The *Drosophila* Myc gene, *diminutive*, is a positive regulator of the *Sex-lethal* establishment promoter, *Sxl-Pe*

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The binary switch gene *Sex-lethal* (*Sxl*) controls sexual identity in *Drosophila*. When activated, *Sxl* imposes female identity, whereas male identity ensues by default when the gene is off. The decision to activate *Sxl* is controlled by an X chromosome counting system that regulates the *Sxl* establishment promoter, *Sxl-Pe*. The counting system depends upon the twofold difference in the gene dose of a series of X-linked transcription factors or numerators. Because of this difference in dose, early female embryos express twice the amount of these transcription factors, and the cumulative action of these transcription factors turns on *Sxl-Pe*. Here we show that the *Drosophila* Myc gene *diminutive* is an X-linked numerator.

Sexual identity in *Drosophila* is controlled by the binary switch gene *Sex-lethal* (*Sxl*) (1). When *Sxl* is on, it imposes female development, whereas male development proceeds by default when it is off. During most of the life cycle, on/off regulation is at the level of alternative splicing (2, 3). In males, *Sxl* transcripts are spliced in the default pattern that incorporates a translation terminating male-specific exon, exon 3. In females, exon 2 is spliced directly to exon 4, skipping exon 3. The resulting female mRNAs encode 36- to 40-kDa proteins that have two RNA recognition motif binding domains. These Sxl proteins direct female-specific splicing of *Sxl* premRNAs expressed from the *Sxl* maintenance promoter, *Sxl-Pm*, and this establishes a positive autoregulatory feedback loop that serves to maintain female identity (4). In addition to maintaining the determined state, *Sxl* orchestrates female development by promoting the female-specific splicing of *transformer* (*tra*) mRNA and turns off X chromosome dosage compensation by blocking *male-specific lethal-2* (*msl-2*) mRNA translation (1). In males, where *Sxl* is off, default splicing of *tra* generates nonproductive mRNAs and male differentiation ensues, whereas expression of Msl-2 permits the assembly of a functional dosage compensation system. As would be expected from its binary activity, loss of *Sxl* has no phenotypic consequences in males, whereas it leads to sex transformation and lethality in females. Conversely, ectopic activation of *Sxl* in males induces female development and is lethal due to the lack of dosage compensation.

Whereas *Sxl* is regulated by alternative splicing during much of development, the initial decision of whether to activate it or not and, thus the choice of sexual identity is made at the level of transcription by regulating the *Sxl* establishment promoter, *Sxl-Pe* (5). *Sxl-Pe* is located in the first intron of the *Sxl-Pm* transcription unit and is the target of the system that measures the X chromosome-to-autosome "ratio" in precellular blastoderm embryos. The counting system consists of zygotically transcribed X-linked "numerators," which function to activate *Sxl-Pe* and correspond to genes encoding transcription factors like *scute* (*sc*) and *runt* (6–8). On the autosomes, there is only one known zygotically transcribed "denominator," *deadpan* (9). In addition there are a series of maternally derived transcription factors. Some like *daughterless* act as cofactors for the numerators (*sc*), whereas others, like *groucho* (*gro*), appear to function as autosomal denominators (10, 11). The activation of *Sxl-Pe* pivots on the difference in dose of the X-linked numerators in 2X female and 1X male embryos. This difference is sufficient to turn on *Sxl-Pe* in female precellular blastoderm embryos, but keep it off in males. Except for ~20 amino acids at the N terminus, the *Sxl-Pe* mRNAs encoded proteins are identical to the *Sxl-Pm* female mRNAs and they activate the positive autoregulatory feedback loop by directing female-specific splicing of the first *Sxl-Pm* transcripts, which appear as *Sxl-Pe* shuts down just before cellularization (5).

Sxl-Pe consists of an evolutionarily conserved 0.4-kb switch element that confers sex-specific transcription and an upstream augmentation element that generates high levels of expression (12, 13). Within these elements are multiple (typically noncanonical) binding sites for numerators like *sc* and *runt* and denominators like *dpn*. Here we report the identification of a previously unknown X-linked numerator, *diminutive* (*dm*), which encodes the *Drosophila* bHLH transcription factor dMyc.

Results

To identify genes important for *Sxl* regulation we screened for deficiencies that suppress or enhance the weak female-specific lethality induced by a dominant negative *hsp83:N′–β-gal* transgene. The chimeric Sxl–β-gal protein expressed by this transgene is assembled into Sxl splicing complexes and interferes with *Sxl* autoregulation (14). Whereas we expected to recover mostly splicing factors in this screen, mutations in known numerators such as *sc* also enhance female lethality. One of the X-linked deficiencies identified in our screen was *Df(1)dm75e19*, which uncovers the 3C-E region. When *Df(1)dm75e19* females were crossed to *N′–β-gal* males, the viability of *Df(1)dm75e19*/+ females was 75% that of their +/+ sibs (n = 196). Female lethal interactions with the transgene are exacerbated by removing a copy of *Sxl.* Transgenic females *trans*-heterozygous for *Df(1)dm75e19* and the *Sxl* deletion, Sxl^{7BO}, are only half as viable as their transgenic Sxl^{7BO} female sibs (n = 96).

***dm* Mutations Are Preferentially Female Lethal.** One gene uncovered by *Df(1)dm75e19* is *diminutive* (*dm*). *dm* encodes the *Drosophila* Myc protein (15–17). In other organisms, Myc, together with its partner Max, is a transcriptional activator. Because *dm* is X linked and is transcribed in precellular blastoderm embryos, a plausible idea is that *dm* is a new numerator. If this is the case, it should be possible to duplicate the female lethal interactions between the *N′–β-gal* transgene and *Df(1)dm75e19* using *dm* mutants. Consistent with this expectation, *N′–β-gal* females that are *trans*-heterozygous for Sxl^{7B0} and the dm^1 allele are two-thirds as viable as their *N′–β-gal* Sxl^{7B0}/+ sisters (n = 644). Although the female

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lethality induced by dm^1 is less than that of *Df(1)dm75e19*, this could be due to the fact that dm^1 is not a null.

We subsequently found that dm^1 and two other hypomorphic alleles, dm^{P0} and dm^{P1}, exhibit partial female-specific lethality (Table 1). Of the three, dm^1 is the strongest and only 6% of dm^1/dm^1 females survive compared with 85% of sibling dm^1/Y males. dm^{P1} and dm^{P0}, which have P-element insertions upstream of the transcription start site (17), also preferentially kill females. Homozygous dm^{P1} females are half as viable as their male dm^{P1} sibs, whereas dm^{P0} females are two-thirds as viable as their male dm^{P0} sibs. Arguing against background effects, female lethality is also observed when the mutants are tested over *Df(1)dm75e19* (Table S1).

Female Lethal Effects of *dm* Are Due to *Sxl* Misregulation. We used two experimental approaches to confirm that *dm* functions in *Sxl* regulation. First, we examined the effects of *dm* on Sxl expression by probing embryos from wild-type or $dm^1/+$ mothers mated to dm^1/Y fathers with Sxl antibodies. We found that the reduction in *dm* activity in progeny of wild-type mothers had a small but obvious effect on Sxl. As expected, half of the embryos (46%, $n = 124$) expressed Sxl, whereas the other half (56%) did not. However, the $dm^1/+$ female progeny from this cross express less Sxl than fully wild-type female embryos probed in parallel. Moreover, whereas Sxl is always expressed uniformly throughout the soma in +/+ females, about 40% of the $dm^1/+$ females (20% of total population) had patchy Sxl expression. Even greater effects on Sxl were evident when $dm^1/+$ mothers were crossed to dm^1/Y fathers (Fig. S1). As before, half (48%, $n = 223$) of the embryos had no Sxl and these are presumed to be males. The remaining embryos fell into three classes: class 1, 13% were uniformly stained although not as strongly as wild-type females; class 2, 15% also had intermediate levels of staining but Sxl expression was uneven; and class 3, 24% had lower levels of Sxl and were patchy. On the basis of the frequency of the different classes (and the results with wild-type mothers crossed to *dm* fathers), we believe that embryos in classes 1 and 2 are $dm^1/+$, whereas embryos in class 3 are dm^1/dm^1.

In the second approach, we recombined dm^{P0} with the gain-of-function allele Sxl^{M4}. Sxl^{M4} has a transposon inserted into the male exon that causes constitutive female splicing of *Sxl-Pm* pre-mRNAs irrespective of the signal from the X/A counting system. Eleven independent dm^{P0} Sxl^{M4} recombinants were mated to dm^{P0}/Y males. As expected, the Sxl^{M4}, dm^{P0}/dm^{P0} females are fully viable (Table S2). Whereas constitutive Sxl expression rescues the female-specific lethality of dm^{P0}, it does not rescue the *dm* bristle or female sterility phenotypes. As expected, the constitutive Sxl^{M4} is male lethal and no dm^{P0} Sxl^{M4} males are recovered from the cross, whereas balancer males are.

***Sxl* and Numerators Show Female Lethal Interactions with *dm*.** The misregulation of *Sxl* evident when *dm* activity is compromised supports the idea that it functions as a numerator. Mutations in numerators show dose-dependent female lethal interactions with *Sxl* and each other. On the basis of the relative severity of these lethal effects, *sc* and *sis-a* have been classified as primary numerators, whereas *runt* (*run*) and the JAK/STAT ligand, *unpaired* (*upd*) are secondary numerators (1). To provide further evidence that *dm* is a numerator and ascertain its relative importance, we first tested for female lethal interactions with *Sxl*. Males carrying *dm* mutations were mated to Sxl^{7B0}/Bal females. Females compromised for only one copy of *dm* are fully viable as are $Sxl^{7B0}/+$ females. However, females *trans*-heterozygous for Sxl^{7B0} and one of the *dm* alleles are only about two-thirds as viable as their *dm*/+ sibs (Table 2). The female lethal interactions between Sxl^{7B0} and the three *dm* alleles are not as strong as those observed for mutations in *sc* or *sis-b*, but are stronger than *run*. In the reciprocal experiment, we introduced dm^- (or *Df(1)dm75e19*) from the mother (Table S3). As expected for a numerator, there is no indication of a significant maternal effect.

Trans-heterozygous combinations between *dm* and other numerators also enhance female lethality (Table 3). The strongest interactions were seen between *sc* and dm^1 with female viability being reduced to less than one-fourth, whereas there were weaker interactions between *sc* and the two other *dm* alleles. Viability is only slightly reduced in the *sis-a* and *dm trans*-heterozygous combinations, with dm^{P1}/*sis-a* being the strongest. run^3 also interacts most strongly with dm^{P1}, whereas it shows no interactions with dm^1.

***dm* Is Required for Activation of *Sxl-Pe* in Females.** Numerators activate *Sxl* by turning on the establishment promoter *Sxl-Pe*. We used two different *Sxl-Pe:LacZ* reporters to determine whether *dm* regulates *Sxl-Pe*. The larger promoter, $Sxl\text{-}Pe_{3.0kb}$, contains both the conserved sex-specific switch element and the upstream augmentation element, whereas the smaller reporter, $Sxl\text{-}Pe_{0.4kb}$, only has the switch element. In wild type, a single copy of $Sxl\text{-}Pe_{3.0kb}$ drives a high level of LacZ expression throughout the soma of female embryos (Fig. 1*A*), whereas no LacZ is detected in males (Fig. 1*B*). We first crossed wild-type females carrying two copies of $Sxl\text{-}Pe_{3.0kb}$ to dm^1 or, as a control, *sc* males. All female progeny have a single copy of $Sxl\text{-}Pe_{3.0kb}$ and are heterozygous for dm^1 or *sc*. Like wild type, LacZ is expressed in ~50% of the embryos; however, as can be seen by comparing the $dm^1/+$ embryo in Fig. 1*C* with wild type in Fig. 1*B*, the level of LacZ expression is reduced. In fact, the $Sxl\text{-}Pe_{3.0kb}$ reporter appears to be almost as sensitive to reduced *dm* activity as it is to the *sc* control (Fig. 1*D*).

In these experiments, all female embryos are heterozygous for either dm^1 or *sc* and all showed a reduction in LacZ expression compared with wild type processed in parallel. In the reciprocal experiment, we mated reporter males to dm^1 or *sc* females. In this case, half the female embryos will be wild type, whereas the other half will be *dm*/+ or *sc*/+. For the *sc* control, females from this cross fall into two equal classes. In one class, which corresponds to +/+ females, LacZ expression is the same as in female progeny of wild-type mothers. In the other class, which corresponds to *sc*/+ females, LacZ expression is reduced like that when the father is *sc* (12). As would be expected if *dm* functions as a numerator, the female progeny of *dm*/+ mothers also fall into two nearly equal classes (Table 3).

Although these experiments show that $Sxl\text{-}Pe_{3.0kb}$ is sensitive to the dose of *dm* (or *sc*), most if not all female embryos (i.e., 50% of the population) still express LacZ. This would be expected because *dm*/+ (or *sc*/+) females are fully viable. On the other hand, under conditions in which female viability is compromised, the percentage of embryos expressing LacZ might be expected to drop below half. This is the case. When $dm^1/+$ females are mated to

Table 1. *dm* mutations are female lethal

	dm/dm, % (*n*)	*dm/FM7* (*n*)	*dm/Y*, % (*n*)	*FM7/Y* (*n*)	(*n*)
$dm^1/FM7$ x dm^1/Y	6 (46)	(731)	85 (624)	(456)	(1,857)
$dm^{P0}/FM7$ x dm^{P0}/Y	46 (233)	(522)	73 (380)	(343)	(1,388)
$dm^{P1}/FM7$ x dm^{P1}/Y	23 (94)	(410)	51 (209)	(374)	(1,087)

Progeny of each type from crossing *dm/Bal* females to *dm/Y* males (as indicated in column 1) were counted. Percentage in second and fourth columns is calculated on the basis of number of *dm/FM7* females.

Table 2. Female lethal interactions between *dm* and other sex determination genes

	Paternal					
Maternal	dm^1/Y	dm^{P0}/Y	dm^{P1}/Y	*sis-a*/Y	sc^{3-1}/Y	$runt^3/Y$
$Sxl^{7BO}/FM7$	60 (2,031)	63 (312)	68 (260)	7 (32)	9 (204)	81 (671)
sis-a/*SM1*	76 (616)	88 (279)	84 (408)	ND	ND	51 (565)
$sc^{3-1}/FM7$	23.8 (2,385)	90.3 (745)	76.1 (1,200)	14 (409)	ND	60 (564)
$runt^3/FM7$	100 (271)	79 (516)	89 (484)	1 (281)	38 (67)	ND

For each cross, paternal genotype is indicated in the top row and maternal genotype in the first column. Percentage of surviving trans-heterozygous females from each cross were calculated relative to balancer females. Percentage of offspring indicated outside parenthesis. Total number of offspring indicated in parentheses. ND, not determined.

dm^1/Y males, half of the females (25% of total) will be dm^1/dm^1. In this cross, *Sxl-Pe*$_{3.0kb}$ is active in only about one-third of the progeny instead of 50%, indicating that it is not turned on in a significant fraction of dm^1 females (Table 3). We also tested *trans* combinations of dm^1 and *sc*. Whereas *Sxl-Pe*$_{3.0kb}$ is still active in ~50% of the progeny, half of these (which are expected to be dm^1/sc) have only a low level of LacZ (Fig. 1 *E* and *F* and Table 3), whereas the remaining embryos resemble *sc*/+ or *dm*/+ females. (Note: As would be predicted from these experiments, Fig. S1 shows that Sxl^{M4} rescues dm^{P0} female lethality by bypassing, rather than correcting, the defect in *Sxl-Pe* activation.)

We next determined whether *dm* exerts its effects on *Sxl-Pe* through the conserved *Sxl-Pe*$_{0.4kb}$ switch element. As indicated in Table S2, *Sxl-Pe*$_{0.4kb}$ also depends upon *dm* for full activity. When $dm/+^1$ or $dm^{P0}/+$ mothers are crossed to wild-type fathers, LacZ expression is reduced in half of the female progeny. When $dm^1/+$ mothers are crossed to dm^1/Y fathers, a significant fraction of the female embryos fail to activate *Sxl-Pe*$_{0.4kb}$ (Table S4). Similar reductions in LacZ expression are evident when either dm^1 or dm^{P1} are combined with a mutation in another numerator (Table S4). For *dm*/*sc* or *dm*/*sis-a* combinations, the promoter is active in less than 50% of the embryos and in many, there is only little LacZ. Similarly, although nearly 50% of the progeny express LacZ when *dm* is crossed with *upd*, about half the females (presumably upd/dm^1) have only a very low level of expression.

Excess *dm* Activates *Sxl* in Males. If *dm* promotes *Sxl-Pe* activity, it should be possible to inappropriately turn it on in males by overexpressing Dm. For this purpose we used a *hairy* (*h*)-*GAL4* driver to activate a *UAS-dm* transgene. *h* exhibits a dynamic expression pattern in blastoderm stage embryos. It is initially active in a broad domain and then resolves into stripes spanning the central two-thirds of the embryo. Females homozygous for *UAS-dm* were mated to *h-GAL4*/*TM3* males. If Dm functions to activate *Sxl-Pe*, then the pattern of Sxl expression should differ from wild type in that there should be four distinct (equal in number) classes instead of just two. This prediction is correct. Embryos in the first and second class resemble wild-type males (no expression) and wild-type females (uniformly high expression) (Fig. 1 *I* and *J*). These embryos are expected to carry *UAS-dm* but not the *h-GAL4* driver. Embryos in the third class express higher levels of Sxl than wild-type females (Fig. 1*K*) and are expected to be females that have both *UAS-dm* and the *h* driver. Finally, the fourth class has a low level of Sxl in the central region of the embryo (Fig. 1*L*). From the level and pattern of expression, these embryos must be males that have *UAS-dm* and *h-GAL4*.

Table 3. Effects of *dm* on *Sxl-Pe*$_{3.0kb}$ activity

Maternal	Paternal	+++, %	++, %	+, %	–, %	*n*
w^1/w^1	w^1/Y; 3.0	47			53	678
$dm^1/+$	w^1/Y; 3.0	25	22		53	623
$dm^1/+$	dm^1/Y; 3.0		31		67	471
w^1/w^1	sc^{3-1}/Y; 3.0		48		52	163
$dm^1/+$	sc^{3-1}/Y; 3.0		27	28	46	79

Sxl-Pe$_{3.0kb}$ activity in progeny from each of the indicated crosses. +++, high level of LacZ; ++, intermediate level; +, low level; –, none. *n* = number scored.

***dm* Is Required to Activate the *Sxl-Pe*$_{GOF}$ Promoter in Males.** To provide additional evidence that *Sxl-Pe* is sensitive to *dm* levels in males, we took advantage of a "gain-of-function" promoter, *Sxl-Pe*$_{GOF}$, which is active in both sexes. It has four copies of a 72-bp sequence from the switch element appended to *Sxl-Pe*$_{0.4kb}$. The multimer shifts the balance between negative (denominator) and positive (numerator) *cis*-acting target sequences and the *GOF* promoter is turned on in 1X/2A embryos in a characteristic spatially restricted pattern (18, 19). Males carrying *Sxl-Pe*$_{GOF}$ were mated to $dm^1/+$ females. As would be predicted if *dm* affects the activity of the *Sxl-Pe*$_{GOF}$ in both sexes, the embryos could be divided into approximately four equal classes on the basis of the pattern and level of LacZ expression. One class resembled wild-type *Sxl-Pe*$_{GOF}$ females, whereas the second resembled wild-type *Sxl-Pe*$_{GOF}$ males (Fig. 1*G*). In the third class, the spatial pattern of LacZ expression is like that of *Sxl-Pe*$_{GOF}$ in wild-type females, but the level of expression is reduced. This class is expected to correspond to $dm^1/+$ females. Similarly, in the fourth class (Fig. 1*H*), the spatial pattern of LacZ resembles that of wild-type *Sxl-Pe*$_{GOF}$ males; however, there is much less LacZ. This class is expected to be dm^1/Y males.

***dm* Responsive Elements in *Sxl-Pe*.** The 0.4-kb *Sxl-Pe* switch element contains two copies of a "D box" (CACGCG) in a conserved 23-bp sequence block ~100 bp upstream of the transcription start site. These D-box sequences are bound by the transcriptional repressor Dpn in vitro, and when they are mutated in the context of a *Sxl-Pe*$_{1.4kb}$ reporter (which like *Sxl-Pe*$_{3.0kb}$ has the switch and augmentation elements), the promoter is inappropriately activated in male embryos (13, 20). Although these findings demonstrate that the two D-box motifs function as target sites for negative regulation by *dpn* (and probably also a maternal bHLH protein), mutations of the two D boxes in the context of the *Sxl-Pe*$_{0.4kb}$ reporter had paradoxical properties (21). Unlike the *Sxl-Pe*$_{1.4kb}$ D-box double mutant, the *Sxl-Pe*$_{0.4kb}$ double mutant was not activated in males, whereas in females it was *less* active than the wild-type *Sxl-Pe*$_{0.4kb}$. The mutant promoter was also more sensitive to a reduction in the dose of *dpn* than wild-type *Sxl-Pe*$_{0.4kb}$.

One explanation for the unusual properties of the *Sxl-Pe*$_{0.4kb}$ mutant is that these two D-box sequences are a regulatory target not only for Dpn but also for some unknown numerator. This numerator and its target sequences would have a more critical function in the context of *Sxl-Pe*$_{0.4kb}$ than they do in the context of *Sxl-Pe*$_{1.4\ kb}$. As the D-box corresponds to one of the non-canonical motifs recognized by Myc:Max complexes (22, 23) a plausible candidate for the mystery numerator would be *dm*.

To test this model, we first asked whether Dm associates with the switch element:D-box region in *Sxl-Pe* during the period

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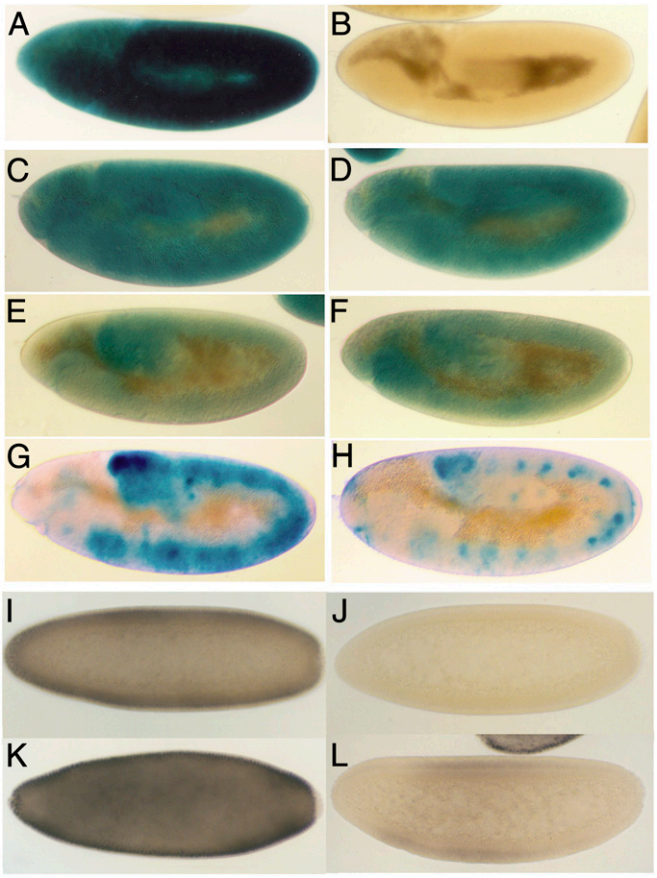

Fig. 1. *dm* regulates *Sxl*. (*A–H*) (LacZ expression) *Sxl-Pe*$_{3.0kb}$ (8) in wild-type female (*A*) and male (*B*) embryos, in *sc*/+ (*C*), or *dm*^{1}/+ (*D*) female embryos, and (*E* and *F*) in *dm*^{1}/*sc*^{1} female embryos. *sc*/+ and *dm*^{1}/+ females were obtained by crossing *w*^{1} females to *sc* or *dm*^{1} males carrying two copies of *Sxl-Pe*$_{3.0kb}$. The *sc*/*dm*^{1} embryos were from a cross between *sc*/*FM7* females and *dm*^{1} males homozygous for *Sxl-Pe*$_{3.0kb}$. Controls were obtained by mating *w*^{1} females to two-copy *Sxl-Pe*$_{3.0kb}$ males and processed in parallel with the experimentals. (*G* and *H*) *Sxl-Pe*$_{GOF}$ (22, 23) wild-type and *dm*^{1}/*Y* male embryos. Males carrying two copies of *Sxl-Pe*$_{GOF}$ were mated to wild-type or *dm*$^{+}$/*FM7* females. (*I–L*) (Sxl expression) Four classes of embryos are generated by crossing females homozygous for *UAS-dm* to *h-GAL4/TM3* males. (*I*) Class I. WT female (*UAS-dm; TM3*). (*J*) Class II, WT male (*UAS-dm;TM3*). (*K*) *UAS-dm/h-GAL4* female. (*L*) *UAS-dm/h-GAL4* male.

when the promoter is being activated by the X/A counting system. For this purpose we immunoprecipitated cross-linked chromatin from 1.0- to 3.5-h embryos with Dm preimmune and immune serum. We used two known *dm* targets, *Nop60b* and *peterpan* (*ppan*) (24), as positive controls, and a sequence 1 kb downstream of the *Sxl-Pe* start site as a negative control. Although there is no enrichment of the *Sxl* downstream region, enrichment of sequences spanning the paired *Sxl-Pe* D boxes is close to that of *Nop60b* and almost twice that of *ppan* (Fig. 2).

As a further test of the model, we appended a multimerized 34-bp sequence spanning the paired D boxes to the minimal *Sxl-Pe*$_{0.4kb}$ reporter (*Sxl-Pe*$_{5xDbox}$). This *Sxl-Pe*$_{5xDbox}$ reporter is expected to behave differently depending upon whether the 34-bp multimer has regulatory targets for just *dpn* (and the postulated maternal bHLH protein, ref. 13) or for both *dpn* and *dm*. If the multimer is only a target for *dpn*, *Sxl-Pe*$_{5xDbox}$ should be strongly repressed in females, induced to the same extent as *Sxl-Pe*$_{0.4kb}$ in the absence of *dpn* and largely unresponsive to *dm*. On the other hand, if the multimer has regulatory targets for both *dpn* and *dm*, the *Sxl-Pe*$_{5xDbox}$ reporter should still be on in females, whereas it should respond in an unusual fashion to changes in the relative levels of *dpn* and *dm*.

The properties of the *Sxl-Pe*$_{5xDbox}$ reporter argue that the multimer contains targets not only for *dpn* but also for *dm*. Instead of being completely silenced, it is still active in close to 50% of the embryos. However, the balance is clearly shifted in favor of repression as it drives much less LacZ expression than the parental *Sxl-Pe*$_{0.4kb}$ (Fig. 3). The multimer promoter also seems to be somewhat less sensitive to a twofold reduction in *dpn* than *Sxl-Pe*$_{0.4kb}$; there is only a small increase in activity in *dpn*^{7}/+ females (Fig. S3) and no activation in *dpn*^{7}/+ males. A quite different result is seen for *dpn*^{7}/*dpn*^{7}. Fig. 3 shows that the parental *Sxl-Pe*$_{0.4kb}$ is up-regulated in *dpn*^{7}/*dpn*^{7} females and weakly activated in males. By contrast, there is a dramatic increase in *Sxl-Pe*$_{5xDbox}$ activity in *dpn*^{7}/*dpn*^{7} embryos. Although *Sxl-Pe*$_{5xDbox}$ is only weakly active in wild-type females, it drives a higher level of LacZ expression in *dpn*^{7}/*dpn*^{7} females than *Sxl-Pe*$_{0.4kb}$. Moreover, the multimer is also strongly activated in *dpn*^{7}/*dpn*^{7} males where the level of expression is even higher than *Sxl-Pe*$_{0.4kb}$ in wild-type females (Fig. 3). The substantial activation of *Sxl-Pe*$_{5xDbox}$ in the absence of *dpn* indicates that the multimer must contain sequences for at least one numerator. Additionally, it would appear that the activity of this numerator(s) is antagonized by Dpn.

To determine whether *dm* might be one of these numerators, we examined the effects of altering the relative dose of *dm* and *dpn*. An extra copy of *dm* in an otherwise wild-type background has even less effect on *Sxl-Pe*$_{5xDbox}$ than a twofold reduction in *dpn*. However, the multimer promoter is strongly activated when females carrying three copies of *dm* are also heterozygous for *dpn*^{7} (Fig. 3). Further supporting a multimer-dependent numerator function are the effects of *dm*^{1} in homozygous *dpn*^{7} embryos. Fig. 3 shows that activation of *Sxl-Pe*$_{5xDbox}$ in *dpn*^{7}/*dpn*^{7} females is reduced in *dm*^{1}/*dm*^{1} females, whereas it appears to be completely eliminated in *dm*^{1} males. In this respect, *Sxl-Pe*$_{5xDbox}$ also differs from the parental *Sxl-Pe*$_{0.4kb}$, which is still weakly active in *dpn*$^{-}$; *dm*^{1} males. The loss of promoter activity in males indicates that *dm* plays a pivotal role in activating *Sxl-Pe*$_{5xDbox}$. On the other hand, the fact that LacZ expression in *dpn*$^{-}$/*dm*$^{-}$ *Sxl-Pe*$_{5xDbox}$ females remains substantially above that in wild type (Fig. 3) indicates that the multimer promoter is still activated by a numerator(s) in the *dm*^{1} mutant. Whereas two copies of the hypomorphic *dm*^{1} would be expected to provide females with more numerator activity than males, it is not clear that this would be sufficient to account for the amount of LacZ that is expressed by the *Sxl-Pe*$_{5xDbox}$ in *dpn*$^{-}$/*dm*$^{-}$ females.

Discussion

The choice of sexual identity pivots on differences in the dose of X-linked numerators that function to activate *Sxl-Pe*. The most important numerators are *sis-a* and *sc* (1). They are expressed throughout the embryo, and mutations in either gene can have quite pronounced effects on *Sxl* activation. The two other previously described numerators, *runt* and *upd*, are spatially restricted in their pattern of expression and have more modest effects on *Sxl*. In the studies reported here, we have identified a previously unknown numerator, *dm*, which encodes the fly dMyc protein.

Several lines of evidence demonstrate that *dm* is a numerator. First, three independent hypomorphic alleles exhibit preferential female lethality. Second, these *dm* mutations show synergistic female lethal interactions with mutations in other known numerators. Moreover, as required for a numerator element, the female lethal effects of *dm* are dependent upon zygotic activity. Third, *dm* activity is required for Sxl protein expression in female embryos. Fourth, it is possible to rescue the female-specific lethal effects of *dm* mutant females with a gain-of-function *Sxl* allele that activates the *Sxl* autoregulatory feed loop independently of the X/A ratio. Fifth, as observed for other X chromosome counting elements, Sxl protein expression can be induced in males by excess Dm. Sixth, *dm* acts as a dose-dependent regu-

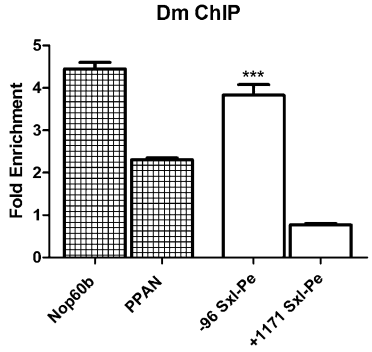

Fig. 2. Dm binds to *Sxl-Pe* in vivo. ChIP on 1- to 3.5-h *Ore R* embryos using anti-Dm or control preimmune serum. Fold enrichment is of ChIP immune versus preimmune serum. Nop60b and PPAN are positive controls previously reported as Dm targets. The −96 Sxl-Pe is upstream of the Sxl-Pe start site and spans the D-box region; the negative control +1,171 is downstream within the coding region. Unpaired *t* test using GraphPad to analyze signal difference at −96 versus +1,171 gives significance at $***P < 0.0001$.

lator of *Sxl-Pe* and the activation of the promoter in female embryos is compromised by mutations in *dm*. Seventh, as expected for a numerator, Dm is found associated with *Sxl-Pe* in blastoderm-stage embryos. On the basis of the effects of *dm* mutations on female viability, Sxl protein expression and *Sxl-Pe* activity, the role of *dm* in X chromosome counting would appear to be more similar to that of the "secondary" numerators *runt* and *upd* than to the "primary" numerators *sc* and *sis-a*. However, the one caveat is that the three *dm* alleles we have studied are hypomorphs rather than nulls.

Because *dm* mutations affect the activity not only of the full-length *Sxl-Pe*, but also of the minimal *Sxl-Pe*$_{0.4kb}$, there must be target sequences for *dm* in the highly conserved region that functions as the sex-specific switch. Supporting this possibility, ChIP experiments show that Dm associates with the *Sxl-Pe* switch element region in early embryos. As the switch element does not contain canonical high-affinity dMyc (Max) binding sites (CACGTG) *dm* must exert its regulatory effects through non-canonical sites. Probably the best candidates for noncanonical Dm sites in the switch element are a pair of closely spaced D-box motifs. These two D-box motifs have unusual properties. Consistent with in vitro studies showing that the D-box motifs are binding sites for Dpn, mutations in the two D-box motifs in the context of a full-length *Sxl-Pe* activate the promoter (11). On the other hand, mutations in the context of the minimal switch element *Sxl-Pe*$_{0.4kb}$ promoter decrease its activity instead of increasing it as expected (21). This contradictory context-dependent behavior of the D-box mutants argues that the D boxes (or sequences overlapping them) are targets not only for Dpn, but also for a numerator such as *dm*. We tested this idea by multimerizing a short sequence spanning the two D-box motifs and appending the multimer to *Sxl-Pe*$_{0.4kb}$. As would be expected if the multimer has Dpn targets, *Sxl-Pe*$_{5xDbox}$ is less active than the parental *Sxl-Pe*$_{0.4kb}$ in wild-type females. However, the multimer must also contain targets for numerators as *Sxl-Pe*$_{5xDbox}$ is much more strongly activated than *Sxl-Pe*$_{0.4\,kb}$ in *dpn*$^{-}$, turning on at very high levels not only in females but also in males. Importantly, ectopic activation in males requires *dm* activity as it is eliminated when the *dpn*$^{-}$ males are hemizygous for the hypomorphic *dm*^{1}. Although this finding argues that *dm* plays a critical role in activating the multimer, we cannot exclude the possibility that the multimer contains target sites for at least one other numerator.

If Dm binds to *Sxl-Pe* via the D boxes in the multimer, it would presumably exert at least a part of its regulatory effects by preventing Dpn (and the maternal repressor) from binding to *Sxl-Pe*. This would be the first instance of a numerator competing with a denominator(s) for the same or overlapping *cis*-acting elements in *Sxl-Pe*. However, even if Dm functions to prevent Dpn binding,

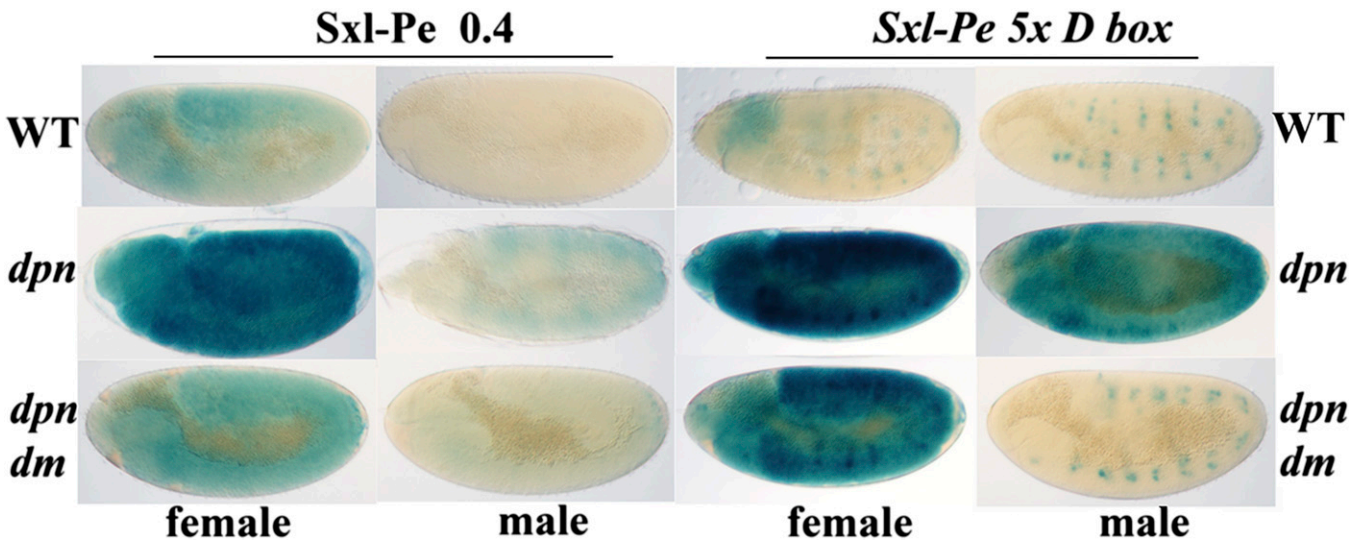

Fig. 3. Regulation of *Sxl-Pe*$_{5xDbox}$ by *dpn* and *dm*. (*Top*) LacZ in wild-type embryos carrying a single copy of *Sxl-Pe*$_{0.4kb}$ of *Sxl-Pe*$_{5xDbox}$ as indicated. For both promoters, embryos fall into two equal classes (corresponding to female and male) on the basis of LacZ pattern: representative embryos are shown. The LacZ positive spots in *Sxl-Pe*$_{5xDbox}$ embryos is due to a sex nonspecific activation in the peripheral nervous system. (*Middle*) LacZ in *dpn*$^{-}$ embryos as indicated. *Sxl-Pe*$_{0.4kb}$: +/+; *dpn*^{7}/*Cyo ftz:LacZ* X +/*Y*: *dpn*^{7} *Sxl-Pe*$_{0.4kb}$/*Cyo ftz:LacZ*. *Sxl-Pe*$_{5xDbox}$: +/+; *dpn*^{7}/*Cyo ftz:LacZ* X +/*Y*: *dpn*^{7} /*Cyo ftz:LacZ*; *Sxl-Pe*$_{5xDbox}$/*Sxl-Pe*$_{5xDbox}$. *dpn*$^{-}$ *Sxl-Pe* reporter embryos lack the *ftz:LacZ* stripes and fall into two equal classes (female and male) that differ in level of LacZ. We used Sxl/LacZ antibody staining to confirm the sexual identify of *dpn* embryos. (*Bottom*) LacZ in *dpn*$^{-}$/*dm*$^{-}$ embryos as indicated. *Sxl-Pe*$_{0.4kb}$: *dm*^{1}/+; *dpn*^{7}/*Cyo ftz:LacZ* X *dm*^{1}/*Y*: *dpn*^{7} *Sxl-Pe*$_{0.4kb}$/*Cyo ftz:LacZ*. *Sxl-Pe*$_{5xDbox}$: *dm*^{1}/+; *dpn*^{7}/*Cyo ftz:LacZ* X *dm*^{1}/*Y*: *dpn*^{7}/*Cyo ftz:LacZ*; *Sxl-Pe*$_{5xDbox}$/*Sxl-Pe*$_{5xDbox}$. *dpn*$^{-}$ *Sxl-Pe* reporter embryos lack the *ftz:Lac* stripes and fall into four equal classes (*dm*/+ or *dm*/*dm* female and +/*Y* or *dm*/*Y* male) that differ in level of LacZ. A control cross using males wild type for *dm* was done in parallel to help distinguish *dm*/+ and +/*Y* from *dm*/*dm* and *dm*/*Y* embryos, respectively. *dm*/+ females have an intermediate (between *dm; dpn* and *dpn* females) level of LacZ, whereas +/*Y; dpn* males have less LacZ than *dm; dpn* females. We used Sxl/LacZ antibody staining to confirm the existence of four classes of *dpn* embryos from this cross and the sexual identity of each class.

this cannot be its only activity as it must also be able to promote transcription in the absence of *dpn* activity. In this context, it is interesting to note that mammalian Myc functions by recruiting the transcription elongation factor P-TEFb to paused polymerases (25). A postinitiation function for Dm would be attractive as it would help explain the effects of one of the global germ cell transcriptional quiescence factors, *nanos* (*nos*), on *Sxl-Pe* (26). In the absence of *nos*, *Sxl-Pe* is inappropriately turned on in the soma of male embryos and in the germ cells of both sexes by a mechanism involving Pol II carboxy terminal domain (CTD) phosphorylation that is independent of the chromosome counting system. Because it was assumed that numerators and denominators regulated *Sxl-Pe* by controlling Pol II recruitment, it was hard to understand how *Sxl-Pe* could ever be activated in *nos* male embryos by a general up-regulation of CTD phosphorylation. However, if Pol II is localized to *Sxl-Pe* in both sexes, and the numerators/denominators control a subsequent step, such as recruitment of P-TEFb, the bypass of the counting system in *nos* embryos is more readily explained.

Experimental Procedures

Sxl-Pe$_{5xDbox}$ was generated by PCR amplifying a 34-bp sequence spanning the two D-box motifs using DMYCBAM5′-CAATTCGCGGGATCCTAGGTAGC and DMYCBGL3′-GCCAGGTAGAAGATCTAAGGAGG. The resulting fragment was ligated, cloned into the BamHI site of pBSK, and a recombinant containing five tandem copies isolated. The tandem copies were excised with EcoRI and XbaI, placed upstream of *Sxl-Pe*$_{0.4kb}$, and then inserted into the EcoEI site of pCasp. Nine independent *Sxl-Pe*$_{5xDbox}$ lines were isolated. All had expression patterns similar to the examples presented here. Two representative lines, *63* and *142*, were used for most of the genetic experiments.

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1. Cline TW, Meyer BJ (1996) Vive la différence: Males vs females in flies vs worms. *Annu Rev Genet* 30:637–702.
2. Bell LR, Maine EM, Schedl P, Cline TW (1988) Sex-lethal, a Drosophila sex determination switch gene, exhibits sex-specific RNA splicing and sequence similarity to RNA binding proteins. *Cell* 55:1037–1046.
3. Bell LR, Horabin JI, Schedl P, Cline TW (1991) Positive autoregulation of *sex-lethal* by alternative splicing maintains the female determined state in *Drosophila*. *Cell* 65:229–239.
4. Cline TW (1984) Autoregulatory functioning of a Drosophila gene product that establish es and maintains the sexually determined state. *Genetics* 107:231–277.
5. Keyes LN, Cline TW, Schedl P (1992) The primary sex determination signal of Drosophila acts at the level of transcription. *Cell* 68:933–943.
6. Cline TW (1988) Evidence that *sisterless-a* and *sisterless-b* are two of several discrete "numerator elements" of the X/A sex determination signal in *Drosophila* that switch *Sxl* between two alternative stable expression states. *Genetics* 119:829–862.
7. Torres M, Sánchez L (1991) The *sisterless-b* function of the *Drosophila* gene *scute* is restricted to the stage when the X:A ratio determines the activity of *Sex-lethal*. *Development* 113:715–722.
8. Duffy JB, Gergen JP (1991) The *Drosophila* segmentation gene *runt* acts as a position-specific numerator element necessary for the uniform expression of the sex-determining gene *Sex-lethal*. *Genes Dev* 5(12A):2176–2187.
9. Younger-Shepherd S, Vaessin H, Bier E, Jan LY, Jan YN (1992) *deadpan*, an essential pan-neural gene encoding an HLH protein, acts as a denominator in Drosophila sex determination. *Cell* 70:911–922.
10. Cronmiller C, Schedl P, Cline TW (1988) Molecular characterization of daughterless, a Drosophila sex determination gene with multiple roles in development. *Genes Dev* 2(12A):1666–1676.
11. Lu H, et al. (2008) Maternal Groucho and bHLH repressors amplify the dose-sensitive X chromosome signal in Drosophila sex determination. *Dev Biol* 323:248–260.
12. Estes PA, Keyes LN, Schedl P (1995) Multiple response elements in the Sex-lethal early promoter ensure its female-specific expression pattern. *Mol Cell Biol* 15:904–917.
13. Yang D, et al. (2001) Interpretation of X chromosome dose at Sex-lethal requires non-E-box sites for the basic helix-loop-helix proteins SISB and daughterless. *Mol Cell Biol* 21: 1581–1592.
14. Deshpande G, Calhoun G, Schedl PD (1999) The N-terminal domain of Sxl protein disrupts Sxl autoregulation in females and promotes female-specific splicing of *tra* in males. *Development* 126:2841–2853.
15. Gallant P, Shiio Y, Cheng PF, Parkhurst SM, Eisenman RN (1996) Myc and Max homologs in *Drosophila*. *Science* 274:1523–1527.
16. Schreiber-Agus N, et al. (1997) Drosophila Myc is oncogenic in mammalian cells and plays a role in the diminutive phenotype. *Proc Natl Acad Sci USA* 94:1235–1240.
17. Johnston LA, Prober DA, Edgar BA, Eisenman RN, Gallant P (1999) *Drosophila* myc regulates cellular growth during development. *Cell* 98:779–790.
18. Kramer SG, Jinks TM, Schedl P, Gergen JP (1999) Direct activation of Sex-lethal transcription by the *Drosophila* runt protein. *Development* 126:191–200.
19. Jinks TM, Polydorides AD, Calhoun G, Schedl P (2000) The JAK/STAT signaling pathway is required for the initial choice of sexual identity in Drosophila melanogaster. *Mol Cell* 5:581–587.
20. Hoshijima K, et al. (1995) Transcriptional regulation of the Sex-lethal gene by helix-loop-helix proteins. *Nucleic Acids Res* 23:3441–3448.
21. Jinks TM, Calhoun G, Schedl P (2003) Functional conservation of the sex-lethal sex determining promoter, Sxl-Pe, in Drosophila virilis. *Dev Genes Evol* 213:155–165.
22. Blackwell TK, et al. (1993) Binding of myc proteins to canonical and noncanonical DNA sequences. *Mol Cell Biol* 13:5216–5224.
23. Kim JW, et al. (2004) Evaluation of myc E-box phylogenetic footprints in glycolytic genes by chromatin immunoprecipitation assays. *Mol Cell Biol* 24:5923–5936.
24. Teleman AA, Hietakangas V, Sayadian AC, Cohen SM (2008) Nutritional control of protein biosynthetic capacity by insulin via Myc in Drosophila. *Cell Metab* 7:21–32.
25. Rahl PB, et al. (2010) c-Myc regulates transcriptional pause release. *Cell* 141:432–445.
26. Deshpande G, Calhoun G, Jinks TM, Polydorides AD, Schedl P (2005) Nanos downregulates transcription and modulates CTD phosphorylation in the soma of early Drosophila embryos. *Mech Dev* 122:645–657.