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Multiple Response Elements in the *Sex-lethal* Early Promoter Ensure Its Female-Specific Expression Pattern

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The choice of sexual identity in somatic tissues of the fruit fly *Drosophila melanogaster* is determined early in embryogenesis by the X-chromosome-to-autosome (X/A) ratio. The system that signals the X/A ratio selects the sexual development pathway by determining the activity state of the binary switch *Sex-lethal* (*Sxl*). In 2X/2A animals, the X/A signalling system turns the *Sxl* gene on, ultimately activating an RNA-splicing autoregulatory feedback loop which serves to maintain the female state during the remainder of development. In 1X/2A animals, this autoregulatory feedback loop is not activated and the male state is subsequently maintained by the default splicing machinery. In the studies reported here, we have examined how the X/A signalling system controls the initial choice of sexual identity through its action on a special early embryonic *Sxl* promoter, *Sxl-Pe*. We show that in the early embryo, the activity of *Sxl-Pe* is controlled in a highly dose-sensitive fashion by the genes on the X chromosome that function as numerator elements and by genes located on the autosomes that function as denominator elements. Functional dissection of *Sxl-Pe* indicates that activating the promoter in females requires the cumulative action of multiple numerator genes which appear to exert their effects through reiterated *cis*-acting target sites in the promoter. Conversely, maintaining the promoter silent in males requires the repressive activities of denominator genes, and at least one of the denominator genes also appears to function through target sequences within the promoter.

In the fruit fly *Drosophila melanogaster*, the activity state of the master regulatory gene, *Sex-lethal* (*Sxl*), determines whether the fly follows the female or male developmental pathway (for reviews, see references 1, 20, 41, 42, 56, and 67). In the somatic cells of the fly, this activity state is set early in development in response to the ratio of X chromosomes to autosomes (X/A [16, 17, 46, 63]). Activation of the *Sxl* gene in 2X/2A animals initiates the female sexual development pathway, while the *Sxl* gene remains off in 1X/2A animals and the male developmental pathway is followed (12, 13, 16–18). Once the activity state of the *Sxl* gene has been selected, it plays a key role in subsequent sexual development by controlling somatic sexual differentiation (17) and dosage compensation (15, 16, 36, 48) and by maintaining the determined state (16, 49). In chromosomally female animals, loss-of-function mutations in *Sxl* result in lethality because of a failure to block the hyperactivation of the X chromosome by the dosage compensation system (15, 16, 36, 48). By contrast, these mutations have no phenotypic consequences in chromosomally male animals (59).

Even though *Sxl* is on in females and off in males, the gene is transcribed in both sexes throughout much of development and its on-off regulation is posttranscriptional, at the level of RNA splicing (3, 60). In males, *Sxl* RNAs expressed from the late or maintenance promoter, *Sxl-Pm*, are spliced to include the third exon (see Fig. 1 and references 3, 60, and 62), which contains in-frame stop codons that truncate the open reading frame. Consequently, only short, nonfunctional Sxl polypeptides are synthesized (8). In females, exon 2 is spliced directly to exon 4, bypassing the male-specific exon, and this generates *Sxl* mRNAs which have uninterrupted open reading frames. The Sxl proteins translated from the female mRNAs contain two domains showing homology to the RRM family of RNA-binding proteins (3), and a number of studies have shown that the Sxl proteins bind pre-mRNA and function as splicing regulators (3, 43, 61, 77). They autoregulate their own expression by directing the female-specific processing of Sxl pre-mRNAs transcribed from *Sxl-Pm* (2, 3, 62). This autoregulatory activity generates a positive feedback loop, and it provides the mechanism for remembering the female-determined state once it has been selected by the X/A signalling system. In males, in which no Sxl protein is present, the splicing machinery includes the male-specific exon in the mature transcripts, and the male state is maintained by default.

Since the female-specific splicing of *Sxl* transcripts requires Sxl protein, this raises the question of how the *Sxl* gene is initially activated in 2X/2A animals. Genetic studies have identified several components of the X/A signalling system that are likely to be involved in controlling the initial activation of the *Sxl* gene in the soma. On the X chromosome are the numerator elements which include *sisterless-a* (*sis-a* [18, 19, 33]), *sisterless-b* (*sis-b* [19, 32, 53, 74]), *sisterless-c* (*sis-c* [21]), and *runt* (30, 76). These genes are required in the zygote for counting the X chromosomes. Loss-of-function mutations in these genes interfere with the selection of the female pathway in 2X/2A animals but have no effect on the choice of the male pathway in 1X/2A animals. The requirement for these counting elements in females appears to be additive. Thus, defects in sex determination are observed not only in female embryos homozygous for either a *sis-a* or a *sis-b* mutation but also in females trans-heterozygous for mutations in these two genes (19). While loss-of-function mutations have no effect on male sex determination, female sexual identity can be inappropriately selected in males when extra copies of these genes are present (e.g., a duplication of both *sis-a* and *sis-b* [19, 33]). The second component of the signalling system consists of autosomal genes that function as denominator elements. Thus far, only one zygotically active denominator element has been identified, the *deadpan* (*dpn*) gene (80), located on the second chromosome. *dpn* acts in a reciprocal manner to the numerator

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genes on the X chromosome. When a loss-of-function mutation in *dpn* is combined with an extra dose of *sis-b*, 1X/2A animals inappropriately choose the female sex determination pathway. Conversely, 2X/2A animals inappropriately select the male pathway when extra copies of *dpn* are combined with a loss-of-function mutation in *sis-b* (80). The third component of the X/A signalling system consists of maternal genes such as *daughterless* (*da* [22, 23, 50]) and *extramachrochaetae* (*emc* [80]). As is the case for the numerator elements on the X chromosome, mutations in *da* interfere with the selection of the female pathway in 2X/2A animals but have no effect on the choice of the male pathway in 1X/2A animals (23). In contrast to the numerator elements, the *da* gene product is deposited in the egg during oogenesis, and it is the maternal, not the zygotic, genotype which is relevant to the sex determination process (14–16, 23). Consequently, *da* (and other maternal genes that promote the selection of the female pathway) is not an X chromosome-counting element, since its activity is not dependent upon the dose of the X chromosome in the zygote, but is rather a required cofactor (19). Mutations in *emc* have the opposite effect from those in *da*; they interfere with the choice of the male pathway in 1X/2A animals but have no effect on sex determination in 2X/2A animals (80). Like *da*, it is the maternal rather than the zygotic genotype of *emc* that is important in sex determination. However, unlike *da*, *emc* (and other maternal genes that promote the selection of the male pathway) could potentially function in a manner analogous to a zygotic autosomal counting element since the maternal contribution of this gene product to both male and female embryos is constant.

It is noteworthy that six of the identified components of the X/A signalling system display homologies to transcription factors. Specifically, the *da* (10, 24), *sis-b* (51, 79), *emc* (31, 35), and *dpn* (6) proteins share sequence homologies with the helix-loop-helix (HLH) family of transcription factors; *sis-a* is a member of the bZip family (33); and *runt* is thought to function as a transcription regulator in segmentation (37) and is homologous to a proto-oncogene responsible for a subtype of acute myeloid leukemia (25, 44). The sequence motifs found in these components of the X/A signalling system suggest that initiation of the *Drosophila* sex determination pathway is controlled at the level of transcription. Indeed previous studies have shown that the *Sxl* gene has a special promoter, *Sxl-Pe* (for embryonic, or establishment promoter), which is located within the first intron of the *Sxl-Pm* transcription unit (46). This promoter is active in early embryos and appears to be the target for the X/A signalling system: transcripts from *Sxl-Pe* accumulate in all somatic cells of female embryos (2X/2A) but are not found in somatic cells of male embryos (1X/2A). Moreover, *Sxl-Pe* provides the mechanism for bypassing the normal requirement for Sxl protein in directing the female-specific splicing of *Sxl* transcripts from the late promoter, *Pm*. Transcripts produced from the early promoter are spliced directly from the embryonic exon, E1, to exon 4, skipping exons 2 and 3 (reference 46, and see Fig. 1). Except for the N-terminal ~25 amino acids, the early transcripts encode proteins identical to the late female transcripts, including the two RRM RNA-binding domains. These embryonic proteins have autoregulatory activity and they function to direct the female-specific splicing of the first transcripts expressed from the late, *Sxl* maintenance promoter. This sets the autoregulatory feedback loop in motion. In 1X/2A animals, in which no embryonic Sxl protein is produced, the first transcripts from the late promoter, *Pm*, are spliced in the default male mode (46).

In the studies reported here, we have examined how *Sxl-Pe* functions in response to the X/A signalling system. We have defined the sequences necessary for sex-specific regulation of *Sxl-Pe* and have shown that this promoter contains multiple, redundant elements that help ensure an appropriate response to the X/A signalling system. We have also investigated how the different components of the X/A signalling system affect the activity of the promoter.

MATERIALS AND METHODS

Fly stocks. All mutations and chromosomes are described in reference 47. Briefly, the da^1 allele is a temperature-sensitive hypomorph with respect to the maternal sex determination function of *da* (11, 23); the $sis\text{-}b^{sc3\text{-}1}$ allele is temperature sensitive for sex determination but not proneural functions (32, 33, 55); the $sis\text{-}a^1$ allele is a lysine-to-glutamine substitution within the DNA-binding motif which greatly reduces its function (33); the *dpn* chromosome used contains the deficiency *Df(2L)193A*; and the $runt^{XA06}$ allele is null (37).

Subcloning. (i) *Sxl-Pe:lacZ* fusion constructs. All of the *Sxl:*β-*galactosidase* (*Sxl:lacZ*) constructs were generated by insertion of the *Sxl-Pe* sequences just upstream of the *lacZ* structural gene within the pCaSpeR-AUG-β-galactosidase vector (72). The *Sxl-Pe* sequences were derived from a 5,289-bp *Sal*I-*Xho*I genomic fragment (see reference 46) which contains the *Pe* transcription start site as well as 3,651-bp upstream and 1,629-bp downstream sequences. The 3′ ends of the *Sxl* sequences within each fusion gene are the *Bgl*II sites located 45 bp downstream of the *Sxl* early transcription start sites, while the 5′ ends varied as described below.

The $Sxl\text{-}Pe_{3kb}$*:lacZ* construct contains the *Sxl-Pe* fragment which extends from the *Bgl*II site to the *Xba*I site located 2,994 bp upstream (see Fig. 4). These *Sxl* *Xba*I-*Bgl*II sequences were also treated with exonuclease III (39) to produce the $Sxl\text{-}Pe_{2.5kb}$, $Sxl\text{-}Pe_{2.2kb}$, $Sxl\text{-}Pe_{2.0kb}$, $Sxl\text{-}Pe_{1.7kb}$, $Sxl\text{-}Pe_{1.4kb}$, and $Sxl\text{-}Pe_{1.1kb}$ promoter fragments (see Fig. 4). The 5′ endpoints were determined by the dideoxy sequencing method (64).

The $Sxl\text{-}Pe_{0.8kb}$*:lacZ*, $Sxl\text{-}Pe_{0.4kb}$*:lacZ*, and $Sxl\text{-}Pe_{0.2kb}$*:lacZ* reporter genes were made by fusing the 829-bp *Pst*I-*Bgl*II, 430-bp *Dra*I-*Bgl*II, and 245-bp *Bam*HI-*Bgl*II *Sxl* sequences, respectively, to the *lacZ* structural gene.

The $Sxl\text{-}Pe_{\Delta Bam}$*:lacZ* reporter gene was generated by digestion of the *Xba*I-*Bgl*II *Sxl-Pe* segment with *Bam*HI followed by blunt-end ligation to remove the internal 1,131-bp *Bam*HI fragment (see Fig. 4).

To produce the $Sxl\text{-}Pe_{\Delta BPdist}$*:lacZ* and $Sxl\text{-}Pe_{\Delta BPprox}$*:lacZ* constructs (see Fig. 4), the ~3.0-kb *Xba*I-*Bgl*II *Sxl-Pe* fragment was partially digested with *Bam*HI, blunt ended, and religated in order to eliminate one of the two *Bam*HI sites within *Sxl-Pe*. The resulting sequences were then digested with *Bam*HI and *Pst*I, blunt ended, and religated to eliminate one of the *Bam*HI-*Pst*I segments. This generated $Sxl\text{-}Pe_{\Delta BPdist}$*:lacZ*, in which the 543-bp distal *Bam*HI-*Pst*I fragment was deleted, and $Sxl\text{-}Pe_{\Delta BPprox}$*:lacZ*, in which the proximal 588-bp *Bam*HI-*Pst*I fragment was deleted.

(ii) *Sxl:bcd*. To fuse the *Sxl-Pe* sequences to the *bcd* structural gene, we first introduced the *bcd* coding sequences, which had been fused to the *globin* leader (bcdTN3 [29]) downstream of the *Bgl*II-*Xba*I 3.0-kb *Sxl-Pe* regulatory sequences. Next, to ensure proper processing, an 800-bp fragment derived from the 3′ untranslated sequences of the α-tubulin gene was inserted in a *Cla*I site located downstream of the *bcd* coding sequences. Finally, a *Not*I-*Kpn*I fragment containing the 5.8-kb *Sxl:bcd* fusion gene was introduced into the corresponding restriction sites within the *sws′* vector described below.

(iii) *Sxl:hb*. The *hb* coding sequences, flanked by 15-bp upstream and 520-bp downstream sequences (*Xba*I-*Eco*RI fragment [71]), which had formerly been fused to the 800-bp 3′ untranslated sequences of α-tubulin (68), were isolated on a *Kpn*I-*Xba*I fragment and then inserted downstream of a 3.7-kb (*Sal*I-*Bgl*II) *Sxl-Pe* fragment. The resultant 7.3-kb *Sxl:hb* fusion gene was removed by *Not*I-*Kpn*I digestion and inserted into the *Not*I-*Kpn*I sites within the *sws′* vector (see below).

(iv) The transformation vector for the *Sxl:bcd* and *Sxl:hb* fusion genes. For the introduction of the *Sxl:bcd* and *Sxl:hb* constructs into flies, we first designed a vector that would increase the probability of obtaining high expression levels of these transgenes. For this purpose, we generated the vector *sws′*, which both allows the amplification of the injected DNA and circumvents position effects. We began with the vector P[>w^{hs}>] (38), which is a modification of the P vector Carnegie 1 (58). This vector contains two copies of an FLP recombination target (FRT) that is derived from the yeast genome and provides site-specific recombination in the presence of the yeast enzyme FLP (a recombinase).

We modified the P[>w^{hs}>] vector to restore the mini-*white* (*w*) promoter in place of the *hsp70* promoter, since it provides a lower level of *w* expression initially and therefore provides a more sensitive measure of the copy number of the vector. In addition, we inserted both a *Not*I site and a *Kpn*I site just upstream of the mini-*w* promoter to facilitate the introduction of the *Sxl:bcd* and *Sxl:hb* fusion genes. Next, the sequences *scs* and *scs′*, derived from the *hsp70* locus (45), were inserted on either side of the FRT sequences. These sequences have been demonstrated to insulate the *w* reporter gene from chromosomal position effects which cause variability in expression levels. The *scs* sequences were isolated on a 1.8-kb *Cla*I fragment (45) and inserted into the unique *Bam*HI site within the vector. Next, the *scs′* sequences were isolated on a 500-bp *Bam*HI-*Eco*RI frag-

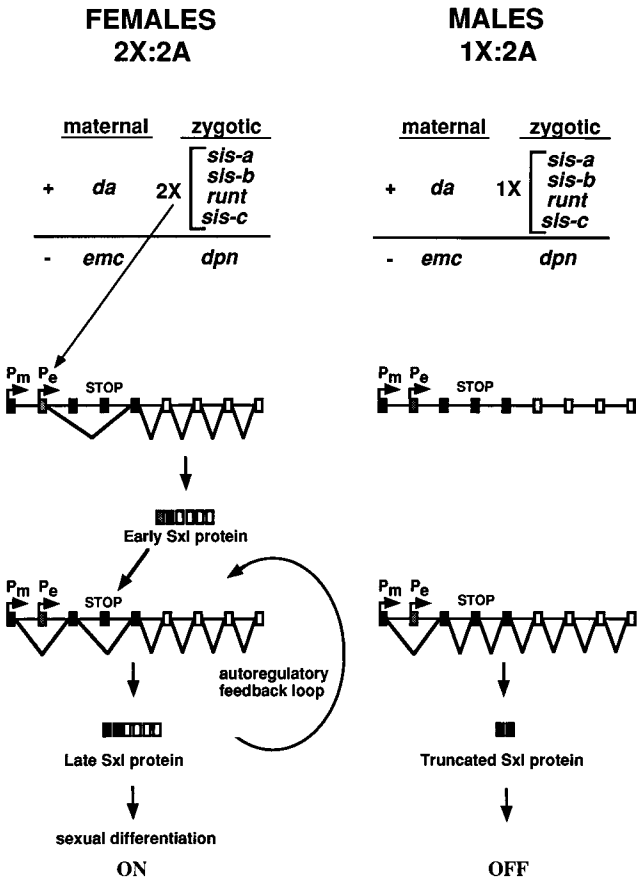

FIG. 1. Model for initiation of the sexual development pathway in *D. melanogaster*. During syncytial blastoderm formation, seven genes (*da*, *emc*, *sis-a*, *sis-b*, *sis-c*, *runt*, and *dpn*), which constitute the X/A signalling system, regulate *Sxl-Pe* in a sex-specific manner, resulting in the production of early *Sxl* RNA in female (shown to the left), but not male (shown to the right), embryos. The factors above the line at the top of the figure (*da*, *sis-a*, *sis-b*, *sis-c*, and *runt*) all have a positive influence on *Sxl-Pe*, as indicated by the plus sign, whereas *dpn* and *emc* have a negative influence on *Sxl-Pe*, as indicated by the minus sign. By cellular blastoderm formation, the late, constitutive promoter of *Sxl* (*Pm*) is activated in both sexes. The presence of the early Sxl protein in female embryos forces the late transcripts produced from *Sxl-Pm* to be spliced in the female mode, whereas in males containing no early Sxl protein, *Sxl* transcripts are spliced in the male, default mode. The female pathway is then maintained by an autoregulatory feedback loop in which functional Sxl protein regulates the sex-specific splicing of its own precursor RNA. The boxes represent *Sxl* exons, and the lines represent *Sxl* introns. The embryonic exon is indicated by the shaded box, and the exons that encode the RNA binding domains of the Sxl protein are indicated by the white boxes.

ment, the *Bam*HI site was changed to an *Eco*RI site with linkers, and then the fragment was inserted into the unique *Eco*RI site within the vector. The final vector consisted of these elements in the following order: P-*scs*-FRT-*Sxl* fusion gene-w^+-FRT-*scs'*-P. This vector was used to introduce the *Sxl:hb* and *Sxl:bcd* fusion genes into flies.

P-element-mediated transformation. We transformed the above DNAs, together with the helper plasmid P-turbo (pUChspΔ2-3wc [73]), into the germ line of either w^{1118} or w^1 *D. melanogaster* stock by standard procedures (57, 66).

β-Galactosidase staining. Embryos (4 to 7 h at 25°C) were stained overnight according to the procedure described in reference 4 or with the modification that the fixing solution consisted of 1 part phosphate-buffered saline–1 part 50% glutaraldehyde–2 parts heptane, instead of formaldehyde. *Sxl-Pe* is active during the syncytial blastoderm stage of development (33, 36, 46, 54), and β-galactosidase enzyme activity in the assay used here is first observed during the syncytial blastoderm stage, reaching maximal levels at 4 to 7 h. The stained embryos were counted and classified according to their β-galactosidase staining pattern: embryos were scored as dark if the staining pattern was a deep blue throughout the entire embryo; intermediate if the LacZ staining pattern was light blue and uniform; light if some regions of the embryo did, but other regions did not, stain; and negative if no blue stain was detectable. For each deletion construct, at least three lines were analyzed in a wild-type background. The levels of staining observed varied slightly between the different lines, probably because of position effects. *Sxl-Pe*$_{0.2kb}$*:lacZ* is the only reporter construct which showed differences in the percentage of positive-staining embryos among the various transgenic lines in a wild-type background (see Results). A representative line of each construct was selected for the analysis reported here.

TABLE 1. The *Sxl:bcd* and *Sxl:hb* fusion constructs cause female lethality

Sex and type	% Flies of indicated class recovered		
	Sxl:bcd[a]	*Sxl:hb*[b]	Vector[c]
Female			
P[w^+]	0.1	0.0	22.2
w^-	34.5	33.8	25.2
Male			
P[w^+]	34.1	32.9	24.9
w^-	31.3	33.3	27.6
Total[d]	1,468	2,125	2,055

[a] Cross (at 25°C; shown as females × males here and in footnotes *b* and *c*): w^{1118}/w^{1118}; +/+ × w^{1118}/Y; P[w^+; *Sxl:bcd*]/+.
[b] w^{1118}/w^{1118}; +/+ × w^{1118}/Y; P[w^+; *Sxl:hb*]/+.
[c] w^{1118}/w^{1118}; +/+ × w^{1118}/Y; P[w^+]/+.
[d] Total is the total number of flies examined in each cross.

RESULTS

Female-specific ectopic expression of developmental regulators driven by *Sxl-Pe*. In our model for the initiation of the *Drosophila* sexual development pathway shown in Fig. 1, the X/A signalling system controls pathway choice by activating the *Sxl* early promoter, *Pe*, in female but not in male embryos (46). Consistent with this hypothesis, we found that a genomic DNA segment extending ~3.0 kb upstream from the first exon of the embryonic transcript was able to direct expression of a *lacZ* reporter gene in 50% of the transgenic embryos. To provide more direct evidence that this promoter is active in female but not in male embryos, we attempted to disrupt early development specifically in females by fusing *Sxl-Pe* to cDNAs for two genes, the maternal morphogen *bicoid* (*bcd*) and the gap gene *hunchback* (*hb*). Both of these genes are essential for the normal development of head and thoracic structures. Maternal *bcd* RNA is deposited at the anterior of the egg during oogenesis (5, 34) and is translated after fertilization, generating an anterior-posterior concentration gradient of Bcd protein (26, 27). This gradient defines position along the anterior-posterior axis of the embryo by controlling the transcription of downstream zygotic genes like *hb* (28, 65, 70, 71).

Since the normal functions of both *bcd* and *hb* require that they be expressed only in the anterior of the embryo, the inappropriate expression of these proteins in other regions of the embryo should be deleterious, if not lethal. Thus, if *Sxl-Pe* is active in the somatic cells of female but not of male embryos, the *Sxl-Pe:bcd* and *Sxl-Pe:hb* fusion genes should have sex-specific effects on development. In the experiments presented in Table 1, transgenic males carrying either the *Sxl-Pe:bcd* or the *Sxl-Pe:hb* fusion gene were crossed to w^- females, and the resulting progeny were analyzed. For both transgenes, the number of *P[w^+]* males is essentially the same as the number of w^- male sibs lacking the transgene. In contrast, both transgenes have lethal effects on females. In the case of *Sxl-Pe:hb*, no surviving transgenic females were observed, while for *Sxl-Pe:bcd*, a few (~1 in 500) *P[w^+]* escaper females were found.

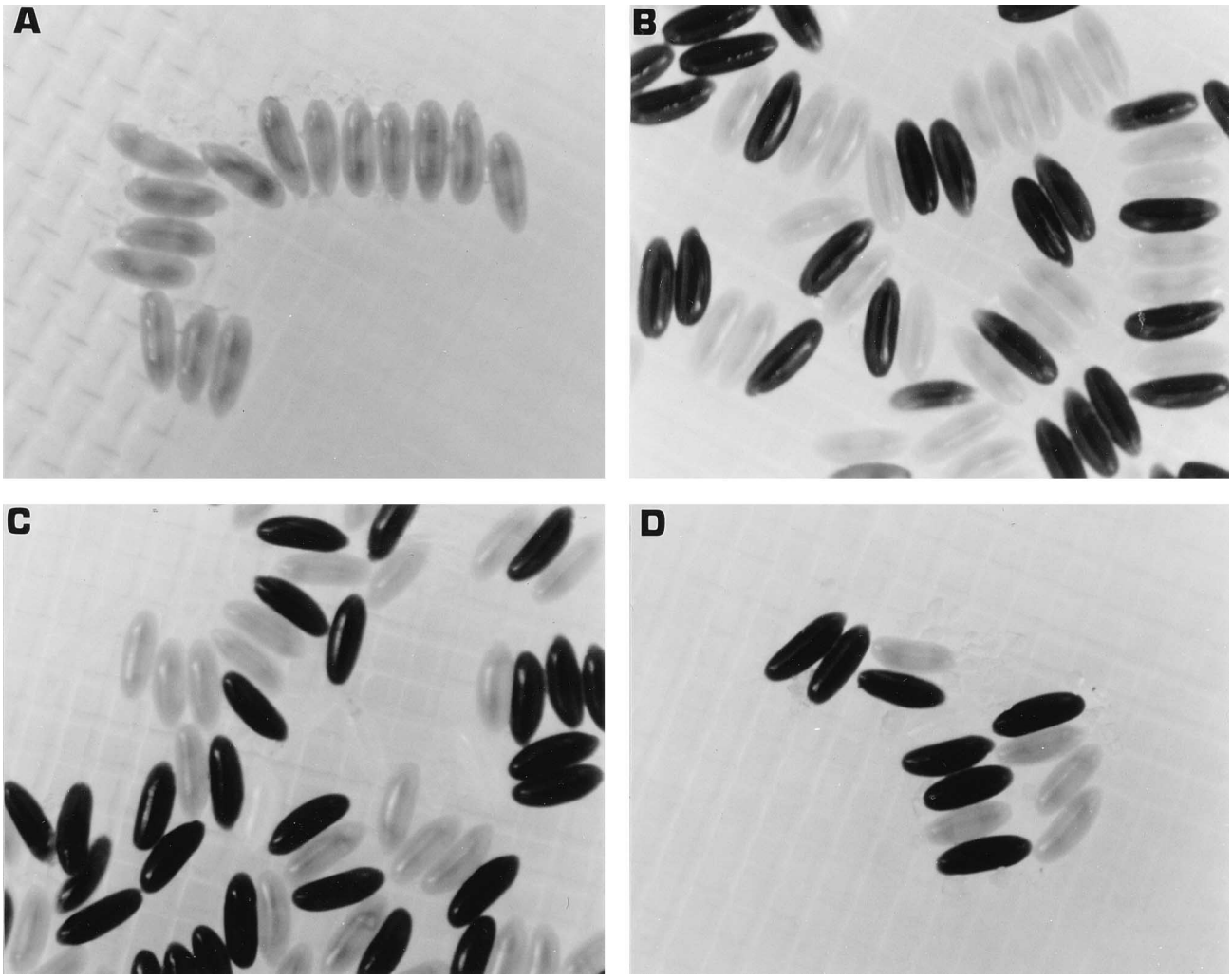

FIG. 2. da^{-}/da^{-} mothers produce embryos that fail to activate *Sxl-Pe*. The 3.0-kb *Sxl-Pe* fragment was placed upstream of the β-galactosidase reporter gene (*Sxl-Pe*$_{3kb}$*:lacZ*), and transgenic fly lines carrying this fusion gene were generated. Mothers homozygous (A) or heterozygous (B) for a da^{1} mutation were crossed to males homozygous for the *Sxl-Pe*$_{3kb}$*:lacZ* reporter gene, and the resultant embryos were assayed for β-galactosidase activity. A field of the stained embryos is shown. In the reciprocal experiment, fathers either heterozygous (C) or homozygous (D) for the da^{1} mutation were crossed to mothers homozygous for the *Sxl-Pe*$_{3kb}$*:lacZ* reporter gene, and the resultant embryos were assayed for the production of β-galactosidase.

To confirm that the sex-specific lethal effect of the *Sxl* fusion genes is the result of the inappropriate expression of *bcd* and *hb* during early embryogenesis, we examined the cuticles of embryos produced from these crosses. We found that approximately one-quarter of the embryos from the *Sxl-Pe:bcd* or the *Sxl-Pe:hb* cross exhibited a *nanos*$^{-}$-like phenotype (data not shown), similar to that observed by Struhl (68) after heat shocking animals carrying an *hsp70:hb* transgene. Taken together, these findings demonstrate that the embryonic promoter fragment from the *Sxl* gene directs female-specific transcription in early embryos.

While embryos containing the *Sxl-Pe:bcd* transgene exhibited a *nanos*$^{-}$-like phenotype, it was typically less severe than that of *Sxl-Pe:hb* embryos. The difference in phenotype may be due to the time during development when *Sxl-Pe* is active. Previous studies indicate that *Sxl-Pe*, like *hb*, is active during the syncytial blastoderm stages (33, 46, 65, 69, 71). By contrast, *bcd* protein first begins to accumulate soon after fertilization (27). Hence, misexpression of *bcd* from *Sxl-Pe* may occur too late for this protein to exert its maximal effect on the transcription of *hb* and other downstream targets.

***Sxl-Pe* activity depends upon the X/A signalling system.** In previous studies, we showed that the accumulation of the *Sxl* early mRNAs depends upon the maternal and zygotic genes implicated in signalling the X/A ratio (46). Consequently, it was important to determine if the X/A signalling system controls the choice of sexual identity by regulating the function of *Sxl-Pe*. For this purpose, we introduced the *Sxl-Pe*$_{3kb}$*:lacZ* reporter gene (in which the 3.0-kb *Sxl-Pe* fragment is fused to the β-galactosidase gene) into backgrounds in which the activity of one or more of the components of the X/A signalling system is altered and then examined early embryos for β-galactosidase expression. We used two criteria for assessing the affect of these genes. First, we examined the spatial distribution of the staining pattern within embryos (Fig. 2 and 3). Second, we classified the embryos on the basis of their staining patterns and calculated the percentage of embryos within each class (Tables 2 and 3).

The maternal product of the *da* gene is required for *Sxl-Pe* activity. The product of the *da* gene is a maternal component of the X/A signalling system, and it must be deposited in the egg for normal sex determination in the embryo (23). Females

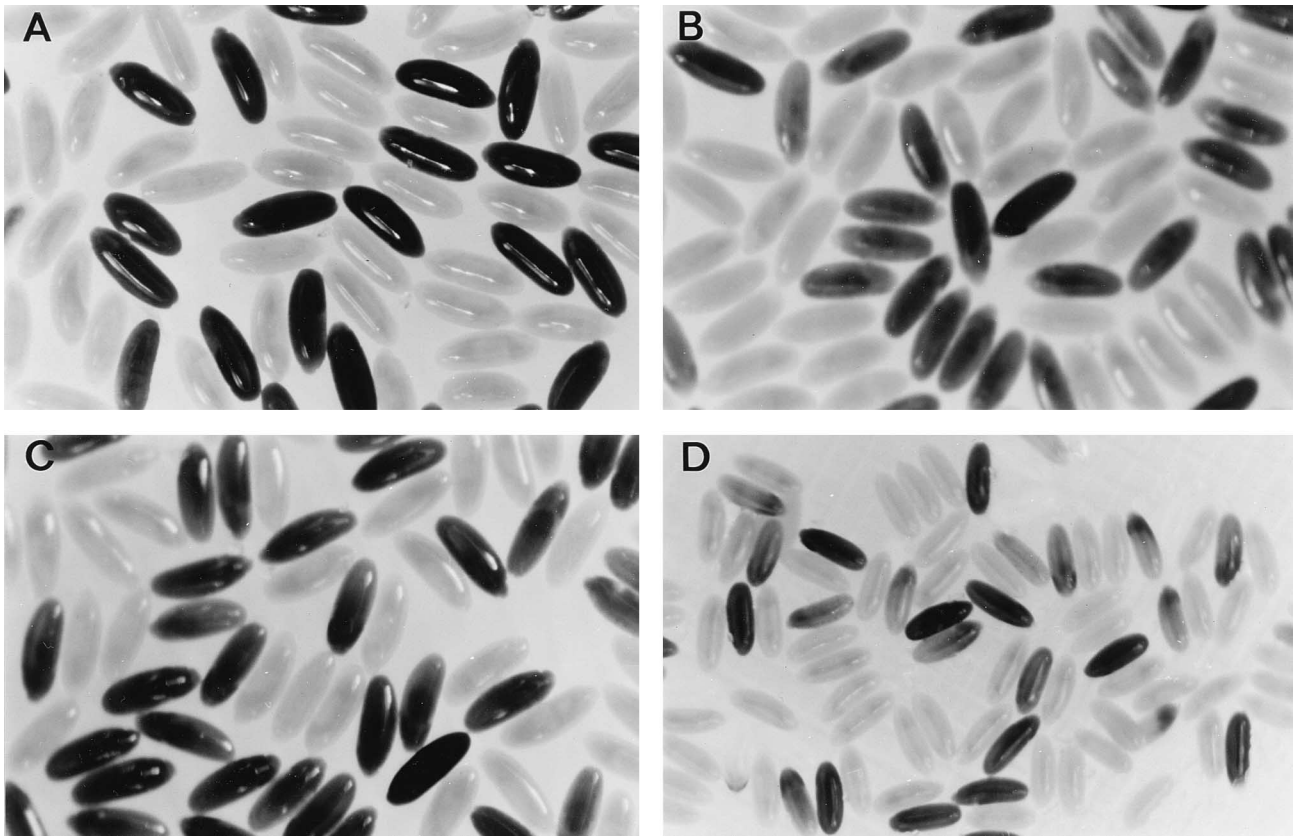

FIG. 3. The *Sxl-Pe*$_{3kb}$*:lacZ* reporter gene responds to *sis* mutations in a dose-sensitive manner. (A) Field of embryos containing one copy of the *Sxl-Pe*$_{3kb}$*:lacZ* reporter gene, stained for β-galactosidase activity in a wild-type background. Cross, *Sxl-Pe*$_{3kb}$*:lacZ*/*Sxl-Pe*$_{3kb}$*:lacZ* × +/+. (B) Embryos containing one copy of the *Sxl-Pe*$_{3kb}$*:lacZ* reporter gene assayed for β-galactosidase activity in a *sis-a*$^{-}$/$^{+}$ background. Cross, +/+; *Sxl-Pe*$_{3kb}$*:lacZ*/*Sxl-Pe*$_{3kb}$*:lacZ* × *y w sis-a*$^{-}$/*Y*; +/+. (C) Embryos containing one copy of the *Sxl-Pe*$_{3kb}$*:lacZ* reporter gene assayed for β-galactosidase activity in a *sis-b*$^{sc3\text{-}1}$/+ background. Cross, +/+; *Sxl-Pe*$_{3kb}$*:lacZ*/*Sxl-Pe*$_{3kb}$*:lacZ* × *sis-b*$^{sc3\text{-}1}$/*Y*; +/+. (D) Embryos containing one copy of the *Sxl-Pe*$_{3kb}$*:lacZ* reporter gene assayed for β-galactosidase activity in a *sis-a*$^{-}$/*sis-b*$^{-}$ transheterozygous background. Cross, *sis-a*$^{-}$/FM7; *Sxl-Pe*$_{3kb}$*:lacZ*/*Sxl-Pe*$_{3kb}$*:lacZ* × *sis-b*$^{sc3\text{-}1}$/*Y*; +/+. Female embryos produced from this cross will have one of two possible genotypes: (i) *sis-b*$^{sc3\text{-}1}$/FM7 or (ii) *sis-b*$^{sc3\text{-}1}$/*sis-a*. These different genotypes are reflected in the different staining patterns: approximately half of the positive embryos stain throughout the embryo (presumably the *sis-b*$^{sc3\text{-}1}$/FM7 embryos), and approximately half stain only in the anterior (presumably the *sis-b*$^{sc3\text{-}1}$/*sis-a* embryos).

homozygous for the mutant *da* allele, *da*^{1}, have no female offspring because of a failure to properly regulate the *Sxl* gene in the zygote. To test the role of maternal *da* in *Sxl-Pe* function, we crossed homozygous *da*^{1} females to males carrying two copies of the *Sxl-Pe*$_{3kb}$*:lacZ* reporter gene and stained the resultant progeny at 4 to 7 h of development. As a control, the reciprocal cross was carried out, i.e., females homozygous for the *Sxl-Pe*$_{3kb}$*:lacZ* reporter were crossed to homozygous *da*$^{-}$ males. The β-galactosidase staining pattern in the control cross was completely normal: 50% of the embryos expressed β-galactosidase (Table 2 and Fig. 2D). Likewise, the staining pattern of embryos derived from *da*^{1}-heterozygous fathers was normal (Table 2 and Fig. 2C). In contrast, none of the embryos produced by homozygous *da*^{1} mutant mothers stained for β-galactosidase (Table 2 and Fig. 2A). While the female progeny of mothers homozygous for the *da*^{1} mutation fail to activate the *Sxl-Pe*$_{3kb}$*:lacZ* reporter, no major effects on β-galactosidase expression were observed in progeny of mothers heterozygous for the *da*^{1} mutation: 50% stained uniformly, with roughly the same intensity as that observed in progeny from wild-type mothers (Fig. 2B and Table 2). These results demonstrate that the *da* gene product is required for the accumulation of the *Sxl* early transcripts (46) because it controls the activity of *Sxl-Pe*. Moreover, it appears that the *da* gene product exerts its regulatory effects on *Sxl* expression by acting on the 3.0-kb *Sxl-Pe* sequences.

TABLE 2. The *Sxl-Pe*$_{3kb}$*:lacZ* reporter gene responds to mutations in *da*

Genotype of[a]:		% Embryos with indicated β-galactosidase staining pattern[b]		Total no. of embryos examined
Mother	Father	Positive	Negative	
da/*da*	+/+	0	100	74
da/+	+/+	51	49	635
+/+	*da*/+	52	48	190
+/+	*da*/*da*	49	51	47

[a] Crosses (at 25°C; shown as mother × father for rows 1 through 4, respectively): *da*^{1}/*da*^{1} × *Sxl-Pe*$_{3kb}$*:lacZ*/*Sxl-Pe*$_{3kb}$*:lacZ*, *da*^{1}/*CyO* × *Sxl-Pe*$_{3kb}$*:lacZ*/*Sxl-Pe*$_{3kb}$*:lacZ*, *Sxl-Pe*$_{3kb}$*:lacZ*/*Sxl-Pe*$_{3kb}$*:lacZ* × *da*^{1}/*CyO*, and *Sxl-Pe*$_{3kb}$*:lacZ*/*Sxl-Pe*$_{3kb}$*:lacZ* × *da*^{1}/*da*^{1}.

[b] See Materials and Methods for the criteria used in the classification of the staining patterns.

The X chromosome numerator elements are required for *Sxl-Pe* activity. Genetic studies have shown that the three known zygotic components of the X/A signalling system which are located on the X chromosome, *sis-a*, *sis-b*, and *runt*, function in a dose-sensitive fashion to control the activation of *Sxl*. A reduction in the number of wild-type copies of one or more

TABLE 3. The *Sxl-Pe$_{3kb}$:lacZ* reporter gene responds to mutations in *sis* and *runt*

Genotype of[a]:		Temp (°C)	% Embryos with indicated β-galactosidase staining pattern[b]				Total[c]	Viability[d]
Mother	Father		Dark	Intermediate	Light	Negative		
sis-b/*sis-b*	*sis-b*/*Y*	18	31	16	2	51	128	100
		22	12	27	10	51	142	92
		25	8	12	31	49	115	15
		29	0	0	12	88	60	5
sis-a/+	*sis-a*/*Y*	25	14	16	20	50	320	
sis-a/+	*sis-b*/*Y*	25	4	16	33	47	338	
runt/+	+/*Y*	25	32	15		53	169	
runt/+	*runt*/*Y*^{Dp} (*runt*/+)	25	4[e]	29[e]	24[e]	43	227	

[a] Crosses (shown as mother × father for the five mating-type rows): *sis-b*$^{sc3-1}$/*sis-b*$^{sc3-1}$; *Sxl-Pe$_{3kb}$:lacZ*/*Sxl-Pe$_{3kb}$:lacZ* × *sis-b*$^{sc3-1}$/*Y*; *Sxl-Pe$_{3kb}$:lacZ*/*Sxl-Pe$_{3kb}$:lacZ* (first row), *y w sis-a*/*FM7*; *Sxl-Pe$_{3kb}$:lacZ*/*Sxl-Pe$_{3kb}$:lacZ* × *y w sis-a*/*Y*; *Sxl-Pe$_{3kb}$:lacZ*/*Sxl-Pe$_{3kb}$:lacZ* (second row), *y w sis-a*/*FM7*; *Sxl-Pe$_{3kb}$:lacZ*/*Sxl-Pe$_{3kb}$:lacZ* × *sis-b*$^{sc3-1}$/*Y*; +/+ (third row), *runt*^{XA06}/*FM6*; +/+ × +/*Y*; *Sxl-Pe$_{3kb}$:lacZ*/*Sxl-Pe$_{3kb}$:lacZ* (fourth row), and *runt*^{XA06}/*FM6*; +/+ × +/*YDp*$^{(mal126runt+)}$; *Sxl-Pe$_{3kb}$:lacZ*/+ (fifth row).

[b] See Materials and Methods for the criteria used in the classification of the staining pattern.

[c] Total indicates the total number of embryos examined.

[d] Viability is the percent *sis-b*$^{sc3-1}$-homozygous females recovered at the various temperatures relative to the permissive temperature (18°C).

[e] The percent positive embryos reported here was corrected (doubled), since only 1/2 of the embryos received the reporter gene from this cross.

of these genes can interfere with the activation of *Sxl* in female embryos. Conversely, the presence of extra copies of these genes in males can lead to inappropriate activation of *Sxl*. To test whether these genes function in sex determination by controlling the activity of *Sxl-Pe*, we introduced the *Sxl-Pe$_{3kb}$:lacZ* reporter into embryos in which the dose (or activity) of one or more of these X-chromosome-counting elements was altered.

sis-b. We tested the effect of the temperature-sensitive *sis-b* allele, *sis-b*$^{sc3-1}$, on the expression of β-galactosidase driven by the *Sxl-Pe$_{3kb}$:lacZ* transgene in early embryos. Previous genetic studies have shown that at 18°C, homozygous mutant *sis-b*$^{sc3-1}$ females exhibit no apparent defects in *Sxl* activation and are fully viable (32, 75). However, raising the temperature results in a progressive reduction in female viability and at 29°C, only 5% of the female offspring survive (Table 3).

We find that even at the permissive temperature, β-galactosidase expression from the *Sxl-Pe$_{3kb}$:lacZ* transgene is abnormal in homozygous mutant *sis-b*$^{sc3-1}$ embryos. Although a majority of the female embryos have near-wild-type levels of β-galactosidase expression at this temperature, a significant portion (32%) show moderate reductions in expression. Since *sis-b*$^{sc3-1}$ females are fully viable at 18°C, it appears that sufficient quantities of the early protein are produced to activate the autoregulatory feedback loop in these animals even though a reduction in *Sxl-Pe* activity can be detected in our assay. Although more extensive perturbations in β-galactosidase expression are evident at 22°C, the effects on female viability are also only marginal at this temperature (Table 3).

At 25°C, the level of *sis-b* activity in *sis-b*$^{sc3-1}$ embryos apparently falls below the critical threshold for activation of the *Sxl* autoregulatory feedback loop, resulting in a dramatic drop in female viability. This drop in female viability is correlated with a reduction in *Sxl-Pe* activity. As indicated in Table 3, only a small proportion of the embryos still show strong staining (8%), while there is a substantial increase in the fraction of embryos (31%) that show only light β-galactosidase expression. β-Galactosidase staining in the light embryos typically disappears in the posterior two-thirds, while staining tends to persist in the anterior one-third. Other embryos have only very light staining in the cephalic region. In spite of this very severe reduction in the level of β-galactosidase expression, the promoter is still active in 50% of the embryos. However, at 29°C, even more severe defects in *Sxl-Pe* activity are observed and a large proportion of the female embryos do not express detectable levels of β-galactosidase (only 12% of all of the embryos express β-galactosidase). These results indicate that the lethal effects of the *sis-b*$^{sc3-1}$ mutation result from a failure to properly activate *Sxl-Pe.*

sis-a. We also examined the effects of a *sis-a* mutation on β-galactosidase expression in transgenic embryos containing *Sxl-Pe$_{3kb}$:lacZ*. In the experiment presented in Table 3 (second row), two classes of female embryos are expected: those that are heterozygous (*sis-a*/*FM7*) and those that are homozygous for the *sis-a* mutation. As in the wild type, β-galactosidase staining could be detected in about 50% of the embryos. However, in contrast to the wild type, in which virtually all embryos express high levels of β-galactosidase, the staining in embryos from this cross was much more variable and the positive embryos could be grouped into three classes. Nearly half of the positive embryos had severely reduced levels of staining, and these presumably correspond to the *sis-a* homozygous mutants. The remaining positive embryos presumably correspond to the *sis-a* heterozygotes. About one-half were darkly stained, while the others showed intermediate levels of staining. These results suggest that like *sis-b*, the *sis-a* gene is required for *Sxl-Pe* activity in female embryos.

Sxl-Pe* activity is dependent on the dose of both *sis-b* and *sis-a. Previous studies by Cline (18, 19) showed that gene dose is critical in the functioning of *sis-a* and *sis-b* in sex determination. The effects of gene dose on the choice of female sexual identity can be most readily demonstrated when the system is sensitized by a mutation in the *Sxl* (or *da*) gene; thus, females heterozygous for a loss-of-function mutation in *Sxl* show a marked reduction in viability as the number of wild-type copies of either *sis-a* or *sis-b* is reduced from two to one. This disruption in female sex determination appears to be a direct consequence of the dependence of *Sxl-Pe* function on the dose of *sis-a* and *sis-b* genes. This can be demonstrated by reduction of the dose of either *sis-a* or *sis-b* in females carrying only a single copy of the *Sxl-Pe$_{3kb}$:lacZ* reporter (Fig. 3). When 2X animals have two copies of both *sis-a* and *sis-b*, female embryos (49% of the total) show a uniform pattern of β-galactosidase staining (Fig. 3A). However, when the 2X animals have only one functional copy of *sis-a* (Fig. 3B), the level of β-galactosidase expression is reduced and becomes somewhat heterogeneous in many of the female embryos. Similar, though not quite as dramatic, effects on *Sxl-Pe* activity are observed in females heterozygous for the temperature-sensitive *sis-b* allele *sis-b*$^{sc3-1}$ (Fig. 3C; this *sis-b* allele is hypomorphic and has a smaller effect on female viability than the *sis-a* mutation at 25°C). The sensitivity of *Sxl-Pe* to the dose of the numerator elements is further demonstrated by the substantial reductions in β-galactosidase expression observed in embryos transheterozygous for a mutation in both *sis-a* and *sis-b* (Fig. 3D and Table 3, third row).

runt. Genetic studies have suggested that the segmentation gene, *runt*, functions as a numerator element in sex determination (30, 76). Consistent with these studies, *runt* mutations

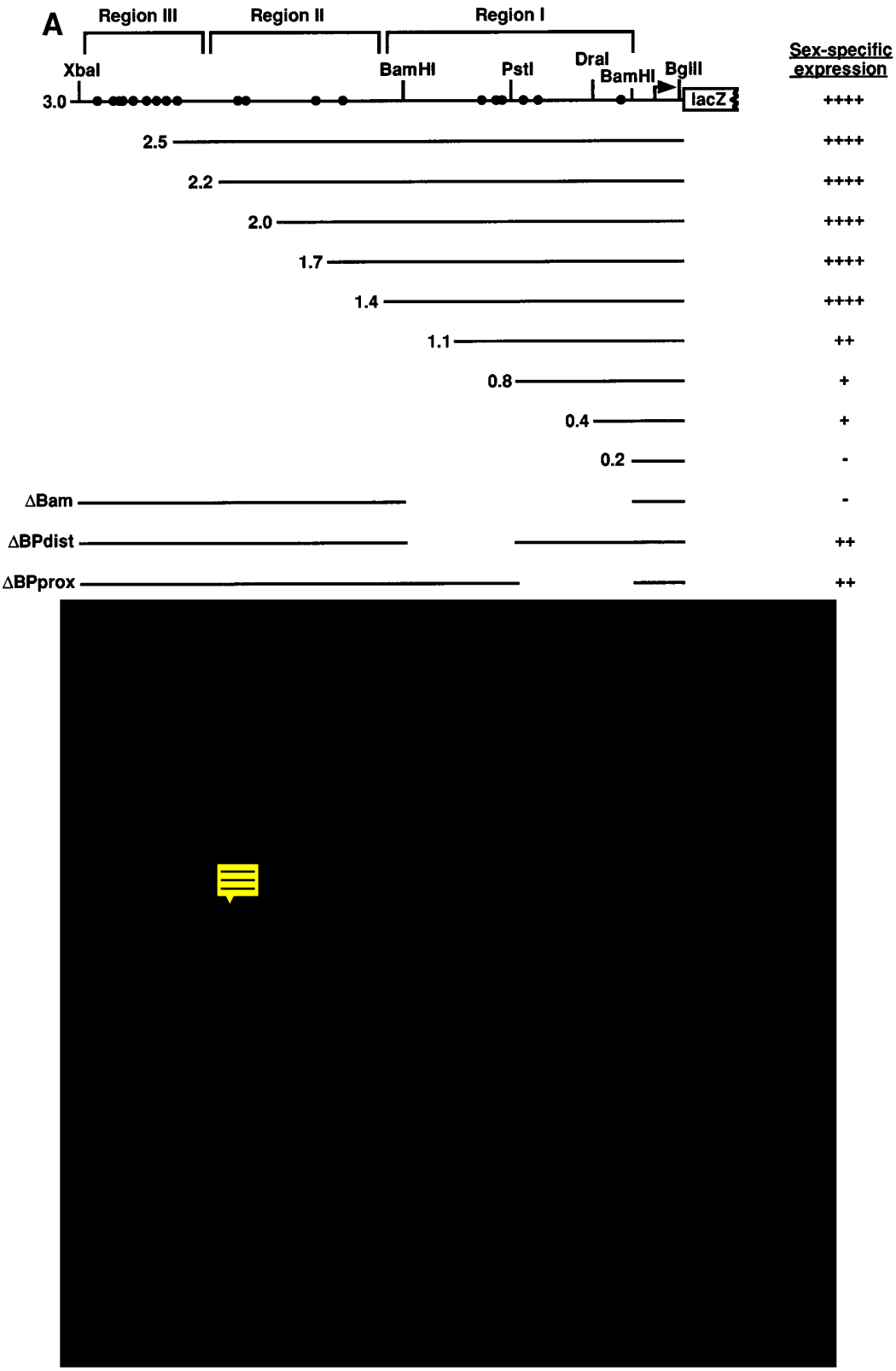

FIG. 4. *Sxl-Pe* deletions. (A) Schematic representation of the region located ~3.0 kb upstream of the *Sxl-Pe* transcription start site and the deletion constructs tested in these studies. Each fragment was fused to the β-galactosidase structural gene, and the resulting fusion constructs were introduced into flies. Embryos from the transformed lines were then assayed for sex-specific β-galactosidase expression. A summary of the results of this analysis obtained from embryos homozygous for the various reporter genes is shown to the right of each promoter construct. Four pluses indicate a dark staining pattern, two pluses indicate an intermediate staining pattern, one plus indicates a light staining pattern, and a minus indicates no staining. The circles represent the presence of the DNA sequence CANNTG, which is the consensus binding site for bHLH proteins. These sites are clustered into three regions, which are indicated above the 3.0-kb fragment. The restriction sites used to generate the constructs are indicated at the top. The designation for each deletion construct is indicated to the left. (B to I) Representative embryos assayed for β-galactosidase activity containing two copies of the *Sxl-Pe*$_{3.0kb}$*:lacZ* (B), *Sxl-Pe*$_{1.4kb}$*:lacZ* (C), *Sxl-Pe*$_{1.1kb}$*:lacZ* (D), *Sxl-Pe*$_{0.8kb}$*:lacZ* (E), *Sxl-Pe*$_{0.4kb}$*:lacZ* (F), *Sxl-Pe*$_{0.2kb}$*:lacZ* (line 7) (G), *Sxl-Pe*$_{\Delta BPdist}$*:lacZ* (H), and *Sxl-Pe*$_{\Delta BPprox}$*:lacZ* (I) reporter genes are shown.

TABLE 4. The region 1.4 kb upstream of the transcription start site of *Sxl-Pe* is important for sex-specific early expression

Identity of *Sxl-Pe* fragment[a]	% Embryos with indicated β-galactosidase staining pattern				Total no. of embryos examined
	Dark	Intermediate	Light	Negative	
3.0	49			51	8,151
1.4	50			50	671
1.1	1	41	6	52	1,832
0.8	1	23	26	51	2,262
0.4	3	21	27	50	924
0.2 (line 2)[b]				100	366
0.2 (line 3)[b]			78	23	939
0.2 (line 7)[b]			100		548
ΔBam				100	332
ΔBP_{dist}		44	7	50	709
ΔBP_{prox}	1	31	17	52	371

[a] Cross (at 25°C): *Sxl-Pe:lacZ*/*Sxl-Pe:lacZ* × *Sxl-Pe:lacZ*/*Sxl-Pe:lacZ*. Numerical values identify the fragments by size (in kilobases). With regard to sizes of the *Sxl-Pe* fragment, see Fig. 4.

[b] For the *Sxl-Pe*$_{0.2kb}$*:lacZ* construct, the results obtained with three independent transformed lines varied considerably and are shown here.

TABLE 5. Mutations in *sis-a* or *sis-b* reduce expression from the various *Sxl-Pe:lacZ* reporter genes

Size of *Sxl-Pe* (kb)	Background[a]	% Embryos with indicated β-galactosidase staining pattern				Total no. of embryos examined
		Dark	Intermediate	Light	Negative	
3.0	+	6	43		50	1,182
	sis-a		9	44	47	1,091
	sis-b		16	27	57	225
1.4	+		44	3	53	466
	sis-a			49	51	273
	sis-b		2	37	61	188
1.1	+			49	51	561
	sis-a			19	80	592
	sis-b			29	71	152
0.8	+		2	45	53	855
	sis-a			2	98	687
	sis-b		1	32	67	397
0.4	+		5	42	52	391
	sis-a			14	86	524
	sis-b			8	92	271

[a] Crosses (at 25°C, shown as females × males): +/+; *Sxl-Pe:lacZ*/*Sxl-Pe:lacZ* × w^{1118}/*Y*; +/+ (+), +/+; *Sxl-Pe:lacZ*/*Sxl-Pe:lacZ* × *y w sis-a*/*Y*; +/+ (*sis-a*), and +/+; *Sxl-Pe:lacZ*/*Sxl-Pe:lacZ* × *sis-b*$^{sc3-1}$/*Y*; +/+ (*sis-b*).

affect *Sxl-Pe* activity. In the experiment shown in Table 3 (fourth row), we examined the effects of reducing the number of wild-type copies of the *runt* gene in female embryos on the activity of the *Sxl-Pe*$_{3kb}$*:lacZ* reporter. As was the case for *sis-a* and *sis-b*, *Sxl-Pe* function is sensitive to the dose of the *runt* gene, and nearly one-third of the female embryos (15% of the total number of embryos) had a reduced and heterogeneous pattern of β-galactosidase expression. Typically, the polar regions of these embryos stained more intensely than the central regions. In the cross used in the second experiment (Table 3, fifth row), female progeny have either no doses or one dose of the *runt* gene. Approximately one-fourth of the embryos containing the *Sxl-Pe*$_{3kb}$*:lacZ* reporter, which presumably correspond to females homozygous for the *runt* mutation, had grossly abnormal β-galactosidase expression patterns with little or no staining in the central region (data not shown). The lack of staining in the central region of the embryo correlates well with the distribution of Sxl protein in *runt* embryos (30). Thus, like *sis-a* and *sis-b*, the *runt* gene functions as a dose-dependent positive regulator of *Sxl-Pe.*

***dpn*$^-$ activates the promoter.** While decreasing the dose of the X-linked zygotic genes *sis-a*, *sis-b*, and *runt* reduces *Sxl-Pe* activity, the reciprocal effect is observed when the dose of the autosomal zygotic gene *dpn* is reduced. When males heterozygous for a *dpn* mutation are crossed to females carrying the *Sxl-Pe*$_{3kb}$*:lacZ* reporter, instead of 50%, more than 60% of the resultant embryos express β-galactosidase (3.0 kb; see Table 6). This increase in the percentage of embryos expressing β-galactosidase most likely reflects the activation of *Sxl-Pe* in about half of the males that inherit the *dpn* mutation. In addition, we find that staining in many of the embryos is considerably more intense than that typically observed in embryos carrying only a single copy of the reporter. These results indicate that *dpn* normally functions as a dose-dependent negative regulator of *Sxl-Pe.*

Functional dissection of *Sxl-Pe.* Of the *trans*-acting factors involved in controlling *Sxl-Pe* activity, the DNA sequence specificity of the two basic HLH (bHLH) proteins, Da and Sis-b, is known (7, 51, 52). These proteins recognize a minimal consensus sequence: CANNTG. As indicated in Fig. 4A, the *Sxl-Pe*$_{3kb}$ promoter fragment contains 19 sites that match this bHLH consensus sequence, and they are arranged in three major clusters. The first cluster (I) is at the proximal end of the promoter fragment and contains six bHLH sites. The second (II), in the middle third of the promoter fragment, contains 4 sites, while the third (III) is at the very distal end of the 3.0-kb promoter and contains nine sites, or nearly half of the total. The reiteration of binding sites for regulatory factors could potentially explain the dose sensitivity of the *Sxl-Pe*$_{3kb}$ promoter. Consequently, it was of interest to ascertain whether *Sxl-Pe* activity is dependent upon the number or position of these reiterated bHLH binding sites or whether other sequences in *Sxl-Pe*$_{3kb}$ are important for the sex-specific activity of *Sxl-Pe*. We generated a series of deletions within the 3.0-kb promoter fragment (Fig. 4A). Each deletion variant was fused to the *lacZ* reporter gene and introduced into flies. The resultant transgenic animals were then examined for β-galactosidase expression.

Regions II and III are not required for *Sxl-Pe* function. The deletions in the constructs *Sxl-Pe*$_{2.5kb}$*:lacZ* and *Sxl-Pe*$_{2.2kb}$*:lacZ* remove either part or all of region III at the distal end of *Sxl-Pe*$_{3kb}$. In spite of the fact that nearly half of the reiterated bHLH consensus sequences are clustered in region III, neither of these deletions has any apparent effect on *Sxl-Pe* function. β-Galactosidase is expressed in 50% of the embryos, and both the level and spatial distribution of β-galactosidase staining (data not shown) are indistinguishable from those observed with the starting 3.0-kb promoter fragment (Fig. 4B). These results suggest that the clustered bHLH sites in region III (as well as any other potential factor-binding sites in this DNA segment) are not required for full *Sxl-Pe* activity.

Quite similar results were obtained for the constructs *Sxl-Pe*$_{2.0kb}$*:lacZ*, *Sxl-Pe*$_{1.7kb}$*:lacZ*, and *Sxl-Pe*$_{1.4kb}$*:lacZ* (Tables 4 and 5 and Fig. 4C). In these, the upstream deletion removes not only all of region III but also part or all of region II. The activities of the two larger reporter constructs appear to be essentially identical to that of the *Sxl-Pe*$_{3kb}$, while the *Sxl-Pe*$_{1.4kb}$*:lacZ* reporter shows a slight reduction in activity (see

FIG. 5. *Sxl-Pe* deletion constructs respond to mutations in the numerator and denominator genes. Representative embryos containing the *Sxl-Pe*$_{1.4kb}$*:lacZ*, *Sxl-Pe*$_{1.1kb}$*:lacZ*, *Sxl-Pe*$_{0.8kb}$*:lacZ*, and the *Sxl-Pe*$_{0.4kb}$*:lacZ* (leftmost to rightmost columns, respectively) reporter constructs in different genetic backgrounds were stained for β-galactosidase activity. The results obtained from embryos containing one copy of the reporter gene (first row) or embryos containing one copy of the reporter gene together with a single *sis-a*$^{-}$ (second row), *sis-b*$^{vc3\text{-}1}$ (third row), or *dpn*$^{-}$ (fourth row) mutation are shown.

below). Thus, it appears that regions II and III (which together contain 13 of the 19 bHLH sites) are not required for full promoter function in a background that is wild type for the known components of the X/A signalling system.

Region I contains sequences essential for full *Sxl-Pe* function. Deletions which extend into region I cause a reduction in promoter function. For example, *Sxl-Pe*$_{1.1kb}$*:lacZ* (Fig. 4D) shows a significant reduction in promoter strength relative to either the starting *Sxl-Pe*$_{3kb}$*:lacZ* or the *Sxl-Pe*$_{1.4kb}$*:lacZ* construct, although it still appears to be active in all female embryos (Tables 4 and 5). Not only is the β-galactosidase staining much less intense in embryos carrying one copy of the 1.1-kb construct, but it is also unevenly distributed (see Fig. 5). In most embryos, there is an anterior stripe of staining with patches on the ventral side and at the posterior. Moreover, as indicated in Table 4, this reduction in promoter activity is also evident when female embryos have two copies of the transgene. In this case, instead of the dark staining observed with either the 3.0- or the 1.4-kb construct, nearly half of the embryos show intermediate (Fig. 4D) or greatly reduced levels of staining.

Further reductions in promoter activity are evident with *Sxl-Pe*$_{0.8kb}$*:lacZ*. Although this 0.8-kb promoter fragment also appears to retain sex specificity (~50% have detectable β-galactosidase staining), its activity is substantially reduced, even in comparison with that of *Sxl-Pe*$_{1.1kb}$*:lacZ*. As illustrated in Fig. 5, we typically observe only very weakly stained patches in the anterior of embryos containing a single copy of this transgene. With two copies of the transgene (Fig. 4E), staining is somewhat darker but still quite heterogeneous. Taken together, these findings suggest that the 0.6-kb DNA segment located between 1.4 and 0.8 kb upstream of the transcription start site contains *cis*-acting elements critical for full *Sxl-Pe* function.

While *Sxl-Pe*$_{0.8kb}$*:lacZ* appears to have much less activity than *Sxl-Pe*$_{1.1kb}$*:lacZ*, this is not true for the next deletion construct, *Sxl-Pe*$_{0.4kb}$*:lacZ* (Fig. 4F). Though this construct has only a 0.4-kb fragment from *Sxl-Pe*, β-galactosidase expression is not only sex specific but is also consistently higher than that observed for the 0.8-kb construct. As illustrated in Fig. 5, we typically observe relatively strong staining in the anterior and along the ventral surface in embryos containing a single copy of the 0.4-kb construct. A plausible interpretation of these results is that the balance between the remaining positive and negative *cis*-acting elements in *Sxl-Pe*$_{0.4kb}$*:lacZ* favors promoter activation. By contrast, in the 0.8-kb construct, the ratio of positive and negative *cis*-acting elements in the *Sxl-Pe* fragment favors promoter repression. Moreover, these findings suggest that *Sxl-Pe* is a composite of multiple positive and negative *cis*-acting elements that together determine the level of promoter activity.

The minimal ~0.2-kb *Sxl-Pe* does not show sex-specific expression. The final construct in this deletion series is *Sxl-Pe*$_{0.2kb}$*:lacZ*. β-Galactosidase expression from this construct is quite position dependent, and in one of the transgenic lines (line 2) no staining was evident even with two copies of the transgene (Table 4). In the second line (line 3), a low level of β-galactosidase expression could be detected in the anterior and on the dorsal side of the embryo when there were two copies of the insert. However, instead of 50% of the embryos expressing β-galactosidase, over 75% showed staining (Table 4). When only a single copy of the transgene is present, fewer than 1% of the embryos show detectable expression. In the third line (line 7), very weak β-galactosidase staining could be

detected in all embryos with either one or two copies of the transgene (Table 4 and Fig. 4G). These results show that although this 0.2-kb DNA segment has at least some residual promoter activity, it is no longer sex specific. Presumably, sequences critical for sex specificity have been removed in this construct.

Internal deletions map sequences essential for *Sxl-Pe* activity to region I. While the results described in the previous sections indicate that regions II and III can be deleted without significantly impairing *Sxl-Pe* function in a wild-type background, it could be argued that these two DNA segments may contain elements (e.g., the reiterated bHLH sites) that are functionally redundant with elements in region I. To test this possibility, we deleted region I from the 3.0-kb *Sxl-Pe* fragment (construct ΔBam), thereby fusing regions II and III directly to the minimal *Sxl-Pe* (construct *0.2kb*). In spite of the fact that this ΔBam construct retains 13 of the 19 bHLH consensus sequences in *Sxl-Pe*, it has no promoter activity. This finding indicates that the sequences in regions II and III are not in themselves sufficient to confer *Sxl-Pe* function and, given the result described above, may contribute little to the sex-specific activity of *Sxl-Pe.*

Next, we tested the effect of internal deletions within region I of the 3.0-kb *Sxl-Pe* sequences. As shown in Fig. 4A, we generated two deletions, one that removed the distal region and a second that removed the proximal section of region I. As anticipated from the previous findings, both deletions significantly impaired *Sxl-Pe* function; however, the sex specificity is retained and 50% of the embryos express β-galactosidase (Table 4). The distal deletion appears to have a somewhat smaller effect on promoter activity than the proximal deletion (see the staining distribution with two copies of the transgene in Table 4). This is also illustrated in Fig. 4H and I, which show the β-galactosidase expression pattern in embryos carrying two copies of each internal deletion. β-Galactosidase expression in the distal deletion (Fig. 4H) is stronger in the anterior and weaker in the posterior of the embryo. The staining pattern obtained for the proximal deletion is further reduced relative to the distal deletion (Fig. 4I). These findings provide additional evidence that key regulatory elements for *Sxl-Pe* function are located within region I.

Enhanced sensitivity of the *Sxl-Pe* deletions to reductions in the dose of *sis-a* or *sis-b*. The results described above indicate that the promoter activity of *Sxl-Pe$_{3kb}$:lacZ* is dependent on the dose of *sis-a* and *sis-b*. As might be expected if *Sxl-Pe* is a composite of reiterated positive *cis*-acting elements, the progressive deletion of DNA sequences from *Sxl-Pe* is correlated with an increased sensitivity to reductions in the dose of X-chromosomal counting elements. Interestingly, however, the various deletion constructs responded somewhat differently to mutations in *sis-a* and *sis-b.*

As indicated in Table 5, *sis-a* or *sis-b^{sc3-1}* mutations reduce the promoter activities of both the 3.0- and 1.4-kb constructs. While both still make the appropriate on/off choice, the predominant staining pattern of the female embryos shifts from intermediate to light. The 1.4-kb construct appears to be slightly more sensitive to a *sis-b* mutation than the starting *Sxl-Pe$_{3kb}$:lacZ.*

While a *sis-a* mutation has essentially the same effect on the promoter activity of *Sxl-Pe$_{1.4kb}$:lacZ* as it does on *Sxl-Pe$_{3kb}$:lacZ*, this is not the case for the 1.1-kb construct. As can be seen in Table 5, only 19% rather than 50% of the *Sxl-Pe$_{1.1kb}$* embryos express detectable levels of β-galactosidase, even when they are heterozygous for a *sis-a* mutation. In the remainder of the embryos, only very weak β-galactosidase staining is typically observed (Fig. 5). In contrast, *Sxl-Pe$_{1.1kb}$:lacZ* appears to be active in virtually all females when there are two wild-type copies of *sis-a* (Fig. 5). This observation suggests that the positive *cis*-acting elements remaining in the 1.1-kb fragment are not sufficient to activate the promoter in all female embryos when the number of wild-type copies of *sis-a* is reduced from two to one. Reducing the dose of *sis-a* has even more dramatic effects on the promoter activity of the 0.8-kb construct (Table 5 and Fig. 5). In this case, β-galactosidase expression is observed in only 2% of the embryos, indicating that the sex-specific promoter activity of the 0.8-kb construct is critically dependent upon wild-type levels of *sis-a.*

A reduction in the dose of *sis-b* also affects the promoter function of the 1.1- and 0.8-kb constructs. *Sxl-Pe$_{1.1kb}$:lacZ* contains all six bHLH consensus sequences present in the larger *Sxl-Pe$_{1.4kb}$:lacZ* construct (region I) and hence might be expected to respond to alterations in *sis-b* activity much as does *Sxl-Pe$_{1.4kb}$:lacZ*. However, it is more sensitive to a reduction in *sis-b* activity than the 1.4-kb construct, and only about 29% of the embryos (58% of the female embryos) express detectable levels of β-galactosidase (Table 5). The embryos which do stain stain very lightly (Fig. 5). In the *Sxl-Pe$_{0.8kb}$:lacZ* construct, three of these bHLH consensus sequences found in region I are deleted. Hence, this promoter might be expected to be considerably more sensitive to reductions in *sis-b* activity than the *Sxl-Pe$_{1.1kb}$:lacZ* construct, which retains all six region I bHLH sites. Surprisingly, the effects of the *sis-b* mutation on the promoter activity of the *Sxl-Pe$_{0.8kb}$:lacZ* construct are even less pronounced than those on the promoter activity of the *Sxl-Pe$_{1.1kb}$:lacZ* construct (Table 5). In this context, it is interesting to note that the *sis-b* mutation has a much smaller effect on either the 1.1- or the 0.8-kb construct than the *sis-a* mutation (Table 5). This difference is particularly noticeable in embryos carrying the *Sxl-Pe$_{0.8kb}$:lacZ* construct, in which β-galactosidase expression is almost eliminated by the *sis-a* mutation.

In a wild-type background, *Sxl-Pe$_{0.4kb}$:lacZ* has a consistently higher level of β-galactosidase expression than does *Sxl-Pe$_{0.8kb}$:lacZ* (Fig. 5). Interestingly, it is also less sensitive to a reduction in the dose of *sis-a*. Instead of only 2%, 14% of the embryos express β-galactosidase. While the level of β-galactosidase expression from the 0.4-kb *Sxl-Pe* fragment is clearly higher in a *sis-a*/+ background than that of *Sxl-Pe$_{0.8kb}$*, this is not true in a *sis-b*/+ background. In a *sis-b* mutant background, the promoter activity of the 0.4-kb construct is markedly reduced relative to that of the 0.8-kb construct and only about 8%, instead of 33%, of the embryos express β-galactosidase. It is conceivable that the *sis-b* mutation has a more pronounced effect on the activity of *Sxl-Pe$_{0.4kb}$* than on those of some of the other constructs because this *Sxl-Pe* fragment retains only one of the region I bHLH consensus sequences. Taken together, these findings suggest that the activity of *Sxl-Pe* in the 0.8-kb construct is more dependent upon wild-type levels of *sis-a* than that of the promoter fragment in the 0.4-kb construct. Conversely, the activity of the 0.4-kb promoter fragment appears to be more dependent upon wild-type levels of *sis-b* than that of the 0.8-kb promoter fragment.

Reducing the dose of *dpn* activates the truncated promoters. Mutations in the autosomal *dpn* gene have two effects on the promoter activity of *Sxl-Pe$_{3kb}$:lacZ*. First, the mutation markedly enhances the level of β-galactosidase expression in females, and second, it appears to activate the promoter in more than one-quarter of the males (Table 6). Less-extensive alterations in promoter activity are observed for the various deletion constructs. The 1.4-kb construct, for example, shows a much less pronounced increase in the level of β-galactosidase expression in female embryos, and the promoter appears to be

TABLE 6. *dpn* or a duplication of both *sis-a* and *sis-b* increases β-galactosidase expression from the *Sxl-Pe:lacZ* reporter gene

Size of *Sxl-Pe* (kb)[a]	Background[b]	% Embryos with indicated β-galactosidase staining pattern				Total no. of embryos counted
		Dark	Intermediate	Light	Negative	
3.0	+[c]	6	43		50	1,182
	dpn	59	4		37	315
	DD(2)Ha	18	12	39	31	320
1.4	+[c]		44	3	53	466
	dpn	9	30	17	43	441
	DD(2)Ha	28	33	8	31	263
1.1	+[c]			49	51	561
	dpn		10	47	43	279
	DD(2)Ha		16	42	43	253
0.8	+[c]		2	45	53	855
	dpn		17	38	45	488
	DD(2)Ha		12	9	78	301
0.4	+[c]		5	42	52	391
	dpn	2	1	50	47	454
	DD(2)Ha		11	20	70	277

[a] See Fig. 4 with regard to variation in sizes.

[b] Crosses (at 25°C; shown as females × males for backgrounds as indicated parenthetically): +/+; *Sxl-Pe:lacZ*/*Sxl-Pe:lacZ* × w^{1118}/*Y*; +/+ (+), *Sxl-Pe:lacZ*/*Sxl-Pe:lacZ* × *Df(193A)*/*CyO* (*dpn*), and +/+; *Sxl-Pe:lacZ*/*Sxl-Pe:lacZ* × *y w Df(1)N71*, *sis-a*$^-$/*Y*; *DD(2)Ha*/+ [*DD(2)Ha*, which is a second chromosome carrying two duplicated regions of the X, one including *sis-a*$^+$ and the other including *sis-b*$^+$ (see reference 19)].

[c] Values in this row are taken from Table 5 and are included here for comparison.

activated in only about 15% of the males. In addition, the deletion constructs appear to be differentially sensitive to the *dpn* mutation. Thus, the enhancement in the level of promoter activity in female embryos by the *dpn* mutation is greater for the 0.8-kb construct than it is for the 1.1-kb construct (compare wild-type and *dpn*$^-$ embryos in Fig. 5). Presumably, this reflects the relative balance of positive and negative *cis*-acting elements that remain in the different deletion constructs (see above).

Duplication of *sis-a* and *sis-b* activates *Sxl-Pe*. The presence of extra copies of the X-chromosomal genes *sis-a* and *sis-b* can lead to the inappropriate choice of female sexual identity in 1X/2A animals (19). This choice of the female pathway appears to be due to the activation of *Sxl-Pe* in the 1X/2A embryos (19, 33). In the experiment shown in Table 6, males carrying a duplication of both *sis-a* and *sis-b* on one of their second chromosomes [*DD(2)Ha*] were crossed to females carrying the different *Sxl-Pe* deletion constructs (see Table 6 for details of this cross). These males are able to tolerate a duplication of both *sis-a* and *sis-b* on their second chromosome only because their X chromosome contains a deficiency for *sis-a*: *Df(1)N71*. In this cross, half of the male progeny receive the *DD(2)Ha* chromosome and therefore have two copies of both *sis-a* and *sis-b* [one copy each from the X and *DD(2)Ha* chromosomes], while the remaining males are wild type and have only one copy of each gene (from the X chromosome). All of the female progeny receive an X from their fathers and therefore receive the *Df(1)N71* X chromosome, which has a deletion of *sis-a*. Half of the female progeny will also inherit the *DD(2)Ha* chromosome, and they will have three wild-type copies of *sis-b* but only two of *sis-a*. The other half of the females will have two wild-type copies of *sis-b* but only one copy of *sis-a*.

The duplication of *sis-a* and *sis-b* appears to activate the *Sxl-Pe*$_{3kb}$*:lacZ* reporter in males, since the proportion of positive embryos produced from this cross increases from 50 to 69% (Table 6). A similar result is obtained for the *1.4kb* deletion construct (69% of the embryos express β-galactosidase). However, *Sxl-Pe*$_{1.1kb}$*:lacZ* is less likely to be activated in males in response to extra copies of both *sis-a* and *sis-b* (only 57% of the embryos express β-galactosidase). While the *Sxl-Pe*$_{1.1kb}$*:lacZ* construct appears to have a low probability of being activated in males, neither of the more extensive promoter deletions, the 0.8- and the 0.4-kb constructs, appear to be activated in males by the duplication of these two X-chromosome counting genes. For both constructs, about 75% of the embryos fail to express β-galactosidase. This corresponds closely to the sum of the male embryos plus the female embryos which inherit the *sis-a* mutation and hence are defective to a greater (*Sxl-Pe*$_{0.8kb}$*:lacZ*) or lesser (*Sxl-Pe*$_{0.4kb}$*:lacZ*) extent in β-galactosidase expression. Although these two deletion constructs do not appear to be activated in males, they are capable of responding in females. As can be seen in Table 6, both constructs show a shift in the β-galactosidase staining from light to intermediate levels. This increase in β-galactosidase expression is most likely in the half of the female embryos that receive the *DD(2)Ha* chromosome and therefore have three copies of *sis-b* and two copies of *sis-a*. These results indicate that the larger *Sxl-Pe*$_{3kb}$*:lacZ* and *Sxl-Pe*$_{1.4kb}$*:lacZ* constructs can be activated in males by simultaneously increasing the dose of both *sis-a* and *sis-b*, while the smaller *Sxl-Pe*$_{0.8kb}$*:lacZ* and *Sxl-Pe*$_{0.4kb}$*:lacZ* promoter constructs cannot be so activated.

DISCUSSION

The differential activation of *Sxl-Pe* controls the choice of sexual identity. It is the ratio of X chromosomes to autosomes (X/A) that determines the choice of sexual identity in the fruit fly *D. melanogaster*. The system that counts this ratio functions to set the activity state of the binary-switch gene *Sxl*. From this perspective, two of the central issues are (i) at what step(s) and (ii) by what mechanism(s) does the system manage to translate a small, twofold difference in X-chromosomal gene dose into a decision that must ultimately be all or none. Our previous studies suggested that the key binary decision is not at the level of sex-specific splicing but rather at the level of sex-specific transcription (46) from the special internal promoter of *Sxl*, *Pe*. *Sxl* transcripts expressed from this promoter accumulate in only half the early embryos, the female embryos, and their accumulation depends upon genes that are known to be components of the X/A signalling system. It is the embryonic Sxl protein produced from these *Sxl-Pe* mRNAs that initially activates the *Sxl* autoregulatory feedback loop by directing the female-specific splicing of the first transcripts from the *Sxl-Pm*.

This transcriptional model for the choice of sexual identity in the fly is strongly supported by our analysis of the function of *Sxl-Pe* in transgenic animals. In the studies reported here, we have shown that *Sxl-Pe* fragments extending from ~3.0 kb upstream to a site located just within the 5′ untranslated region of the embryonic exon, E1, can direct the early embryonic expression of heterologous transcripts including *lacZ*, *bcd*, and *hb*. That this expression is sex specific is demonstrated by the female-specific lethality induced by both the *Sxl-Pe:bcd* and the *Sxl-Pe:hb* transgenes (Table 1). Two other lines of evidence argue that the choice of sexual identity is controlled by regulating the activity of *Sxl-Pe*, rather than by affecting the processing and/or stability of the *Sxl* early-embryonic mRNAs. First, the expression of the heterologous β-galactosidase

mRNA from the *Sxl-Pe*$_{3kb}$*:lacZ* construct depends upon the known components of the X/A signalling system. Loss-of-function mutations in the X-chromosome numerator genes *sis-a*, *sis-b*, and *runt* (Table 3) reduce β-galactosidase expression, while mutations in the denominator element *dpn* increase expression (Table 6). Moreover, as expected for a promoter whose activity is dependent on counting of X chromosomes and autosomes, varying the dose of the numerator and denominator genes affects β-galactosidase expression (Fig. 3). Second, the levels of β-galactosidase expression from *Sxl-Pe* can be substantially reduced by deletions that remove regions of the promoter upstream of the embryonic transcription start site (Fig. 4). Even more critically, these deletions alter the responses of the promoter to both the numerator and denominator genes (Fig. 5 and Tables 5 and 6). Taken together, these results argue that the X/A signalling system selects the male or female pathway by controlling the activity of *Sxl-Pe.*

While the on/off regulation of *Sxl-Pe* appears to be the critical binary decision in the selection of sexual identity, our experiments indicate that the promoter is quite sensitive to small fluctuations in the dose of X-chromosomal and autosomal counting elements. Given the sensitivity of *Sxl-Pe*, other, downstream steps in the pathway that activate the *Sxl* autoregulatory feedback loop may help ensure that this initial on/off choice is correct (46). For instance, the initial activation of the *Sxl* autoregulatory feedback loop may require a critical concentration threshold of early Sxl protein to safeguard against inappropriate activation in males if small amounts of early Sxl protein are produced.

Functional properties of *Sxl-Pe.* Our analysis of the functional properties of *Sxl-Pe* also provides some insights into the mechanisms that translate a twofold difference in the X/A ratio into an on/off response by this promoter.

First, the deletion analysis indicates that the sequences most critical for *Sxl-Pe* function are located within a ~1.4-kb DNA segment (Fig. 4). Although the region immediately upstream of this essential 1.4-kb DNA segment contains most of the consensus binding sites for two of the bHLH components of the X/A signalling system (*sis-b* and *da*), these upstream sequences can be deleted without noticeably affecting promoter function in a wild-type background. In contrast, deletions that remove parts of the 1.4-kb DNA segment substantially reduce or even eliminate promoter activity even when the more distal sequences are still present (Fig. 4).

Second, *Sxl-Pe*$_{1.4kb}$ appears to consist of multiple *cis*-acting elements that are capable of responding to the X/A signalling system. Thus, all of the promoter constructs, with the exception of *Sxl-Pe*$_{0.2kb}$, show sex-specific activity. On the other hand, the amplitude of the response to the female signal is reduced as sequences are removed. Hence, a uniform high level of expression in female embryos, but not the choice of on or off, requires the additive effects of combining all of the *cis*-acting elements in the 1.4-kb promoter.

Third, *Sxl-Pe* appears to contain reiterated copies of *cis* elements capable of responding to the different numerator and denominator elements. This is demonstrated by the effects of changing the dose of genes encoding components of the X/A signalling system. For example, the activity of even the smallest (sex-specific) promoter construct, *Sxl-Pe*$_{0.4kb}$*:lacZ*, can be altered by reducing the dose of the numerator genes *sis-b* and *sis-a* or of the denominator *dpn* (Fig. 5). This deletion construct, as well as the larger promoter constructs (*Sxl-Pe*$_{1.4kb}$*:lacZ* and *Sxl-Pe*$_{3kb}$*:lacZ*), also responds to elevated levels of *sis-b* or of *sis-b* and *sis-a* in females. However, even two copies of the strong numerators *sis-a* and *sis-b* are not sufficient to activate expression of the smaller promoter constructs, *Sxl-Pe*$_{0.4kb}$*:lacZ* and *Sxl-Pe*$_{0.8kb}$*:lacZ*, in males. Since the activity of these two constructs is dependent upon *sis-a* and *sis-b* in females, they must contain *cis* elements that are capable of responding to both of these numerator elements. The only difference between females (in which the 0.8- and 0.4-kb constructs are active) and males containing an extra copy each of *sis-a* and *sis-b* (in which the 0.8- and 0.4-kb constructs are inactive) is the dose of the other numerator elements (e.g., *runt* and *sis-c*). These additional numerator elements are present in two copies in 2X/2A female but not 1X/2A male animals. This argues that the *Sxl-Pe*$_{0.4kb}$*:lacZ* and *Sxl-Pe*$_{0.8kb}$*:lacZ* constructs are on in females because they contain *cis* elements which can interact not only with *sis-a* and *sis-b* but also with these other numerator elements. Finally, *Sxl-Pe*$_{1.4kb}$*:lacZ* and *Sxl-Pe*$_{3kb}$*:lacZ* can be activated in males simply by increasing the dose of *sis-a* and *sis-b* from one to two. A plausible interpretation of this finding is that these constructs contain a sufficient number of additional target sites for *sis-a* and *sis-b* for the promoter to be activated in males by an increase in the levels of these two proteins, in spite of the fact that other numerators, like *runt* and *sis-c*, are present in only a single male dose.

Fourth, the reiterated *cis*-acting elements in *Sxl-Pe* appear to be composed of targets not only for positive (numerator) factors that can activate expression but also for negative (denominator) factors that can reduce or eliminate expression. This conclusion is supported by the finding that the promoter activity of the smaller *Sxl-Pe*$_{0.4kb}$*:lacZ* construct is greater than that of the larger *Sxl-Pe*$_{0.8kb}$*:lacZ* construct (Fig. 5). In addition, the differential sensitivity of the promoter constructs to alterations in the doses of components of the X/A signalling system argues that these reiterated *cis*-acting elements are not evenly distributed throughout the promoter. For example, *Sxl-Pe*$_{0.8kb}$*:lacZ* is much more sensitive to a reduction in the dose of *sis-a* than either the 1.1- or the 0.4-kb construct (Table 5).

Taken together, these observations suggest a plausible model for the control of *Sxl-Pe* activity by the X/A ratio. In this model, *Sxl-Pe* is maintained silent in male embryos by both (i) the low concentration of all the X-linked numerator elements and (ii) the presence of repressive denominator elements. These negative regulators include not only the classical denominators, which are active in the zygote, but also maternal genes, like *emc*, whose products are deposited in the egg during oogenesis and subsequently function to repress *Sxl-Pe* in the zygote. Some of the denominator elements are likely to repress the activity of *Sxl-Pe* indirectly by forming inhibitory complexes with positive factors that activate *Sxl-Pe. emc* is a member of the HLH family of proteins yet lacks the DNA binding region and therefore may form inactive complexes with the bHLH numerator *sis-b* or may compete with *sis-b* for the maternal *da* gene product (9, 78), thereby increasing the concentration of this numerator required to form active transcription complexes in females. From the properties of the different deletion constructs, it seems likely that certain other denominator elements must interact directly with sites distributed throughout the promoter to keep it silent in males. *dpn* (and *hairy* [53]) is a good candidate for a negative regulator that may interact directly with one if not several target sequences in the promoter. The binding of denominator proteins could repress transcriptional activity directly by interacting with components of the transcriptional apparatus. Alternatively, they could prevent the formation of stable numerator protein complexes, perhaps by interfering with cross-talk (protein-protein interactions) between numerators bound to the different reiterated low-affinity target sites (see below). That *dpn* plays a key role in the on/off regulation of *Sxl-Pe* is evidenced by the fact that a small (two-

fold) reduction in *dpn* function can dramatically increase the staining pattern generated by all of the constructs (Fig. 5).

In females, the repressive effect of the denominators apparently must be overcome in two ways. First, the numerator elements must reach a concentration threshold which will enable them to form active transcription complexes (e.g., *da–sis-b* heterodimers). The concentration difference of numerator elements between males and females is likely to be maximized both by the nonlinear effects that can be introduced by the formation of concentration-dependent protein-protein complexes and by the formation of inactive complexes with denominator elements such as *emc* (40, 54), as discussed above.

Second, once this concentration threshold has been obtained, the amplitude of the response of *Sxl-Pe* must depend upon the number of positive binding sites that are occupied in females. Since addition of extra copies of *sis-b* (and probably the other numerator elements) can substantially enhance promoter activity in females, these target sites appear to be typically underutilized even in 2X/2A animals. This result suggests that the reiterated target sequences may be only low-affinity binding sites for the numerator proteins. If this is the case, protein-protein interactions between bound numerator elements are required to stabilize the interaction of these factors with their binding sites. While this is a plausible working model for the on/off regulation of *Sxl-Pe*, further studies are likely to reveal interactions of numerator and denominator elements at *Sxl-Pe* and elsewhere within the cell that are even more intricate than those envisioned here.

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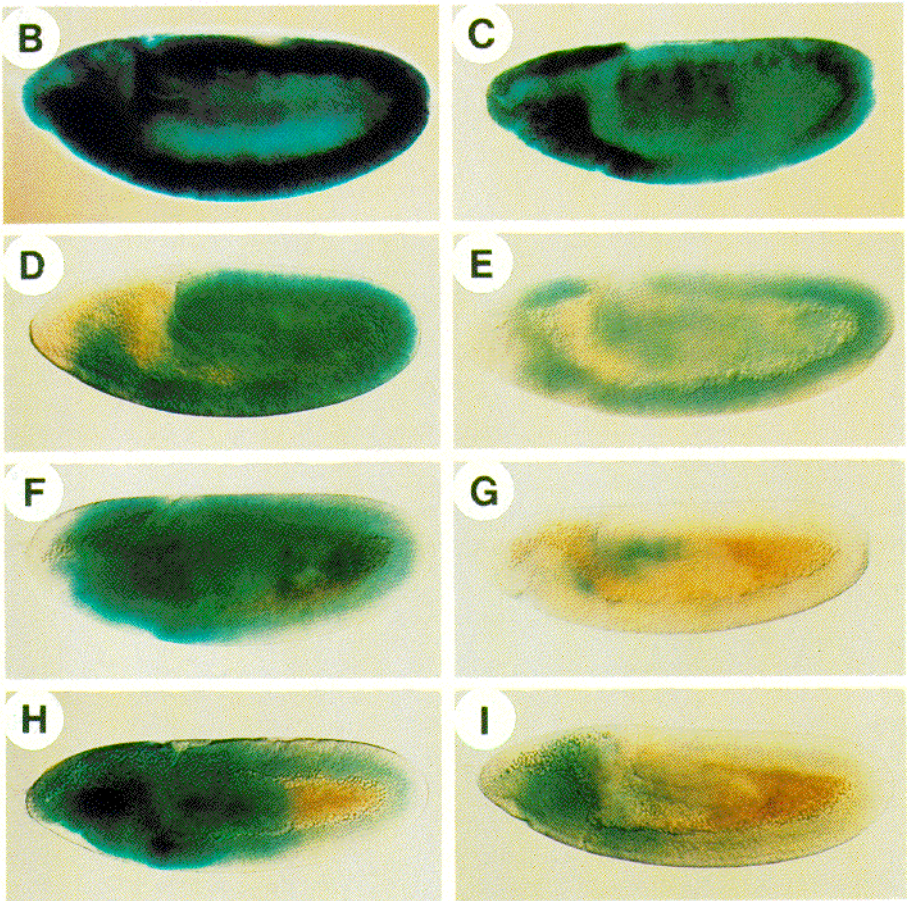
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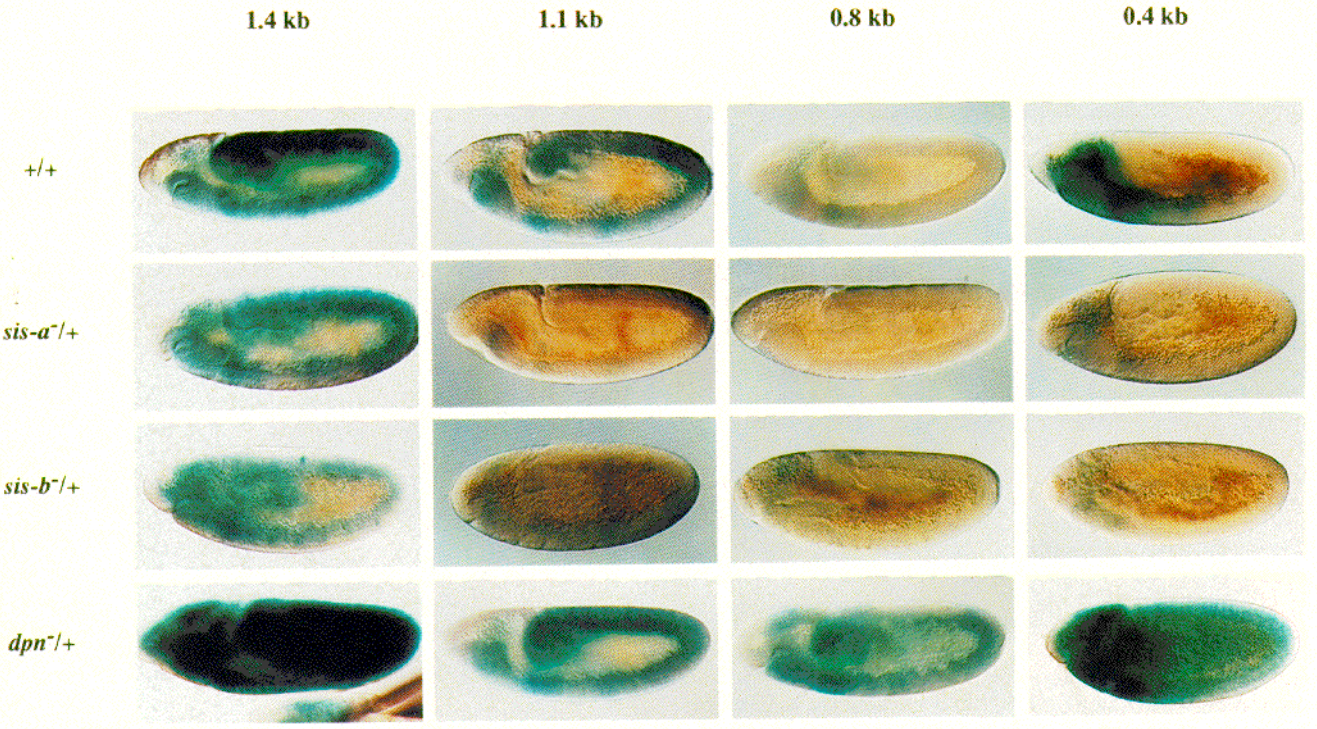
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