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A search for additional X-linked genes affecting sex determination in *Drosophila melanogaster*

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Abstract

The gene *Sex-lethal (Sxl)* plays a pivotal role in *Drosophila* sexual development. Once activated in response to the X : A ratio signal in XX embryos, *Sxl* participates in appropriate implementation of all known aspects of sexual differentiation. We have attempted to identify new X-linked genes involved in sex determination, especially those involved in the regulation of *Sxl*. Since misregulation of *Sxl*, or that of the genes that regulate it, leads to female-specific lethality, or synergistic female-lethal gene interactions, or both, we used these criteria to screen about 10,000 EMS-treated chromosomes for (i) recessive female-specific lethality or (ii) enhanced female lethality in *trans*heterozygous combination with *Sxl*. Four potentially useful mutations—Sxl^{dlf}, *fl-35*, *fl-46*, *l-43*—were recovered and a few of their properties were characterized. Approximate map positions of these mutations were determined by meiotic mapping. To understand their probable position(s) in the hierarchy of genes regulating sex determination, we studied dose-dependent interactions between them and mutations in genes known to affect sex determination by generating double and triple heterozygotes. These studies suggest that (i) Sxl^{dlf} is not defective in the 'early' regulation or functions of *Sxl*, and (ii) *fl-35*, *fl-46* and *l-43* are unlikely to be a part of the X : A ratio signal, i.e. they are not needed for the transcriptional activation of *Sxl*. On the other hand, they could be affecting post-transcriptional processing of *Sxl* transcripts.

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Introduction

In *Drosophila melanogaster*, one of the early steps in the pathway leading to sex determination and dosage compensation is the female-specific activation of a switch gene named *Sex-lethal* (*Sxl*; reviewed by Cline 1993). The activity state of *Sxl* determines whether an embryo develops into a female or a male. We set up screens to isolate X-linked mutations in genes whose activity may be required for regulation of *Sxl*. This screen yielded four mutations of potential interest. Genetic characterization of these mutations is presented here.

It has been known for some time that the primary sex determination signal generated by the ratio of the number of X chromosomes to the number of sets of autosomes (the X : A ratio) acts to set the functional state of *Sxl* (Cline 1993). An X : A ratio of 1.0 results in transcriptional activation of the early-acting, female-specific promoter of *Sxl*, and this is essential for female sexual differentiation. When the X : A ratio is 0.5, as in XY : AA embryos, *Sxl* is not activated and male development ensues. The X : A ratio is thought to be assessed by means of discrete chromosomal sites called counting elements. 'Numerator' elements are those counting elements that are located on the X chromosome. They increase the probability of activation of *Sxl*, and thereby behave as feminizing genes. Four such genes are known: *sisterless-a* (*sis-a*; Cline 1986; Erickson and Cline 1993), *sisterless-b* (*sis-b*; Torres and

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Sanchez 1989; Erickson and Cline 1991), *runt* (*run*; Duffy and Gergen 1989) and *sisterless-c* (*sis-c*; Cline 1993). X-linked numerator elements are measured in reference to the autosomal 'denominator' component of the X : A ratio. Denominator elements, by negatively regulating *Sxl*, appear as masculinizing genes that can antagonize the action of the feminizing numerator elements. A candidate denominator gene, *deadpan* (*dpn*), has been known for some time (Younger-Shepherd *et al.* 1992; Barbash and Cline 1995). Appropriate activation of *Sxl* also requires maternal inputs. Three genes, *extra macrochaetae* (*emc*) (Younger-Shepherd *et al.* 1992; Cline 1993), *daughterless* (*da*) (Caudy *et al.* 1988; Cronmiller *et al.* 1988) and *hermaphrodite* (*her*) (Pulz *et al.* 1994), are needed for proper activation of *Sxl*. All these genes cause recessive female-specific lethality, or show dominant, dose-dependent and sex-specific interactions among themselves and with *Sxl*, or both.

Once *Sxl* has been activated, the X : A signal is not necessary for maintenance of its activity during subsequent development because female-specific activity of *Sxl* is autoregulated by alternative RNA splicing (Bell *et al.* 1991). Three genes, *sans fille* (*snf*; Flickinger and Salz 1994), *female lethal-2-d* [*fl(2)d*; Granadino *et al.* 1990] and *virilizer* (*vir*; Hilfiker *et al.* 1995), are known to participate in the sex-specific splicing of *Sxl* transcripts. By its interaction with downstream genes, *Sxl* participates in three key developmental pathways: somatic sex determination (reviewed by Baker 1989; Mckeown 1992; Steinmann-Zwicky *et al.* 1990; MacDougall *et al.* 1995), germline sex determination (reviewed by Pauli and Mahowald 1990; Steinmann-Zwicky 1992) and dosage compensation (reviewed by Kuroda *et al.* 1993; Baker *et al.* 1994; Bashaw and Baker 1996). Dosage compensation is a vital process that ensures dose equivalence of almost all X-linked genes by hypertranscribing the single X chromosome in males. Recessive loss-of-function mutations of *Sxl* in females, and its dominant gain-of-function mutations in males, result in lethality due to inappropriate levels of transcription of X-linked genes.

To identify the new X-linked genes that may be involved in sex determination and dosage compensation, we tested about 10,000 EMS-mutagenized chromosomes for phenotypes leading to (i) female-specific lethality and (ii) synergistic lethal interactions in *trans*heterozygous combination with *Sxl*. This screen yielded four mutations of interest: Sxl^{dlf}, an allele of *Sxl*; *fl-35* (*female lethal-35*) and *fl-46* (*female lethal-46*), two mutations showing female-lethality only when in *trans*heterozygous combination with *Sxl*, but without any apparent effect on male or female viability; and *l-43* (*lethal-43*), a sex-nonspecific lethal mutation showing synergistic female lethality in *trans*heterozygous combination with *Sxl*. In this paper we present results on the isolation and genetic mapping of these four mutations, as well as data on their interaction with known sex determination and dosage compensation genes.

Materials and methods

Stocks and growth conditions: The X chromosome to be mutagenized carried three recessive markers: *yellow* (*y*), *chocolate (cho)* and *singed* (*sn*). Males to be treated with the mutagen were collected from a stock that had been made isogenic for the X chromosome. Six mutations of *Sxl*, namely Sxl^{f1}, Sxl^{7BO}, Sxl^{f9}, Sxl^{fLS}, $Sxl^{fm7,M1}$ and $Sxl^{M1,fm3}$, and mutations at *sis-a*, *sis-b*, *da* and *fl(2)d* were used in experiments to study complementation and genetic interactions. Unless mentioned otherwise, flies were cultured at 25°C in 250-ml milk bottles or in 30-ml glass vials on standard cornmeal–sucrose–yeast medium. Details of the fly stocks and mutations used in this study are to be found in Lindsley and Zimm (1992).

Induction and isolation of mutations: A 0.025 M solution of ethylmethane sulphonate (EMS) made in 1% sucrose was used as mutagen (Lewis and Bacher 1968). Two-day-old to three-day-old males were selected in batches of about 150 and allowed to feed on EMS solution for about 12 hours. Treated flies were transferred to bottles containing fresh food and allowed to recover for about 5 hours. They were allowed to mate with +/*FM7* virgin females. Flies were transferred to fresh food bottles on the fourth and seventh day after mating. Single *Bar*-eyed females (*y cho sn**/*FM7*) from among the F_1 progeny were mated to *FM7* males. Over 10,000 such crosses were set up. In the F_2 progeny, *y cho sn**/*FM7* females are obtained; if the induced mutation is not a lethal, *y cho sn** males are also obtained. About 8000 mutagenized, male-viable lines were generated in this manner. Each of these 8000 male-viable *y cho sn** lines were crossed to *y cho sn**/*FM7* females and the progeny of these matings were screened for female lethality. Two lines showing female lethality were obtained. Genetic analysis of one of these lines, named Sxl^{dlf}, is presented here. About 2000 male-lethal chromosomes were also obtained in the F_2 progeny. To test whether these sex-nonspecific lethal lesions define genes with potential roles in the sex determination pathway, heterozygous females carrying this chromosome (*y cho sn**/*FM7*) were mated to Sxl^{f1} males and their progeny screened for synergistic female-lethality of the double-mutant combinations. Out of the 2000 lines tested in this manner, three lines, named *fl-35*, *fl-46* and *l-43*, which exhibited lethality in *trans*heterozygous combinations with Sxl^{f1}, were obtained. Each of these three lines (*fl-35*, *fl-46* and *l-43*) and Sxl^{dlf} was backcrossed thrice to an *FM7* stock to eliminate other lesions that might have been induced by EMS. Sex-specific lethality or interaction with Sxl^{f1} was reconfirmed after these backcrossings. The unmutagenized *y cho sn* chromosome was used in control crosses. This chromosome, by itself, does not affect female viability (sex ratio = 1.09) or interact with Sxl^{f1}; in the progeny of the cross between *y cho sn*/*FM7* females and Sxl^{f1} males, 270 *y cho sn*/Sxl^{f1} females, 264 *FM7*/Sxl^{f1} females, 286 *y cho sn* males and 152 *FM7* males were present. Reciprocal crosses

yielded 537 $Sxl^{f1}/y\,cho\,sn$ females, 409 $FM7/y\,cho\,sn$ females, and 609 and 408 males of genotype Sxl^{f1} and *FM7* respectively.

Recombination mapping: Approximate map positions were determined for these four mutations by calculating their map distances from *y* (map position 1-0.0), *cho* (1-5.5) and *sn* (1-21.0). Each mutant chromosome was made heterozygous with the Oregon-R wild-type chromosome by crossing $y\,cho\,sn^*/FM7$ females with Oregon-R males. Wild-type F_1 females ($y\,cho\,sn^*/+++$) were mated to *FM7* males. F_2 recombinant chromosomes were tested for female-specific lethality or interaction with Sxl^{f1} and for segregation of these phenotypes in relation to *y*, *cho* and *sn*.

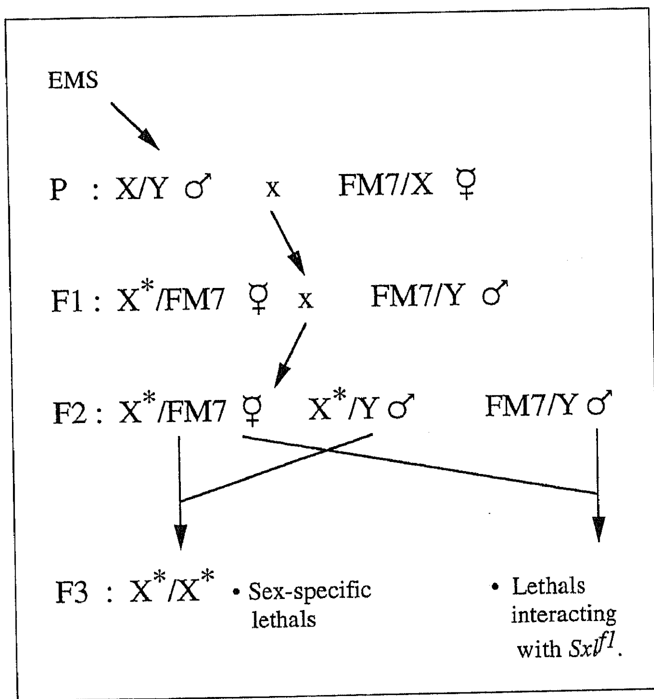

Figure 1. The mating scheme used to isolate mutations with sex-specific phenotypes. Full genotypes: X= *y cho sn*; X* is mutagenized *ycho sn* chromosome; $Sxl^{f1} = cm\ Sxl^{f1}\,ct^6$; and $FM7 = y^{31d}\,sc^8\,w^a\,sn\,X^2\,v^{of}\,g^4\,B$.

Results

In an XX embryo, *Sxl* is the earliest known gene to be turned on in response to the X:A signal. As mentioned earlier, female-specific lethality and dose-dependent female-lethal interactions with its regulators are powerful indicators of inappropriate regulation of *Sxl*. We used these well-established properties of the known sex determining genes as an assay system to find additional X-linked genes participating in this developmental process. In a genetic screen set up for this purpose (see Materials and methods and figure 1) four lesions of potential interest were recovered. Results of genetic characterization of these mutations, their preliminary map positions as determined by meiotic methods, and the results of genetic tests aimed at placing these four mutations in the sex determination pathway are presented here.

Novel X-linked lesions with female-lethal mutant phenotypes

$\boldsymbol{Sxl^{dlf}}$: Heterozygous females ($Sxl^{dlf}/FM7$) when crossed with Sxl^{dlf} males did not give rise to homozygous (Sxl^{dlf}/Sxl^{dlf}) females (table 1). In control crosses $Sxl^{dlf}/FM7$ females were mated to *y cho sn* males. Among the progeny of this cross, all four expected classes of flies were viable (number of males is not shown in table 1).

Sxl^{dlf} was mapped in the *cho–sn* interval, approximately 2 cM centromere-distal to *sn*. This information, considered together with the observations that it did not complement $Df(1)HA32$, *Sxl* (6E4-5; 7A6) [$Df(1)HA32/Sxl^{dlf} = 0$; $FM7/Sxl^{dlf} = 314$] or Sxl^{7BO} (table 1), and that *Sxl* is the only gene known that results in female-specific lethality in this cytogenetic interval lead us to conclude that the 'new' mutation is an allele of *Sxl*.

Sxl^{dlf} does not complement Sxl^{f1} or Sxl^{7BO}. Sxl^{f1} is a null mutation and Sxl^{7BO} is a male-viable chromosome in which almost the entire *Sxl* locus is deleted. $Sxl^{dlf}/FM7$ females were crossed to Sxl^{f1} and Sxl^{7BO} males. In the

Table 1. Complementation tests between Sxl^{dlf} and other *Sxl* alleles.

	Paternal genotype							
Maternal genotype	Sxl^{dlf} 1	Sxl^{f1} 2	Sxl^{7BO} 3	Sxl^{f9} 4	Sxl^{fLS} 5	$Sxl^{fm7,M1}$ 6	$Sxl^{M1,fm3}$ 7	Sxl^{+} 8
Sxl^{dlf}	0	0	0	412 (98%)	0	0	0	394 (108%)
FM7	828	879	806	417	616	675	438	362

Females of the genotype $y\,cho\,Sxl^{dlf}\,sn/FM7$ were crossed with males of the genotype (1) *y cho sn*, (2) $cm\ Sxl^{f1}\ ct^6$, (3) $y\,pn\,cm\,Sxl^{p7bo}$, (4) $cm\,Sxl^{f9}v$, (5) $y\,Sxl^{fLS}oc\,v\,f^{36a}$, (6) $cm\,Sxl^{fm7,M1}ct^6\,v$, (7) $w\,Sxl^{M1,fm3}sn$, and (8) $cm\,Sxl^{+}\,ct^6$. Per cent viability (shown in parentheses) was calculated by dividing the number of female progeny carrying Sxl^{dlf} and the indicated paternal chromosome by the number of females carrying *FM7* and the indicated paternal chromosome. Numbers without parentheses are numbers of surviving females.

progeny, females of the genotypes Sxl^{dlf}/Sxl^{fl} and Sxl^{dlf}/Sxl^{7BO} did not survive. To test complementation behaviour of additional *Sxl* alleles with Sxl^{dlf}, we combined Sxl^{dlf} with Sxl^{f9}, Sxl^{fLS}, $Sxl^{fm7,M1}$ and $Sxl^{M1,fm3}$. Sxl^{f9} is an 'early' defective allele which is unable to establish the female-determined state but is able to promote normal female development when activated in *trans* by a late-defective allele such as Sxl^{fLS} (Cline 1984). Sxl^{dlf} complements Sxl^{f9} but not Sxl^{fLS} or two other alleles, $Sxl^{fm7,M1}$ and $Sxl^{M1,fm3}$, both defective in late functions of the gene (table 1), suggesting that the early aspects of *Sxl* regulation and function are intact in Sxl^{dlf}. It therefore appears that it is the late functions of the gene that have been rendered defective by the mutation.

fl-35: Although *fl-35* was picked as an X-linked recessive lethal which did not complement Sxl^{fl} and Sxl^{7BO}, while 'cleaning' the chromosome we obtained a male-viable chromosome which exhibited a phenotype identical to that of the original mutation, suggesting that it still retained the *Sxl*-interacting lesion. To find out whether this mutation is a female-specific lethal, *fl-35* males were mated with *fl-35/FM7* females. In the progeny of this cross, 623 *fl-35/fl-35* females, 1179 *fl-35/FM7* females, 1427 *fl-35* males and 734 *FM7* males were obtained. Females homozygous for *fl-35*, although less viable than *fl-35/FM7* females and *fl-35* males, were without any obvious morphological changes and were fertile. These data suggest that *fl-35* is a male-viable and female-viable mutation which shows female-specific lethality only when in *trans* with Sxl^{fl}. Results of complementation analysis of *fl-35* with the other alleles of *Sxl*, namely Sxl^{f9}, Sxl^{fLS}, $Sxl^{fm7,M1}$ and $Sxl^{M1,fm3}$, are presented in table 2. *fl-35* complements all these *Sxl* alleles, but the viability of double heterozygotes is poor.

The map position of *fl-35* is apparently neither in the *y–cho* nor the *cho–sn* intervals. The mutation was mapped to a location 5 cM centromere-proximal to *sn* (1-21.0). The period during which lethality due to interaction between *fl-35* and Sxl^{fl} occurs was determined by analysing the progeny of *fl-35/+* females and Sxl^{f1} males. Out of 261 eggs collected, 123 developed into adult males. No adult females were obtained. Fiftyfour embryos did not hatch and 84 died as first-instar larvae, indicating that developmental arrest occurs at embryonic or larval stages and about 39% of the $fl\text{-}35/Sxl^{f1}$ progeny die as embyos. In another experiment, *fl-35/fl-35* females were crossed to Sxl^{f1} males. Out of 738 eggs collected from this cross, 416 undeveloped embryos, no females, and 314 adult males were obtained, again indicating that lethality is primarily embryonic and that there is an effect of maternal *fl-35* genotype on the lethal period. Most of the progeny of mothers homozygous for *fl-35* die as embryos whereas the progeny of *fl-35/+* mothers show developmental arrest at embryonic as well as larval stages.

fl-46: *fl-46* also was initially picked up as a sex-nonspecific lethal exhibiting reduced female survival in *trans*heterozygous combination with Sxl^{f1}. Data on interactions of *fl-46* with *Sxl* alleles are given in table 2. Flies doubly heterozygous for *fl-46* and either Sxl^{fl} or Sxl^{7BO} showed very poor survival, 3.5% in the case of $fl\text{-}46/Sxl^{f1}$ and 2.1% in the case of $fl\text{-}46/Sxl^{7BO}$.

fl-46 does not map to the *y–cho* and *cho–sn* intervals. It was mapped to a location about 5 cM away from the *sn* locus. As in the case of *fl-35*, two male-viable chromosomes were obtained during experiments performed to 'clean' the genetic background. *fl-46/FM7* flies were crossed to males carrying one of the two male-viable derivatives. In one set of crosses, 430 *fl-46/fl-46* females, 485 *fl-46/FM7* females, 407 *fl-46* males and 229 *FM7* males were obtained. *fl-46/fl-46* homozygotes when crossed to Sxl^{f1} males yielded 875 males and 14 females at 25°C. Females homozygous for

Table 2. Interactions between *fl-35*, *fl-46* and *l-43* and *Sxl* alleles.

Maternal genotype	Paternal genotype Sxl^{fl} 1	Sxl^{7BO} 2	Sxl^{f9} 3	Sxl^{fLS} 4	$Sxl^{fm7,M1}$ 5	$Sxl^{M1,fm3}$ 6	Sxl^{+} 7
(a) *fl-35*	0	0	391 (40%)	688 (78%)	475 (47%)	522 (29%)	678 (100%)
FM7	920	816	985	885	1009	1795	676
(b) *fl-46*	54 (4%)	24 (2%)	332 (68%)	682 (69%)	508 (45%)	492 (54%)	843 (80%)
FM7	1547	1140	492	988	1140	912	1062
(c) *l-43*	78 (7%)	288 (25%)	566 (94%)	160 (37%)	140 (19%)	88 (12%)	648 (98%)
FM7	1091	1136	600	432	752	726	660

Females of the genotype (a) *y cho sn fl-35/FM7*, (b) *y cho sn fl-46/FM7* and (c) *y cho sn l-43/FM7* were crossed to males of the genotype (1) $cm\ Sxl^{f1}\ ct^{6}$, (2) $y\ pn\ cm\ Sxl^{p7bo}$, (3) $cm\ Sxl^{f9}v$, (4) $y\ Sxl^{fLS} oc\ v\ f^{36a}$, (5) $cm\ Sxl^{fm7,M1} ct^{6}\ v$, (6) $w\ sxl^{M1,fm3}\ sn$ and (7) $cm\ Sxl^{+}\ ct^{6}$. Per cent viability (shown in parentheses) was calculated by dividing the number of female progeny carrying *fl-35*, *fl-46* and *l-43* and the indicated *FM7* and paternal X chromosome by the number of female progeny carrying indicated *FM7* and paternal X chromosome. Numbers without parenthetical figures are numbers of surviving females.

fl-46 mutant chromosomes were without any obvious phenotypic defects. Like the *fl-35* mutation, *fl-46* also is a male-viable, female-viable lesion showing female-lethality only in *trans* combination with *Sxl*.

Out of 1179 eggs obtained in a cross between *fl-46/fl-46* females and Sxl^{f1} males, six developed into adult females and 418 developed into adult males, 479 died as embryos, and over 270 died as first-instar larvae, indicating that the lethality of $fl\text{-}46/Sxl^{f1}$ flies can occur at embryonic or larval stages.

l-43: This sex-nonspecific lethal mutation was recovered on the basis of its female-lethal interaction with Sxl^{f1}. Females doubly heterozygous for *l-43* and Sxl^{f1} were about 14-fold less viable than flies heterozygous for Sxl^{f1}. The results of complementation analysis of *l-43* with mutations in *Sxl* are given in table 2. *l-43* shows reduced viability in combination with Sxl^{fLS}, $Sxl^{fm7,M1}$ and $Sxl^{M1,fm3}$. Female viability of Sxl^{7BO} (a deficiency for *Sxl*) and *l-43* double heterozygotes was not as strongly affected as in the case of $Sxl^{f1}/l\text{-}43$ females either because of the effects of genetic background or possible allele specificity of the interaction. *l-43* was mapped to a position about 10 cM centromere-proximal to *sn*.

The lethal period was determined from crosses set up at 25°C between *l-43*/+ females and Sxl^{f1} males: out of a collection of 1475 eggs, 384 undeveloped embryos, over 630 dead first-instar larvae, 228 adult females, and 216 adult males were obtained, indicating that lethality occurs at embryonic and larval stages and that about two-thirds of $l\text{-}43/Sxl^{f1}$ and *l-43* progeny die as larvae.

Tests of allelism among Sxl^{dlf}, fl-35, fl-46 and l-43

The following set of crosses was done in order to find out if the four mutations are allelic to each other. Females heterozygous for each of these mutations were crossed to males carrying one of the other mutations. For instance, $Sxl^{dlf}/FM7$ heterozygotes were crossed to *fl-35* males and in the F_1 progeny survival of $Sxl^{dlf}/fl\text{-}35$ females relative to that of $Sxl^{dlf}/FM7$ females was determined. This was done for all possible mutant combinations (table 3). All mutant combinations were viable and viability of doubly heterozygous flies was as good as that of heterozygous flies in all cases except in the progeny of crosses between *l-43/FM7* females and *fl-35* males. In this case, survival of *l-43/fl-35* females was about 19% of that of controls (table 3, section d2). Double heterozygotes for *fl-46* and *fl-35* also showed a moderate reduction in female viability. In two crosses, viability of *fl-35/fl-46* females was 63% and 52% of control values (table 3, sections b3 and c2). Females doubly heterozygous for any two of these four mutations were crossed to Sxl^{f1} males and the numbers of females and males obtained in the progeny are shown in table 3 (females/males in lower part of each sector). These data suggest that Sxl^{dlf}, *fl-35* and *l-43* are not allelic to each other. However,

Table 3. Complementation among Sxl^{dlf}, *fl-35*, *fl-46* and *l-43*.

Maternal genotype	Paternal genotype 1 Sxl^{dlf}	2 *fl-35*	3 *fl-46*
(a) $Sxl^{dlf}/FM7$	—	70%	79%
	—	(494)	(609)
		28/214	ND
(b) *fl-35/FM7*	81%	—	63%
	(458)	—	(418)
			33/1541
(c) *fl-46/FM7*	92%	52%	—
	(317)	(490)	—
(d) *l-43/FM7*	105%	19%	71%
	(266)	(808)	(395)
	230/717	ND	313/850

Complete genotype: Sxl^{dlf} = *y cho* Sxl^{dlf} *sn*; *fl-35* = *y cho sn fl-35*; *fl-46* = *y cho sn fl-46*; *l-43* = *y cho sn l-43*; and *FM7* = $y^{31d} sc^8$ *dm B*. ND = not determined. Sxl^{dlf}/*fl-46* (c1) and *l-43/fl-35* (d2) females showed poor $y^{31d} sc^8$ *dm B* fertility. Numbers of females with the maternal balancer chromosomes and the indicated paternal chromosome are given in parentheses. These were used as control values to calculate per cent viability of females doubly heterozygous for the maternal chromosomes carrying the indicated mutation and indicated paternal chromosome. See text for the lower part of each sector. Males carrying *l-43* are not viable.

in the progeny of *fl-35/fl-46* females and Sxl^{f1} males, 33 females and 1541 males were obtained. These numbers are within limits of what one would expect from the survival rates of combinations of *fl-35* and *fl-46* with Sxl^{f1}, indicating that *fl-35* and *fl-46* are very likely to belong to the same complementation group (table 3, section b3).

Sxl^+ rescues female lethality caused by interaction between Sxl^{f1} and Sxl^{dlf}, fl-35, fl-46 and l-43

If female-specific lethality or female-lethal interactions between the new mutations and *Sxl* are indeed due to misregulation of *Sxl*, then by providing Sxl^+, in *trans*, it should be possible to rescue the lethal phenotypes. In experiments set up to test this possibility, we found that the duplication chromosome $Tp(1;3)sn^{13a1}$ (6C; 7C9-D1; 79E, Lefevre 1981), which includes the Sxl^+ locus, rescues lethality owing to non-complementation or interaction between Sxl^{f1} and Sxl^{dlf}, *fl-35*, *fl-46* and *l-43*, suggesting that female lethality was most likely due to a reduction in product levels of *Sxl* in the double heterozygotes (table 4).

Genetic interactions among the known sex determining genes and the 'new' lesions

Dominant interactions among otherwise recessive mutations have been observed both upstream (Cline 1980, 1984, 1986, 1988; Younger-Shepherd *et al.* 1992) and downstream (Baker and Ridge 1980) of *Sxl*. For instance, XX flies heterozygous for null mutations at *sis-a*, *sis-b* or *run* show no phenotypic effects, but flies doubly heterozygous for one

Table 4. Rescue of female lethality.

Zygotic genotype	Female survival
(a) Sxl^{f1}/Sxl^{f1}	1. Without Dp-Sxl^{+} : 0% (690) 2. With Dp-Sxl^{+} : 41% (530)
(b) Sxl^{dlf}/Sxl^{f1}	1. Without Dp-Sxl^{+}: 0% (586) 2. With Dp-Sxl^{+}:53% (366)
(c) *fl-35*/Sxl^{f1}	1. Without Dp-Sxl^{+} : 0% (421) 2. With Dp-Sxl^{+} : 59% (238)
(d) *fl-46*/Sxl^{f1}	1. Without Dp-Sxl^{+} : 1% (538) 2. With Dp-Sxl^{+} : 47% (548)
(e) *l-43*/Sxl^{f1}	1. Without Dp-Sxl^{+} : 6% (412) 2. With Dp-Sxl^{+} : 28% (164)

Crosses done: (a) 1. $cm\,Sxl^{f1}\,ct^{6}/FM7 \times cm\,Sxl^{f1}\,ct^{6}/Y$
2. $cm\,Sxl^{f1}\,ct^{6}/FM7 \times cm\,Sxl^{f1}\,ct^{6}/Y;\ Tp(1;3)sn^{13a1}/+$

(b) 1. $cm\,Sxl^{dlf}\,ct^{6}/FM7 \times cm\,Sxl^{f1}\,ct^{6}/Y$
2. $cm\,Sxl^{dlf}\,ct^{6}/FM7 \times cm\,Sxl^{f1}\,ct^{6}/Y;\ Tp(1;3)sn^{13a1}/+$

(c) 1. *y cho sn fl-35/FM7* × $cm\,Sxl^{f1}\,ct^{6}/Y$
2. *y cho sn fl-35/FM7* × $cm\,Sxl^{f1}\,ct^{6}/Y;\ Tp(1;3)sn^{13a1}/+$

(d) 1. *y cho sn fl-46/FM7* × $cm\,Sxl^{f1}\,ct^{6}/Y$
2. *y cho sn fl-46/FM7* × $cm\,Sxl^{f1}\,ct^{6}/Y;\ Tp(1;3)sn^{13a1}/+$

(e) 1. *y cho sn l-43/FM7* × $cm\,Sxl^{f1}\,ct^{6}/Y$
2. *y cho sn l-43/FM7* × $cm\,Sxl^{f1}\,ct^{6}/Y;\ Tp(1;3)sn^{13a1}/+$

Numbers in parentheses in the above table are the numbers of females with the indicated maternal balancer chromosomes and the paternal X chromosome [see crosses (a) to (e) above]. These numbers were used as control values to calculate per cent survival of females with the maternal X chromosome carrying the indicated mutation and the paternal X chromosome. About 50% of the females belonging to each class are expected to be carrying $Tp(1;3)sn^{13a1}$.

of these genes and Sxl^{f1} do not survive. A property of the X:A signal elements is that there is reciprocal sex specificity to their dosage effects. When either of the numerator elements *sis-a* or *sis-b* is duplicated, there is no significant effect on male survival, but duplication of both genes is lethal for males (Cline 1988). *dpn*, which is an autosomal denominator gene, and hence normally present in two doses in both sexes, shows sex-specific, dose-independent interaction with *sis-a*. Males with decreased copies of dpn^{+} and increased copies of $sis\text{-}a^{+}$ show reduced viability. On the other hand, females with increased copies of dpn^{+} and decreased copies of $sis\text{-}b^{+}$ die (Younger-Shepherd *et al.* 1992). *snf*, which regulates the processing of *Sxl* transcripts, shows female-lethality in combination with Sxl^{f1} (Oliver *et al.* 1988; Steinmann-Zwicky 1988; Salz 1992). Although *snf* shows interaction with *sis-a*, this interaction is not as strong as that with Sxl^{f1} (Steinnmann-Zwicky 1988), suggesting that synergistic interactions may be stronger among genes belonging to the same class (i.e. early-transcriptional activators vs late-RNA-processing regulators). Similarly, flies heterozygous for recessive mutations in the three genes controlling somatic sex differentiation, *tra*, *tra-2* and *ix*, are phenotypically indistinguishable from wild type, but XX flies heterozygous for all three of them develop as intersexes (Baker and Ridge 1980). The *da* mutation is recessive only when the females have a wild-type dose of *Sxl* or *sis-a*. In view of these results, interaction between a newly identified gene and genes regulating either the transcriptional activation of *Sxl* or the sex-specific splicing of *Sxl* pre-mRNA may be taken as evidence of a role of the new gene in one or the other of these steps. We therefore studied interactions of Sxl^{dlf}, *fl-35*, *fl-46* and *l-43* with known sex determination genes to determine their probable location in the sex determination pathway. The effect on female viability of Sxl^{dlf}, *fl-35*, *fl-46* and *l-43*, when in combination with either *sis-a*, *sis-b*, *da*, *fl(2)d*, *tra-2* or *mle* was determined. Unless otherwise mentioned, crosses were set up at 25°C.

Interactions with sis-a and sis-b: It may be recalled that *sis-a* and *sis-b* are numerator elements of the X:A signal. They code for transcription factors that work in a dose-dependent manner to determine the activity state of *Sxl*. Females heterozygous for Sxl^{dlf}, *fl-35*, *fl-46* and *l-43* were mated to *sis-a* and *sis-b* males and per cent survival of doubly heterozygous females was calculated in each case.

Sxl^{dlf} does not interact with *sis-a* or *sis-b*. The survival rates of Sxl^{dlf}/*sis-a* and Sxl^{dlf}/*sis-b* were 84% and 93% respectively, indicating that the viability of doubly heterozygous females is comparable to the viability of females heterozygous for Sxl^{dlf}, *sis-a* or *sis-b* alone (table 5). Therefore, as suggested earlier, Sxl^{dlf} does not seem to be defective in the early activation steps of the gene.

Although viability of females doubly heterozygous for *sis-a* or *sis-b* with *fl-35* and *fl-46* was not as drastically reduced as in the case of *sis-a/sis-b* double heterozygotes, they did show reduced survival of females (table 5). However, different results were obtained when a deletion chromosome [*Df(1)RA37*] uncovering *sis-a* was employed in these tests. In crosses between *Df(1)RA37*, *sis-a/FM7* females and *fl-35* or *fl-46* males, viability of *Df(1)RA37*, *sis-a/fl-35* females was 84% [*Df(1)RA37*, *sis-a/fl-35*, 539; *FM7/fl-35*, 646] and that of *Df(1)RA37*, *sis-a/fl-46* females was 79% [*Df(1)RA37*, *sis-a/fl-46*, 482; *FM7/fl-46*, 607]. These results do not suggest a significant zygotic interaction between *fl-35* or *fl-46* with *sis-a*. Taken together, these observations indicate that *fl-35* and *fl-46* may not form a part of the X:A ratio system; in other words, they do not appear to be necessary for transcriptional activation of *Sxl*. *l-43* shows reduced female viability in combination with *sis-a* (31%) but its interaction with *sis-b* (69% being the viability of *sis-b/l-43* heterozygotes) is not strong, suggesting that this gene may make, at most, a small contribution to transcriptional activation of *Sxl*.

Interaction with da: Activation of *Sxl* in the zygote by *sis* genes needs maternal activity of *da*. Decreased da^{+} activity in the maternal germline, just as lowering the X:A ratio in the zygote (for example by lowering the levels of $sis\text{-}a^{+}$ and $sis\text{-}b^{+}$), decreases the probability of stable activation of

Table 5. Interactions with *sis-a* and *sis-b*.

Maternal genotype	Paternal genotype	
	sis-a	*sis-b*
(1) *sis-a/FM7*	0/532 (0%)	62/541 (12%)
(2) *sis-b/FM7*	34/442 (8%)	0/371 (0%)
(3) X/*FM7*	378/418 (90%)	480/561 (86%)
(4) *Sxl^dlf^/FM7*	412/492 (84%)	425/457 (93%)
(5) *fl-35/FM7*	638/1168 (55%)	476/816 (58%)
(6) *fl-46/FM7*	528/1610 (33%)	876/1578 (56%)
(7) *l-43/FM7*	240/777 (31%)	520/752 (69%)

Complete genotypes: *sis-a=y sis-a*; *sis-b*=$sc^{10\text{-}1}$; X = *y cho sn*; Sxl^{dlf} = *y cho* Sxl^{dlf} *sn*; *l-43* = *y cho sn l-43*; *fl-35* = *y cho sn fl-35*; *fl-46* = *y cho sn fl-46*. Numbers of females heterozygous for the indicated maternal balancer chromosome and *sis-a* and *sis-b* are given as denominators. These values were used as controls to calculate per cent survival (in parentheses) of females doubly heterozygous for the maternal chromosome carrying the indicated mutation and *sis-a* or *sis-b*. In each of these cases, the numerator is the actual number of doubly heterozygous females.

Table 6. Interactions with *da*.

Paternal genotype	Maternal genotype
	da/SM1
	females/males
(1) X	385/452 (0.85)
(2) Sxl^{f1}	364/582 (0.63)
(3) Sxl^{dlf}	504/527 (0.96)
(4) *fl-35*	617/490 (1.26)
(5) *fl-46*	644/637 (1.01)

Complete genotypes: X=*y cho sn*; Sxl^{f1}=*cm* Sxl^{f1} ct^6; Sxl^{dlf}=*y cho* Sxl^{dlf} *sn*; *fl-35* =*y cho sn fl-35*; *fl-46* =*y cho sn fl-46*. Maternal genotype: da^1/*SM1*, al^2 *Cy* cn^2 sp^2.

Sxl^+ (Cline 1980). Mutations defective in an early activation step of *Sxl* (e.g. Sxl^{f9}) or null mutations (Sxl^{f1}) display synergistic female-lethality in combination with *da*, whereas Sxl^{fLS}, which is a mutation affecting the late functions of *Sxl*, does not interact with it. To test whether any of the four mutations isolated in this study interact with *da*, the viability of females (daughters) heterozygous for Sxl^{dlf}, *fl-35* and *fl-46* whose mothers were heterozygous for *da* was studied (table 6). No such interaction between *da* and Sxl^{dlf}, *fl-35* or *fl-46* was detected in these experiments. Furthermore, to see the effect in triple-mutant combinations, females doubly heterozygous for Sxl^{dlf}, *fl-35*, *fl-46* and *da* were crossed to Sxl^{f1} males and sex ratios of the progeny in each of these crosses were calculated. No significant effect on female viability was observed in these crosses as well (data not shown).

In the progeny of crosses between *fl-43*/+; *da*/+ females and Sxl^{f1} males, 101 females and 149 males were obtained (sex ratio 0.68). These numbers are within normal limits in the light of the reduction in female viability expected from the interaction between *da* and Sxl^{f1} and *fl-43* and Sxl^{f1}. Taken together, these data support the conclusions drawn from our earlier observations that Sxl^{dlf}, *fl-35* and *fl-46* are unlikely to be X : A ratio elements; these results also lessen the possibility of *l-43* being a component of the X : A ratio signal.

Interaction with fl(2)d: *female lethal-2-d* [*fl(2)d*] is needed for the female-specific splicing of *Sxl* RNA (Granadino *et al.* 1990). Larvae homozygous for *fl(2)d* express *Sxl* transcripts characteristic of males, suggesting the involvement of *fl(2)d* in the autoregulatory loop of *Sxl*. Two alleles of this gene are known: *fl(2)d1* and *fl(2)d2*. To test whether the four mutations isolated in the present study interact with either of the two *fl(2)d* alleles, doubly heterozygous combinations of these mutations were made. Since the female-lethality caused by *fl(2)d* is greater at 29°C (Granadino *et al.* 1992), these crosses were performed at this temperature. None of the four mutations exhibits enhanced female-lethality in *trans*heterozygous combination with *fl(2)d* (table 7). Female-specific regulation of *Sxl* occurs by blockage of the male-specific default splice site (Horabin and Schedl

Table 7. Interactions with *fl(2)d*.

Maternal genotype	Paternal genotype	
	fl(2)d1	*fl(2)d2*
(1) X/*FM7*	409/478 (86%)	397/443 (90%)
(2) *Sxl^dlf^/FM7*	512/550 (93%)	350/469 (75%)
(3) *fl-35/FM7*	451/390 (116%)	245/294 (83%)
(4) *fl-46/fl-46*	285/270 (107%)	308/232 (133%)
(5) *l-43/FM7*	227/215 (106%)	216/278 (78%)

Crosses in rows (1) to (5):
(1) *y cho sn/FM7* × *cn fl(2)d bw/CyO*, *Cy*, dp^{lvl} *pr* cn^{22}
(2) *y cho* Sxl^{dlf} *sn/FM7* × *cn fl(2)d bw/CyO*
(3) *y cho sn fl-35/FM7* × *cn fl(2)d bw/CyO*
(4) *y cho sn fl-46/y cho sn fl-46* × *cn fl(2)d bw/CyO*
(5) *y cho sn l-43/FM7* × *cn fl(2)d bw/CyO*

Numbers of females heterozygous for *FM7* and *fl(2)d1* and *fl(2)d2* in rows (1), (2), (3) and (5), and for *CyO* and *fl(2)d1* and *fl(2)d2* in row (4), are given as denominators. These values were used as controls to calculate per cent survival (in parentheses) of females doubly heterozygous for the maternal chromosome carrying the indicated mutations and *fl(2)d1* and *fl(2)d2*. In each of these crosses, the numerator is the actual number of doubly heterozygous females observed.

Table 8. Epistasis with *tra-2*.

Maternal genotype	Paternal genotype
	Sxl^{f1}/Y; *tra-2/+*
(a) X/*FM7*; *tra-2/+*	565/415 (136%)
(b) $Sxl^{dlf}/FM7$; *tra-2/+*	0/537 (0%)
(c) *fl-35/FM7*; *tra-2/+*	0/660 (0%)
(d) *fl-46/FM7*; *tra-2/+*	26/635 (4%)
(e) *l-43/FM7*; *tra-2/+*	15/492 (3%)

Complete genotypes: X = *y cho sn*; Sxl^{dlf} = *y cho* Sxl^{dlf} *sn*; *fl-35* = *y cho sn fl-35*; *fl-46* = *y cho sn fl-46*; *l-43* = *y cho sn l-43*; Sxl^{f1} = *cm* Sxl^{f1} ct^6; *tra-2* = *cn tra-2 bw*; *FM7* = y^{31d} sc^8 *wa sn* X^2 v^{of} g^4 *B*.
In each case, the denominator is the number of females heterozygous for the indicated maternal balancer X chromosome and paternal X chromosome carrying Sxl^{f1}. These were used as control values to calculate per cent survival (in parentheses) of females doubly heterozygous for the maternal X chromosome carrying the indicated mutation and the indicated paternal X chromosome. Approximately 25% of the females of each class are expected to be *tra-2/tra-2*.

1993). In addition to *Sxl* (which encodes a splicing factor), there are other components likely to be involved in this regulatory step. Although the interactions among genes forming the X:A signal are reasonably well understood, we do not yet know the nature of the synergistic interactions among genes needed for processing of *Sxl* transcripts. Therefore the fact that *fl-35*, *fl-46* and *l-43* do not interact with *fl(2)d* does not necessarily suggest that they do not have a role in sex-specific RNA splicing of *Sxl*.

Epistatic interaction with tra-2: The deduced order of genes in the pathway controlling somatic sex is X:A > *Sxl* > *tra* ≥ *tra-2* > *dsx* > *ix*; the terminal sex differentiation genes act subsequently (Baker 1989). There is a possibility that the lethality caused by interaction of Sxl^{f1} with Sxl^{dlf}, *fl-35*, *fl-46* and *l-43* is affected by the state of activity of genes downstream of *Sxl*. Lethality could be influenced by some aspect of sex-specific physiology or biochemistry. Therefore epistatic interactions of these 'new' mutations with *tra-2*, a somatic sex determination gene, were studied. Female lethality due to interactions between Sxl^{f1} and Sxl^{dlf}, *fl-35*, *fl-46* and *l-43* was found to be epistatic over *tra-2* (table 8). This suggests that lethality is independent of activity of the genes downstream of *Sxl* and does not depend on any aspect of the somatic sex of flies.

Discussion

We conducted EMS mutagenesis experiments, to isolate 'new' genes in the sex determination pathway. These experiments involved induction and isolation of lesions on the X chromosome that exhibited (i) female-specific lethality or (ii) reduction in female viability in heterozygous combination with *Sxl* loss-of-function mutations. This screen yielded four new lesions that met the criteria described above.

Approximate map positions of these mutations were determined by meiotic recombination. Sxl^{dlf} was mapped to the same genetic and cytogenetic interval in which *Sxl* is located. Both *fl-35* and *fl-46* mapped about 5 cM centromere-proximal to *sn*. The proximity of their genetic locations and similarities in phenotype (male viability and female viability, and comparable level of interaction with *Sxl*) suggest that these two are allelic to each other. However, at this level of resolution, it is difficult to distinguish between two allelic mutations and two closely linked mutations. *l-43* was mapped about 10 cM centromere-proximal to *sn*. Since the strength of synergistic interactions between Sxl^{f1} and *fl-35*, *fl-46* and *l-43* is to some degree affected by genetic background, any procedure based on recombination mapping can provide only an estimate of their location. More precise localization of these mutations would require that they be mapped in relation to additional X-chromosomal markers and in relation to the X-chromosomal deficiencies and duplications.

When there is abnormal regulation of *Sxl*, the consequent lethality is expected to occur at embryonic and larval stages. Genetic background and environmental factors can influence the timing of developmental arrest. Experiments designed to determine the period at which lethality occurs gave the following results: in most cases lethality was spread over late-embryonic and larval stages. This is indicative of misregulation of *Sxl*.

Of the four mutations described in this paper, Sxl^{dlf} is most likely defective in the 'late' regulation of the gene. This defect might affect autoregulation of *Sxl* activity which occurs by blockage of the default (male-specific) splicing of primary transcripts (Horabin and Schedl 1993). *fl-35*, *fl-46* and *l-43* did not show significant interaction with *da*, *sis-a* or *sis-b* (except that *l-43/sis-a* flies showed somewhat reduced viability), suggesting that none of these three genes is likely to be a part of the X:A signal. Chromosomes carrying *fl-35* and *fl-46* do not affect viability of male or female flies but exhibit dominant lethality in females in combination with Sxl^{f1}. Activation of *Sxl* is sensitive to the zygotic and, to a small extent, maternal dosage of *fl-35* and *fl-46*. Whether *fl-35* and *fl-46* are essential for *Sxl* function cannot be unequivocally determined until the phenotypes of the null alleles of these two genes are identified. A non-essential requirement of *fl-35* and *fl-46* is conceivable if there are one or more additional genes functioning in a manner similar to *fl-35* and *fl-46* and contribute to the regulation of *Sxl*. Such redundant functions have been known to yield viable phenotypes of mutations in genes with predicted vital functions in other developmental pathways. For instance, no lethal mutations were isolated in *Drosophila fasciclin* (*fas 1*) gene which codes for a neural cell adhesion molecule (Elkins *et al.* 1990). A mutant phenotype was observed only when a mutation in *fas 1* is combined with *abelson*, a mutation in the gene for tyrosine

kinase. *l-43* shows synergistic female-lethality in combination with Sxl^{f1}. This mutation seems to affect the late regulation of *Sxl* because it shows synergistic lethality in combination with the 'late'-function-defective mutations of *Sxl*, namely Sxl^{fLS}, $Sxl^{fm7,M1}$ and $Sxl^{M1,fm3}$ (table 2). In contrast, viability when in combination with Sxl^{f9}, an 'early'-function-defective mutation, is normal.

As discussed above, the most likely effects of the mutations identified in this screen are at one of the steps leading to *Sxl* activation. However, a recent finding shows that *Sxl* controls dosage compensation by negatively regulating *msl-2* in females (Bashaw and Baker 1995; Kelley *et al.* 1995). This regulation occurs at the translational level, making it likely that there are additional genes which can collaborate with SXL protein to bring about dosage compensation, i.e. to prevent hypertranscription from both X chromosomes in females. Loss-of-function mutations of one such gene in a genetic background in which SXL is present at reduced levels could lead to female-lethality due to *msl*-mediated hypertranscription of both X chromosomes in females. Another possibility, especially in the case of *l-43*, which is a sex-nonspecific lethal, is that it affects neuronal development. Several *Sxl* regulators that have a role in neurogenesis are known (Erickson and Cline 1991; Anand and Chandra 1994). Needless to say, additional studies are required to find out the precise sex-specific as well as sex-nonspecific roles of the genes defined by *fl-35*, *fl-46* and *l-43* in *Drosophila* development.

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