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A lesson from *flex*: consider the Y chromosome when assessing *Drosophila* sex-specific lethals

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SUMMARY

Bhattacharya et al. (Bhattacharya, A., Sudha, S., Chandra, H. S. and Steward, R. (1999) *Development* 126, 5485-5493) reported that loss-of-function mutations in the *flex* (*female-specific lethal on X*) gene caused female-specific lethality because *flex*$^+$ acts as a positive regulator of the master switch gene *Sex lethal* (*Sxl*). *Sxl* is essential for female development. Key to their conclusion was the ability of *flex* mutations to suppress the male lethality caused by *Sxl*^{M} mutations, which inappropriately activate *Sxl* female-specific expression. Here we report our contrary findings that *flex* mutations fail to suppress even the weakest *Sxl*^{M} alleles, arguing against the proposed regulatory relationship between *flex* and *Sxl*. Instead we show that the lethal *flex* phenotype depends on the absence of a Y chromosome, not on the presence of two X chromosomes. *flex* lethality is caused by a defect in the functioning of the X-linked rDNA locus called *bobbed*, since this defect is complemented by the corresponding wild-type rDNA complex on the Y.

Key words: Y chromosome, *Drosophila melanogaster*, *Sex lethal* (*Sxl*), *female-specific lethal on X* (*flex*), rDNA, *bobbed* (*bb*)

INTRODUCTION

Sex-specific lethals – mutations whose lethal effects depend on X-chromosome dose – have been instrumental in elucidating the mechanisms of *Drosophila* sex determination and X-chromosome dosage compensation. Sex-specific lethality is the hallmark of mutations that disrupt the functioning of *Sex lethal* (*Sxl*) or of the X-chromosome dosage-compensation processes that it regulates (reviewed by Cline and Meyer, 1996). *Sxl* was named for its female-specific lethal (loss-of-function) and male-specific lethal (gain-of-function) mutant alleles (Cline, 1978).

Full length SXL proteins are normally present only in females (XX), in which SXL imposes the female mode of development and X-chromosome dosage compensation throughout the life of the animal. The male mode of development and dosage compensation follow in the absence of SXL. A pulse of SXL protein made very early in development in response to the double dose of X chromosomes in females engages a positive autoregulatory feedback loop involving *Sxl* pre-mRNA splicing that ensures the continued production of SXL proteins in females (reviewed by Cline and Meyer, 1996). Female SXL protein causes cells to exclude a translation-terminating, male-specific exon from *Sxl* mRNA that would otherwise prevent synthesis of full-length SXL proteins. Engagement of the *Sxl* feedback loop in males, or failure to engage in females, leads to death from dosage compensation upsets.

In this journal, Bhattacharya et al. (Bhattacharya et al., 1999) recently reported mutations in an X-linked gene *female-specific lethal on X* (*flex*) that appeared to cause sex-specific lethality: mutant *flex* females (XX) died, while mutant males (XY) were fully viable. The authors reported that SXL protein failed to appear in *flex* mutant animals. The block seemed not to be in the transcriptional or pre-mRNA splicing controls known to be responsible for *Sxl* sex-specific functioning, but rather in a subsequent regulatory step not previously disrupted by mutation: either *Sxl* mRNA translation or SXL protein stability. Most convincing were data showing that mutations in *flex* suppressed the dominant, male-lethal phenotype of even the strongest gain-of-function mutant *Sxl* alleles, such as *Sxl*^{M4}, and that rescued males were fertile. *Sxl*^{M} mutations disrupt the region of *Sxl* involved in sex-specific alternative pre-mRNA splicing (Bernstein et al., 1995). Depending on their strength, they reduce or eliminate the positive autoregulatory requirement for female SXL protein to generate female *Sxl* mRNAs, thereby causing full-length SXL protein to accumulate in males, disrupting their development.

We were surprised that such an effective suppressor of *Sxl*^{M} male lethality had not been recovered long ago in extensive screens for male-viable derivatives of mutagenized *Sxl*^{M} alleles, screens that had yielded scores of full and partial loss-of-function mutations in *Sxl* itself, a gene approximately as mutable as *flex* (Cline, 1984, and unpublished). More puzzling still was the failure of *flex* mutants to fulfill the strongest genetic prediction for, in the authors' words, "a positive regulator of Sxl, which is essential for female-specific splicing, and is required for the expression of the SXL": *flex*$^-$ XX

somatic clones should have been phenotypically male because they lacked SXL protein, but instead they were female. This paradox was not addressed.

A key question regarding sex-specific lethality was not addressed in the *flex* study: was the viability difference between XX and XY *flex* mutant animals really due to the higher X-chromosome dose in females, or instead to the females' lack of a Y chromosome? We show here that the latter explanation is correct: lethality of *flex* mutant chromosomes can be attributed to defects in or effects on the X-linked rDNA gene cluster, *bobbed* (*bb*), since homozygous mutant females are viable in the presence of Ybb^+. *bb* is the only locus in *D. melanogaster* with alleles on both the X and the Y chromosome (FlyBase, 1999). Consistent with an explanation for lethality that does not involve *Sxl*, we see no effect of *flex* mutations on the phenotype of even the weakest gain-of-function mutant *Sxl* alleles. We report these negative findings here not only out of concern that the record on *flex* and *Sxl* be set straight in a timely fashion, but also to remind readers that Y-conditional lethality is remarkably common for *D. melanogaster* and must not be confused with X-chromosome dose-dependent ('sex-specific') lethality.

MATERIALS AND METHODS

Flies were raised at 25°C (unless otherwise specified) in uncrowded conditions on a standard cornmeal, yeast, sucrose and molasses medium. The criterion for survival was eclosion. Mutations and chromosomes not referenced in the text are described in FlyBase (1999).

RESULTS AND DISCUSSION

flex does not suppress Sxl^M alleles and thus cannot be the kind of positive *Sxl* regulator proposed

To exploit this potential new tool for sex determination research and to explore what we saw as paradoxes, we requested the Sxl^M*flex* double mutant chromosomes from Bhattacharya et al. Three of the four basic *flex* mutations were sent promptly, but we were told that the Sxl^M combinations and the fourth allele were not available. The work below followed from our inability to generate viable double-mutant Sxl^{M4} $flex^2$ or Sxl^{M1} $flex^2$ males through crosses identical to those published, a problem we immediately communicated to the authors.

Since failure of *flex* to suppress Sxl^M alleles would invalidate the authors conclusions, we sought to exclude incorrect explanations for our different results. Of particular concern was the possibility that our dominant lethal Sxl^M alleles had picked up extraneous X-linked lethals, notwithstanding our having maintained these alleles heterozygous with intragenic Sxl deficiencies specifically to minimize this possibility (see Bernstein et al., 1995). First, we explored *flex* suppression of the $snf^{1621}Sxl^{M1}$ double mutant combination instead of Sxl^{M1} alone. The unusual antimorphic allele snf^{1621} partially suppresses Sxl^{M1} male lethality by itself, to the extent that double-mutant males are highly viable and fertile when grown at 18°C but are inviable at 25°C (Salz, 1992; Steinmann-Zwicky, 1988). We have maintained homozygous $snf^{1621}Sxl^{M1}$ lines for years, and have used them to generate a variety of male-viable Sxl^{M1} derivatives simply by shifting the mutagenized stock to 25°C (unpublished). These stocks are free of extraneous lethals as indicated by their temperature-conditional viability and the unconditional viability of Sxl^- males derived from them by mutagenesis. Second, we examined the effect of *flex* on the phenotype of Sxl^{M12}, an unusually weak gain-of-function allele that visibly disrupts male development by expressing female-specific *Sxl* activities in that sex, but only at a low level that allows males to survive to adulthood (Cline et al., 1999). Both of these approaches established that *flex* has no suppressing effect on Sxl^M alleles.

Row B of Table 1 illustrates the heat-sensitive male lethality of the $snf^{1621}Sxl^{M1}flex^+$ combination. No double-mutant males were recovered at 25°C (0 versus 203 controls in row A), but at 18°C they were recovered in even greater abundance than the reciprocal nonrecombinant class of $snf^+Sxl^-flex^-$ control males (162 versus 119 in row A). Data in Row C show that recombination must have generated the $snf^{1621}Sxl^{M1}flex^-$ class (row D), but the $flex^-$ mutation in these males did not ameliorate the heat-sensitive lethality of the $snf^{1621}Sxl^{M1}$ combination (0 versus 79 at 25°C; compare to 82 versus 50 at 18°C). In the course of making various *flex* stocks, we did confirm that all three *flex* chromosomes sent to us were recessive lethal in XX females (data not shown). In Table 1, this lethality is apparent from the lower recovery in females of the closely linked *forked* (*f*) marker regardless of temperature (compare rows E and F); however, the genetic distance between *flex* and *f* calculated from this lethal effect was 9.6 (35/366) and 8.7 (17/196) cM at 25°C and 18°C, respectively, significantly greater than the 4.5 cM reported by Bhattacharya et al. This disparity is potentially important (see below).

Table 2 demonstrates the lack of Sxl^M suppression by an

Table 1. *flex* does not rescue $snf^{1621}Sxl^{M1}/Y$ males at 25°C

Inferred genotype* (A-B, C-D and E-F are reciprocal classes)		Marker phenotypes	Animals recovered at 25°C‡	Animals recovered at 18°C‡
A. (*Sxl - f* parental)	snf^+ Sxl^- >95% $flex^2/Y$	w~ ec cv cm^+ ct f males	203	119§
B. (*Sxl - f* parental)	snf^{1621} Sxl^{M1} >95% $flex^+/Y$	w^+ ec^+ cv^+ cm ct^+ f^+ males	0	162
C. (*Sxl - f* recombinant)	snf^+ Sxl^- >95% $flex^+/Y$	w~ ec cv cm^+ ct f^+ males	79	50§
D. (*Sxl - f* recombinant)	snf^{1621} Sxl^{M1} >95% $flex^2/Y$	w^+ ec^+ cv^+ cm ct^+ f males	0	82
E. >95% $flex^2$		f females	35	17
F. >95% $flex^+$		f^+ females	331	179

*% *flex* estimates are based on the reported map position at 61.2 (4.5 cM centromere proximal to *f*).
‡Progeny from the cross: snf^{1621} *cm* Sxl^{M1}*/w ec cv* $Sxl^{M6,fPa\text{-}w+mC}ct^6$ *f* $flex^2$ ♀♀ × ♂♂ *w cv sn f* $flex^2/Y$ at the temperature indicated.
§Reduced control viability is likely a *cv* marker effect.

Table 2. *flex* does not suppress male abdominal malformations caused by Sxl^{M12}

flex genotype* (inferred from *f*)	hemisternite 4 bristle number ±s.e.m. (range)	hemisternite 5 bristle number ±s.e.m. (range)	hemisternite 6 bristle number ±s.e.m. (range)	% hemitergites 2-6 with some etching
A. $flex^{+}$	8.0±0.3 (1-12)	7.0±0.4 (0-15)	2.8±0.4 (0-9)	58%
B. $flex^{2}$	7.2±0.4 (0-11)	6.0±0.2 (0-12)	2.7±0.3 (0-7)	70%

*25 cv^{+} ct^{6} f^{+} (hence Sxl^{M12} and >95% $flex^{+}$) males and 25 cv^{+} ct^{6} f^{-} (hence Sxl^{M12} and >95% $flex^{2}$) males were scored from the cross: *w* Sxl^{M12} ct^{6}/*w cv sn f* $flex^{2}$ ♀♀ × ♂♂ *w* Sxl^{M12} ct^{6}/*Y*.

even more sensitive measure. Sxl^{M12} is like Sxl^{M1} and Sxl^{M4} in having a transposon insertion in the region of sex-specific alternative splicing that leads to expression of female-specific mRNAs in males, but the level of expression of those inappropriate products is sufficiently low and occurs sufficiently late in development to allow nearly all males to survive to the adult stage (Cline et al., 1999). Curiously, abnormalities are confined to the adult abdomen, where tergite etching and reduction of bristle number on sternites two to five signal dosage compensation upsets, and where growth of bristles on sternite six (normally devoid of bristles in males) signals feminization (see row A). Comparison of rows A and B shows that if there is any effect of loss of $flex^{+}$, it is to make these abnormalities slightly worse, not better.

flex mutants are Y suppressed lethals mutant for *bb*, not sex-specific lethals

If the lethality of homozygous *flex* females cannot be attributed to effects on *Sxl* expression, what is the cause? Section 10/2.3.8 of Ashburner's encyclopedic compilation of *Drosophila* genetic lore (Ashburner, 1989) points out that Y-suppressed and Y-enhanced lethal mutations are remarkably common and can be traced to two different aspects of this sex chromosome: the rDNA genes that it carries, and its influence on position-effect variegation. So far as is known, Y-conditional lethals have nothing to do with sex determination or dosage compensation. For this reason, one of the first issues to be addressed in the analysis of any new putative sex-specific lethal mutation is whether its phenotype is affected by the Y chromosome. The same tests can also help one avoid mistaking X-Y translocations for XX-specific lethals.

For such tests, stocks centers maintain special lines in which males have no free Y, but instead carry only a single compound sex chromosome made from the fusion of an X and a Y. Mutant markers on the compound X-Y allow one to follow this *C(1;Y)* chromosome unambiguously. Females in these lines also carry a single sex chromosome, one generated by a fusion of two X chromosomes. Because animals with both or neither compound chromosome are inviable, these unusual sex chromosomes are confined to opposite sexes and hence are stable. To generate XO males, one mates the appropriate male or female to the opposite sex from the compound sex chromosome line. Using *C(1;Y)* males, we determined that all three mutant *flex* chromosomes were lethal to males in the absence of a Y chromosome (data not shown).

Even more important, the converse holds as well: a Y chromosome rescues homozygous *flex* mutant females. In the course of crossing females from balanced *flex* stocks, we recovered rare *flex/flex* mutant females from which we established homozygous *flex* mutant lines. Because the female:male ratio in these mutant lines was approximately 1:2, we suspected that the viable mutant *flex* females were XXY, with the Y chromosome responsible for rescue. We confirmed our suspicion by mating *C(1;Y)* males to such females and showing that approximately half the *flex* mutant sons survived, and the survivors were fertile (data not shown). These males must have inherited both a free X and Y chromosome from their mothers, since without a Y, males are invariably sterile and *flex* mutant males would not live.

By far the best characterized Y-suppressed lethal effect involves complementation of defects in the X-linked rDNA gene complex (the *Xbb* locus) by the corresponding bb^{+} complex on the Y (reviewed by Ashburner, 1989; Ritossa, 1976). Table 3 shows that the three flex alleles failed to complement a mutant Ybb^{-} chromosome, indicating that a defect in Xbb^{-} would account for the inviability of XX and XO *flex* mutant animals and the rescue of XX *flex* females by a wild-type Y. This table also illustrates a caveat in manipulations of the Y: extra Y chromosomes often segregate in stocks that employ X-chromosome balancers. By decreasing the fidelity of X-chromosome segregation in females, balancer chromosomes lead to the production of XXY daughters who receive both Xs from their mother and a Y from their father. These females in turn transmit a Y to their daughters at an even higher rate. Such an extraneous Ybb^{+} is likely responsible for the three surviving $flex^{1}$ males in row A of Table 3, all of whom were subsequently shown not to carry a Ybb^{-} chromosome using test crosses to females carrying *flex*.

Is *flex* nothing more than *bb*, or might *flex* be more interesting, perhaps a specific regulator of Xbb^{+}? Bhattacharya et al. (1999) reported a meiotic map position for *flex* at 61.2 which would suggest a gene different from *Xbb* (located at 66.0), but the only data presented to position *flex* was a complementation test with *Df(1)JA27*, a deletion of chromomeres 18A5 to 18D1-2. Until the *Df* chromosome used is shown to be bb^{+}, the data remain inconclusive. Incidental data in our investigation of the Sxl^{M}-*flex* interaction in Table 1 would place *flex* 9.3 cM (52/562) from *f*, the position expected for *bb* at 66.0.

None of our findings account for the other observations reported by Bhattacharya et al. indicating disruption of *Sxl*

Table 3. All three $flex^{-}$ chromosomes are defective for the rDNA locus *bobbed*

Cross*	*flex* allele	Progeny recovered (not all classes listed)		
		$flex^{-}/Ybb^{-}$ males	*Balancer*/Ybb^{-} males	$flex^{-}$/+ females
A	1	(3‡)	65	116
B	2	0	104	142
C	3	0	149	196

*+/*Df(YS)bb*$^{-}$ ♂♂ × ♀♀ as follows: (A) *f* $flex^{1}$/*Binsinscy, y w* sn^{X2} *B*; (B) *w cv sn f* $flex^{2}$/*Binsinscy*; and (C) *y pn v f* $flex^{3}$/*Binsinscy*.

‡Although *f*, these males carry Ybb^{+} – see text.

regulation: (1) failure of anti-SXL antibody to detect SXL in *flex/flex* embryos, (2) increased staining in XX embryos by anti-H4Ac16 antibody that detects the acetylated histone H4 associated with hyperactive X chromosomes present in animals lacking female SXL proteins; and (3) hybridization to RNA in *flex* mutant female embryos and mutant adult germline clones of a probe to the male-specific *Sxl* exon. Effects on *Sxl* regulation so early in development caused by a deficit of zygotically transcribed rRNA would be of considerable interest. However, these experiments are at odds with the authors' observation that *flex/flex* mutant clones in the forelegs of females are not masculinized.

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