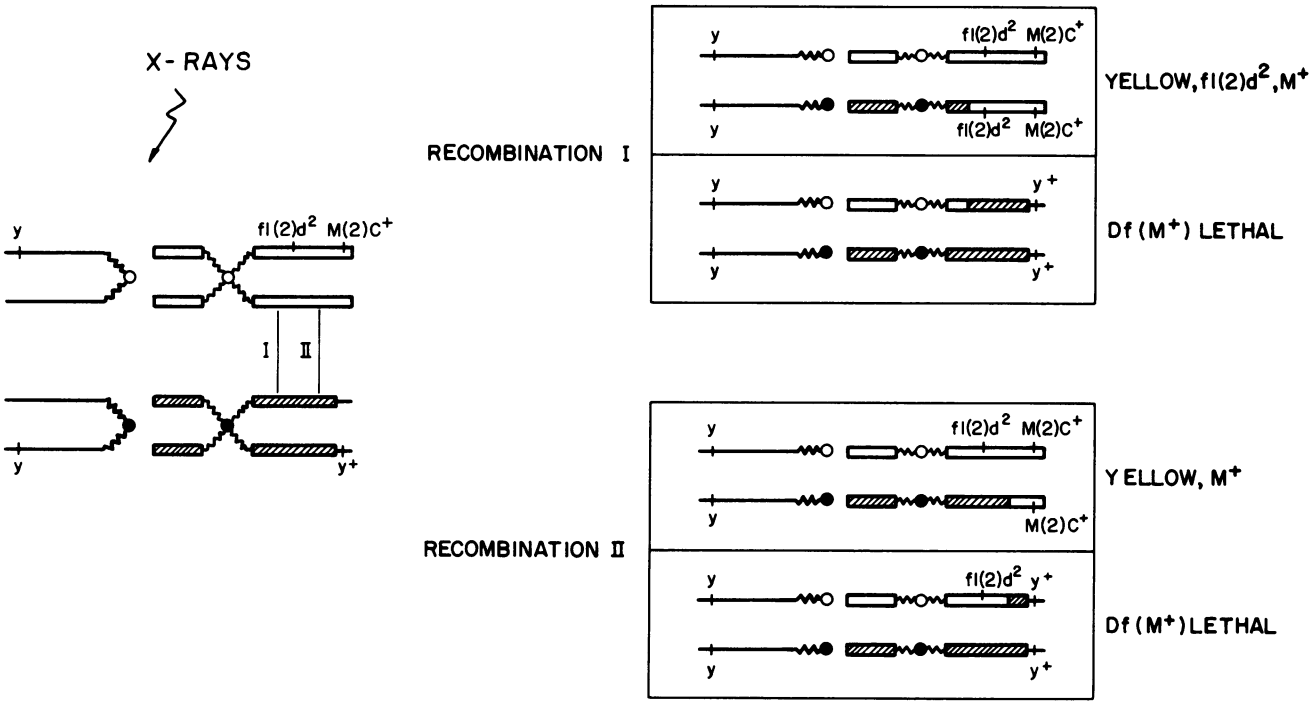

Fig. 1. Schematic representation of the experimental female genotype and consequences in daughter cells after mitotic recombination in different chromosomal regions. Thin line, rod-X chromosome; open and hatched bars, second chromosome; wavy line, heterochromatin. Chromosomes and the location of markers are not drawn to scale. The genotypes of the irradiated females were *y/yw*; *cn fl(2)d² bw M(2)C⁺/T(1,2)sc^s2, y⁺ M(2)C; mwh jv/+* (experimental) and *yw/yw; M(2)C⁺/T(1;2)sc^s2, y⁺ M(2)C; mwh jv/+* (control). Only recombination between the 2R chromosomes is considered. Mitotic recombination in the third chromosome produces *mwh-jv* clones that have been used as an internal control.

Table I shows the number and frequencies of the *yellow* clones obtained, and Figure 2 shows two examples. In the experimental females, the *yellow* clones developed either male or female structures. In the control females, however, all the *yellow* clones gave rise to female structures. The frequencies of the total number of *yellow* clones (with female or male phenotype) in the experimental genotype (24 clones in 110 flies) and in the control genotype (24 clones in 136 flies) are practically identical. Thus the *fl(2)d²* mutation is not behaving as a cell lethal, and consequently we should have recovered all the *yellow* clones that were induced in the experimental females. In both the experimental and the control animals the frequency of the *mwh-jv* clones was practically the same. The size of the *yellow* clones was bigger than that of the *mwh-jv* clones (data not shown), as expected for being *Minute⁺* (for size comparisons we used the clones in the notum). In all cases there was a complete correspondence between the mutant marker and the male phenotype. This shows that clones of male structures resulted from mitotic recombination, and that the expression of their genotypic sex is cell autonomous. With the exception of the tergites (see below), in all sexual dimorphic regions the number of the *yellow* male clones ($n = 15$) was three times the number of the female ones ($n = 5$). This difference is in agreement with the mitotic recombination map for the 2R chromosome (García-Bellido, 1972), given the position of the *fl(2)d* gene (70.08 m.u; San Juán and Sánchez, unpublished data) relative to the y^+ duplication at the tip of the 2R chromosome.

The lower frequency of the male clones in the tergites relative to the rest of the sexual dimorphic regions may be explained by the different growth dynamics of histoblasts and imaginal discs. Imaginal discs grow logarithmically during the larval period, but histoblasts do not divide, and only resume cell division at the onset of puparation (García-Bellido and Merriam, 1971a; Guerra *et al.*, 1973). Although irradiation took place at the larval stage, the clone homozygous for *fl(2)d²* appeared after histoblasts had resumed cell division; during the larval period histoblasts increase in size (Madhavan and Schneiderman, 1977) and sufficient *fl(2)d⁺* product could be synthesized to allow *fl(2)d²* homozygous cells to proceed with the female

Table I. Clonal analysis of *fl(2)d²* following irradiation at 48–72 h of development

Irradiated genotype	Structure	Number and frequency (%) of *yellow* clones	
		Female phenotype	Male phenotype
Experimental	Foreleg	1 (0.5)	4 (1.8)
(No. of flies = 110)	5th and 6th tergites	3 (2.7)	1 (0.9)
	Genitalia	2 (1.8)	5 (4.5)
	Analia	2 (1.8)	6 (5.4)
Control	Foreleg	7 (3.3)	0
(No. of flies = 136)	5th and 6th tergites	9 (6.6)	0
	Genitalia	4 (2.9)	0
	Analia	4 (2.9)	0

Experimental genotype stands for females heterozygous for *fl(2)d²*, and control genotype stands for wild-type females. Cross: *yw; Dp(1;2)sc^s2,y⁺ M(2)C/+; mwh jv* males were crossed to *y; cn fl(2)d² bw M(2)C⁺/SM5* (experimental) or *yw; M(2)C⁺/SM5* (control females).

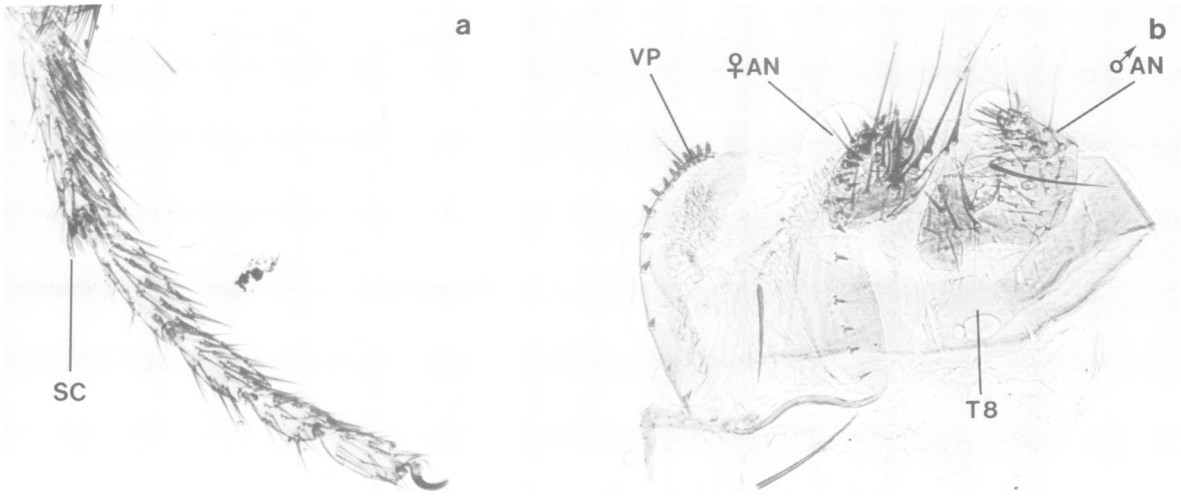

Fig. 2. Photographs (×200) showing male clones in the experimental females. Symbols: sex comb (SC), vaginal plate (VP), 8th tergite (T8), analia (AN). The sex comb bristles exhibited normal morphology and in some cases were even rotated. As a rule, the sex comb bristles replaced the corresponding female bristles in the region of the 7th and 8th transversal rows of the basitarsus. The male genital clones were accompanied by a complete set of female genitalia. Male clones in the anal plates were correlated with the absence of the corresponding female part.

developmental programme, i.e. there would be a 'perdurance effect' (García-Bellido and Merriam, 1971b) for the $fl(2)d^+$ product.

In conclusion, the clonal analysis of $fl(2)d^2$ has shown that this mutation affects sex determination: XX cells homozygous for $fl(2)d^2$ follow the male developmental programme in spite of their female chromosomal constitution. Thus, with respect to the process of sex determination, the $fl(2)d^2$ mutation mimics the behaviour of the loss-of-function mutations at the *Sxl* gene.

Loss-of-function fl(2)d mutations affect dosage compensation

To test the effect of the *fl(2)d* mutations on dosage compensation we followed the experimental approach of Breen and Lucchesi (1986). In salivary glands from $fl(2)d^1$ homozygous and heterozygous female and male larvae, we measured the amount of a specific X-linked transcript, that arising from the *sgs-4* gene, which is dosage compensated (McGinnis *et al.*, 1983), relative to the amount of RNA transcribed from the autosomal *sgs-3* gene (Meyerowitz and Hogness, 1982). These two RNAs are coordinately expressed in those glands from the middle of the third larval instar to the prepupal stage. The results are shown in Figure 3. No significant ($P > 0.05$) differences were found for the *sgs-4/sgs-3* ratio value of $fl(2)d^1/+$ females and males homozygous or heterozygous for $fl(2)d^1$. However, a significant ($P < 0.05$) increase in that ratio was found in females homozygous for $fl(2)d^1$. This indicates that $fl(2)d^1$ affects dosage compensation, i.e., it causes a hypertranscription of the two X chromosomes of females homozygous for that mutation. Thus, the $fl(2)d^1$ mutation mimics the behaviour of loss-of-function mutations at the *Sxl* gene with respect to the process of dosage compensation.

Loss-of-function fl(2)d mutations alter the sex-specific splicing pattern of the Sxl RNA

We have proposed that the *fl(2)d* gene is needed in females throughout development for the maintenance regulatory

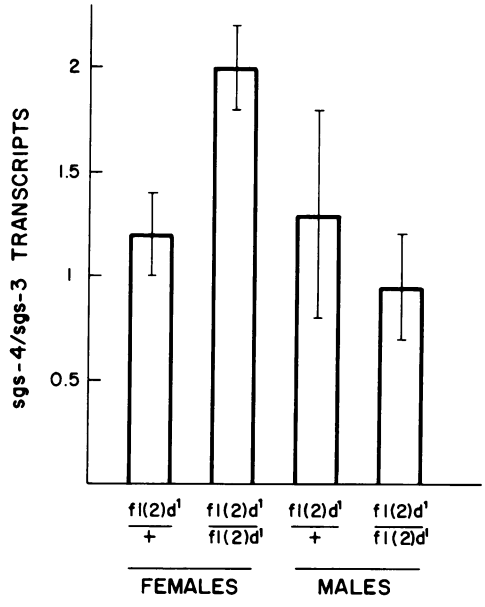

Fig. 3. Analysis of the level of *sgs-4* and *sgs-3* transcripts in female and male larvae homozygous or heteozygous for the $fl(2)d^1$ mutation. The bars represent the 95% confidence intervals. The experiment was performed four times. A statistial analysis of the results showed non-significant differences for the *sgs-4/sgs-3* ratio between the four replicas of the experiment. Then we pooled all the data to get a better estimate for the *sgs-4/sgs-3* ratio value.

pathway of the *Sxl* gene. The experiments reported in this section are aimed at exploring the mechanisms of such interaction.

We have analysed by Northern blots the late *Sxl* transcripts from $fl(2)d^1$ female and male larvae. As a control we have analysed wild-type female and male larvae. Two *Sxl* probes for hybridization have been used: the h^1-probe, that contains some of the exons common to both sexes, and the g^2-probe, that contains the male-specific exon (Bell *et al.*, 1988). Figure 4A shows that when the h^1-probe was used,

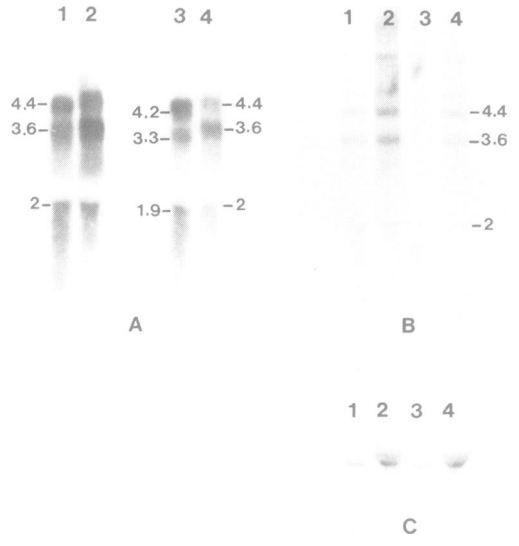

Fig. 4. Late *Sxl* transcripts of female and male larvae homozygous for *fl(2)d*^{1}. Blots contain 25 μg of total RNA from larvae of the indicated genotype. **Lane 1**, female larvae homozygous for *fl(2)d*^{1}. **Lane 2**, male larvae homozygous for *fl(2)d*^{1}. **Lane 3**, wild-type female larvae. **Lane 4**, wild-type male larvae. **A**. hybridization with the h^1 probe that contains exons common to both sexes. **B**. hybridization with the g^2 probe that contains the male-specific exon. **C**. hybridization of filter 4B with the actin probe DM-A3. The distortion in the migration of the lower mol. wt RNAs is due to the presence of rRNA in the RNA preparation.

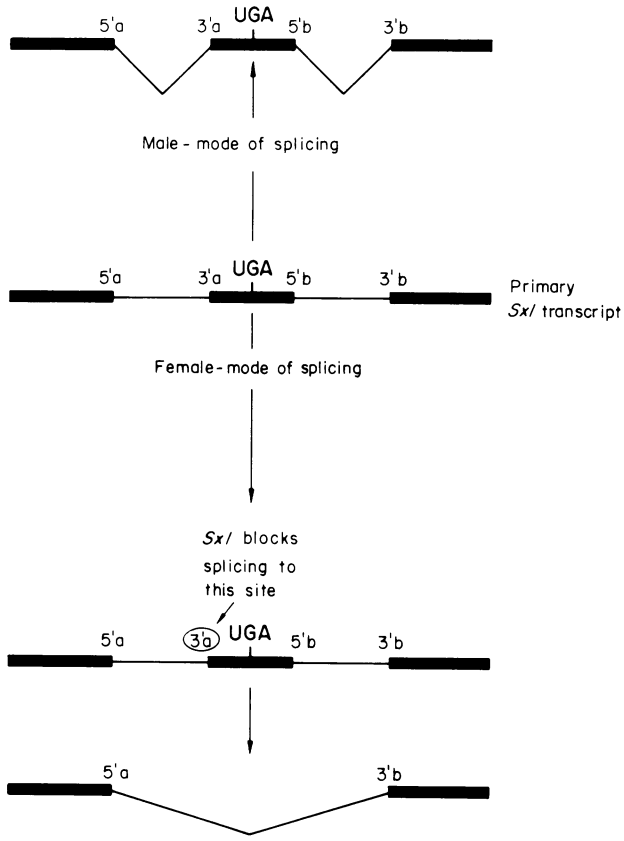

Fig. 5. Schematic representation of the sex-specific alternative splicing of *Sxl* RNA (from Bell *et al.*, 1988; and Sosnowski *et al.*, 1989). Only the portion of the *Sxl* gene that contains the common 2nd and 4th exons and the male-specific exon is shown.

the *fl(2)d*^{1} (lane 2) and wild-type (lane 4) male larvae have the late *Sxl* transcripts characteristic of males. As expected, the wild-type female larvae (lane 3) contain the *Sxl* transcripts characteristic of females; but the *fl(2)d*^{1} females (lane 1) contain the *Sxl* transcripts typical of males. This result was verified using the male-specific g^2-probe (Figure 4B). This probe did not hybridize with RNA from wild-type female larvae (lane 3), but it did hybridize with RNA from wild-type (lane 4) and mutant (lane 2) male larvae, and from *fl(2)d*^{1} female larvae (lane 1). These results demonstrate that *fl(2)d*$^+$ activity is required for the female-specific splicing pattern of the *Sxl* RNA, thus showing the involvement of the *fl(2)d* gene in the maintenance regulatory pathway of *Sxl*.

Discussion

Our results show that the loss-of-function mutations at either the *fl(2)d* gene or the *Sxl* gene are equivalent with regard to the two processes controlled by *Sxl*: sex determination (Table I) and dosage compensation (Figure 3). Since *Sxl* activity is controlled at the level of RNA splicing (Bell *et al.*, 1988), we examined the *Sxl* transcripts in *fl(2)d* mutants. We found that in *fl(2)d* mutant females the *Sxl* RNA follows the splicing pathway typical of males, consequently these females lack *Sxl*$^+$ function. These results indicate that the *fl(2)d* gene is needed for the female-specific splicing of the *Sxl* RNA.

It seems unlikely that the *fl(2)d* gene would code for a component of the general splicing mahcinery: in both *fl(2)d* mutant females and males, the *Sxl* RNA does not show an aberrant splicing pattern, but follows the pattern typical of wild-type males. Moreover, clones homozygous for *fl(2)d*^{2} are fully viable in both females (this report) and males (B.Granadino and L.Sánchez, unpublished data), contrary to the expected cell lethal behaviour of a mutation affecting the generic splicing machinery. On the other hand, the specific requirement of *fl(2)d*$^+$ function for the female mode of splicing of the *Sxl* RNA would explain the suppression of the lethality of *Sxl*^{M1} males by *fl(2)d* mutations; *Sxl*^{M1} is a mutation which expresses the *Sxl* functions characteristic of females independently of the X:A ratio signal, which determines its dominant male-specific lethal phenotype (Cline, 1978). The *Sxl*^{M1} males that are mutant for *fl(2)d* survive to 60% of their expected value (San Juán, Granadino and Sánchez, in preparation).

The *Sxl* gene regulates somatic sexual differentiation in females by controlling the female-specific splicing of the *transformer* (*tra*) RNA (Boggs *et al.*, 1987). It has been demonstrated that the female-specific splicing of the *tra* RNA occurs by blocking the non-sex-specific 3′ splice site; this blockade depends on both the particular sequence around that splicing site and the *Sxl*$^+$ function (Sosnowski *et al.*, 1989; Inoue *et al.*, 1990). The sex-specific splicing of *Sxl* RNA is diagrammed in Figure 5. Due to the similarity between the sequences in the neighbourhood of the non-sex-specific 3′-splicing site of the *tra* RNA and the sequences in the vicinity of the male-specific 3′ splicing site (3′a) of the *Sxl* RNA, it has been suggested that the sex alternative splicing of the *Sxl* RNA might result from a blockade of this 3′a site, which would also require *Sxl*$^+$ function (Sosnowski

et al., 1989; Inoue *et al.*, 1990). In addition, this would explain the positive autoregulatory function of the *Sxl* gene (Cline, 1984). Regardless of whether the *fl(2)d* product is directly involved in *Sxl* RNA splicing or if there exists another gene mediating the *fl(2)d* action, both Sxl^+ and $fl(2)d^+$ functions may be required to block the 3'a site. In this case, it should further be assumed that blockade of the 3'a site causes the 5'b site to become non-functional. This could be due to its inaccessibility, as a consequence of a conformational change of the *Sxl* RNA. This would prevent two donor sites (5'a and 5'b) competing for the same 3'b acceptor site. In *fl(2)d* females, the 3'a blockade would not occur and the primary *Sxl* RNA would follow the male mode of splicing, incorporating the UGA-containing exon. Consequently, these mutant females die because they lack the Sxl^+ proteins required for their development.

We cannot discard the alternative hypothesis that the 3'a blockade requires Sxl^+ function, whereas the $fl(2)d^+$ function would control the use of any of the other three splicing sites involved in *Sxl* RNA sex-specific splicing. The *fl(2)d* function could favour the splicing between the 5'a and 3'b sites either by changing the specificity of one of these two sites or by blocking the 5'b site. This would imply that the *Sxl* splicing function of *fl(2)d* has to be female-specific. Otherwise, the splicing that is normally observed in males might be affected.

As outlined in the Introduction, *fl(2)d* mutations also affect male viability. This cannot be attributed to a lack of *Sxl* activity, since this function seems dispensable for male development (Salz *et al.*, 1987). A possible explanation is that *fl(2)d* has a dual function: it is needed for sex determination and dosage compensation through its involvement in the female-specific splicing of *Sxl* RNA, and it is needed for a vital process which is independent of *Sxl* (A.San Juán, B.Granadino and L.Sánchez, in preparation). It may be possible that in this latter case also, the action of *fl(2)d* is exerted through its involvement in the splicing of a gene(s) controlling that vital process. This process would not be related to basic metabolic cell functions, because the *fl(2)d* mutations are not cell lethals. According to this proposal, the female lethality and the male semilethal phenotype of $fl(2)d^1$ would be due to this mutation affecting the *Sxl* splicing function more than the vital function. In contrast, the $fl(2)d^2$ mutation would strongly affect both functions, thus explaining the lethality of both females and males. In the case of the $fl(2)d^1/fl(2)d^2$ combination, the *Sxl* splicing function would be more affected than the vital function. This would explain why the $fl(2)d^1/fl(2)d^2$ combination is lethal in females while semilethal in males. The study of new *fl(2)d* alleles will help us to understand the function of this gene with regard to the vital process.

Materials and methods

Flies were cultured on standard food at 25°C unless otherwise stated. For a description of the genetic markers see Lindsley and Grell (1968) and Lindsley and Zimm (1982, 1985, 1987).

Clonal analysis of fl(2)d

The irradiation dose was 10 Gy and was supplied by a Philips X-Ray machine at a rate of 5 Gy/min.

The sexual phenotype of the marked clones was assessed in sexually dimorphic regions of the fly: sex comb region on basitarsus of foreleg, 5th and 6th tergites and external derivatives of the genital disc. For a description of the morphology of these structures see Bryant (1978). Male clones in a female foreleg basitarsus were defined by the presence of sex comb bristles. In the tergites, the male clones were defined by the presence of marked bristles associated with male pigmentation in the unpigmented area of the female 5th and 6th tergites. In the external derivatives of the genital disc, male genital elements, as well as large male clones in the anal plates, can easily be recognized. Small anal clones, however, could only be identified as male when they comprised the ventral part of the male anal plate where size and density of bristles clearly differ from the female pattern.

Analysis of sgs-4 and sgs-3 transcripts

The level of *sgs-4* and *sgs-3* transcripts of female and male larvae homozygous (experimental genotype) or heterozygous (control genotype) for the $fl(2)d^1$ mutation were analysed. Larvae were grown at 29°C. To generate them *cn* $fl(2)d^1$ *bw/T(2;3) Xa*, ap^{Xa} $L^{rm}Bc$ $Pu^2/+$ females were crossed with males homozygous for *cn* $fl(2)d^1$ *bw*. The larvae homozygous for $fl(2)d^1$ were recognized by their Black-cell$^+$ phenotype and white Malpighian tubules, while the $fl(2)d^1/+$ larvae presented the Black-cell phenotype.

Salivary glands from each type of larvae were dissected in Ringer's solution (NaCl 8.6 g/l, KCl 0.3 g/l and $CaCl_2.2H_2O$ 0.33 g/l, pH 7.0). Total salivary gland RNA was extracted following the experimental procedure of Case and Daneholt (1978). Levels of *sgs-4* and *sgs-3* transcripts was quantitated by the dot-blot technique using as probes pRH0.75 and adm124E8, respectively (Meyerowitz and Hogness, 1982; McGinnis *et al.*, 1983), kindly supplied by S.K.Beckendorf. RNA blotting and hybridization were performed as described by Maniatis *et al.* (1982). To quantify the degree of hybridization we measured the spots of the film with a Molecular Dynamics Computing Densitometer, model 300 A.

Northern analysis of Sxl transcripts

Female and male larvae homozygous for $fl(2)d^1$ were obtained from the same cross used for the analysis of the salivary gland transcripts. The culture temperature was also 29°C.

RNA preparation from frozen larvae, electrophoretical fractionation of total RNA and blotting to Nylon membranes were performed as described (Maniatis *et al.*, 1982; Campuzano *et al.*, 1986). Blots were prehybridized at 60°C for 2 h in 30 ml of 50% formamide in 5× SSC, 0.25% sodium dodecylsulfate (SDS), 5× Denhardt's solution, 0.25 mg/ml *Escherichia coli* tRNA and 0.1 mg/ml denatured salmon sperm DNA. Hybridization was carried out at 60°C for 20 h in 10 ml of prehybridization solution containing 7×10^{10} d.p.m./μg of single-stranded [^{32}P]RNA probe, prepared as described below. Filters were washed twice with 2× SSC, 0.1% SDS at room temperature, and four times with 0.1× SSC, 0.1% SDS at 65°C.

Mol. wt markers run in a parallel lane were *E.coli* 23S and 16S rRNA (Brosius *et al.*, 1978, 1980). High specific activity single stranded RNA probes were synthesized *in vitro* using T7 RNA polymerase as described by Melton *et al.* (1984). Genomic fragments from λS1 (Maine *et al.*, 1985) were subcloned in pGem-1 and pGem-2 and used as templates for preparing the male (g^2) and non-sex-specific (h^1) RNA probes, respectively. λS1 was kindly supplied by T.W.Cline. The hybridization with the actin probe DM-A3 (Fyrberg *et al.*, 1980) was carried out as described (Campuzano *et al.*, 1986).

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