Developmental distribution of female-specific *Sex-lethal* proteins in *Drosophila melanogaster*

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The binary switch gene *Sex-lethal* (*Sxl*) must be on in females and off in males to allow the proper elaboration of the appropriate sexual developmental pathway in *Drosophila melanogaster*. Previous studies suggested a mechanism in which the on/off regulation of *Sxl* occurs post-transcriptionally at the level of RNA splicing. A critical prediction of this model is that functional *Sxl* proteins are absent in males but present in females. In this report we show that the expected full-length proteins are only present in female animals. Multiple forms of *Sxl* protein are found in females, some of which are expressed in a stage- and tissue-specific pattern. Consistent with a role of *Sxl* proteins in regulating alternate splicing, the proteins are localized in the nucleus where they exhibit a punctate staining pattern. Surprisingly, several minor *Sxl* proteins appear to be present in specific tissues of both sexes of adults. The possible origin of these species is discussed. We also show that *Sxl* expression in the early embryo is sex specific and depends on maternal *daughterless* and zygotic *sisterless-b* activity in accordance with the established roles of these genes as positive regulators of *Sxl*. The onset of *Sxl* expression in the germ line occurs later than that in the soma.

[Key Words: RNA splicing; sex specific; *Sex-lethal* proteins; sex determination; *Drosophila melanogaster*]

Received October 11, 1990; revised version accepted December 28, 1990.

Sexual development in *Drosophila melanogaster* is programmed by the binary switch gene *Sex-lethal* (*Sxl*) (for review, see Baker and Belote 1983; Cline 1985, 1988b; Hodgkin 1990; Steinmann-Zwicky et al. 1990). In females *Sxl* must be turned on early in embryogenesis. Its activity is then required throughout the remainder of the life cycle for the proper elaboration of the female developmental pathway. Loss-of-function mutations in *Sxl* are female lethal due to inappropriate activation of the dosage compensation system (Lucchesi and Skripsky 1981; Cline 1983a, 1984; Gergen 1987) and cause masculinization of diplo-X (chromosomally female) cells due to a failure to turn on downstream genes in the sexual differentiation pathway (Cline 1979, 1983a, 1984; Sanchez and Nöthiger 1982). In contrast, the elaboration of the male developmental pathway requires that *Sxl* remain off throughout the life cycle. Loss-of-function mutations in *Sxl* have no apparent effects on male development, and males carrying a deletion for the gene are viable and fertile (Salz et al. 1987). However, there is a class of dominant gain-of-function *Sxl* mutations in which the *Sxl* gene is constitutively active. These mutations are male lethal due to the inappropriate inactivation of the dosage compensation system. They cause

feminization of haplo-X (chromosomally male) cells due to activation of downstream genes in the sexual differentiation pathway (Cline 1979, 1983a; Maine et al. 1985).

The on/off state of the *Sxl* gene is set in the early zygote in response to the X/A ratio (Sanchez and Nöthiger 1983; Cline 1984; Gergen 1987). When the X/A ratio is 1 (in females), the *Sxl* gene is activated, while it is not activated when the X/A ratio is 1 : 2 (in males). Two classes of genes have been identified that are required to transduce this primary signal to *Sxl*. The first group, which includes *daughterless* (*da*), generates maternally synthesized components in the egg that appear to function as cofactors in the *Sxl* activation process after fertilization (Cline 1983a; Cronmiller and Cline 1986). They are required to initiate *Sxl* gene activity but do not themselves determine whether *Sxl* will be activated. This decision requires a second class of genes that function in the zygote to assess the X/A ratio. Two X-linked genes that act as counting elements, *sisterless-a* (*sis-a*) and *sisterless-b* (*sis-b*), have been identified (Cline 1986, 1988a). When the dose of these genes is high as in diplo-X animals, the *Sxl* gene is turned on; when the dose is low as in haplo-X animals, the *Sxl* gene remains off.

Once the state of *Sxl* activity is set in somatic cells early in embryogenesis, this state is maintained throughout the remainder of the life cycle independently of the system that initially signaled the X/A ratio (Sanchez and Nöthiger 1983; Cline 1984, 1988b; Maine et al. 1985). In males the off state is maintained by default. In females

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the on state is maintained by a positive autoregulatory activity of *Sxl* itself (Cline 1984; Bell et al. 1991). In addition to its autoregulatory function, *Sxl* controls female development by activating downstream regulatory genes involved in sexual differentiation of the soma and the germ line and by inactivating male-specific dosage compensation functions.

Molecular studies revealed that the *Sxl* gene encodes a complex set of RNAs that show sex, stage, and tissue specificity (Bell et al. 1988; Salz et al. 1989). The first zygotic *Sxl* transcripts are only present for a very brief period early in embryogenesis when the activity state of *Sxl* is initially selected (Salz et al. 1989). These "early" *Sxl* RNAs appear to be derived from a promoter that is distinct from the one used during most of the life cycle, and it has been suggested that the expression of these early transcripts would be a likely target for upstream genes involved in assessing the X/A ratio (Salz et al. 1989). The embryonic RNAs are then replaced by a set of "late" transcripts that are transcribed from a distinct promoter (Salz et al. 1989). In spite of the fact that *Sxl* must be on in females and off in males, this late promoter appears to be constitutive and late transcripts are found in both sexes. Thus, once the pathway has been initiated, the on/off regulation of *Sxl* must be post-transcriptional. Structural analysis of the late female and male RNAs reveals that on/off regulation involves sex-specific differences in splicing. All of the male-specific RNAs have an additional exon that introduces several in-frame stop signals that should terminate translation prematurely. In contrast, this translation-terminating exon is spliced out in the female-specific late *Sxl* RNAs. This leaves a large ORF that would allow the synthesis of *Sxl* gene products that include two protein domains sharing homology with a family of RNA-binding proteins (RNP domains). These findings have suggested a model in which regulation of *Sxl* activity throughout most of the life cycle is determined by sex-specific splicing (Bell et al. 1988; Salz et al. 1989).

A critical prediction of our model for on/off regulation of *Sxl* during development is that full-length *Sxl* protein must be present in females and absent in males. During the late phase, the differences between females and males should reflect post-transcriptional regulation of *Sxl* at the level of RNA splicing. Early in embryogenesis the difference between females and males should reflect the mechanisms involved in the initial activation of the *Sxl* gene. In this case, expression of appropriate levels of *Sxl* protein in the female embryo should be dependent on the functioning of genes upstream in the pathway that are involved in the transduction of the X/A signal. To test this model, we have used anti-*Sxl* antibodies to examine the sex-, stage-, and tissue-specific distribution of *Sxl* proteins.

Results

Full-length Sxl proteins are only present in females

As shown in Figure 1, A and B, sex-specific alternate splicing of the late *Sxl* transcripts generates female and

male mRNAs that differ substantially in their predicted protein-coding capacities. The female transcripts have an ORF that starts in exon 2 and extends for ~350 amino acids. The exact length of this ORF varies among different classes of *Sxl* female RNAs and depends on the pattern of splicing and polyadenylation in the 3' portion of the nascent RNA (only one example is shown in Fig. 1A, the corresponding sequence of which is reported in Bell et al. 1988). Differential processing of the late *Sxl* transcripts mainly affects protein-coding sequences at the carboxyl terminus, and all classes of female transcripts are predicted to encode both RNP domains (M. Samuels et al., in prep.). While the ORF for the different classes of male transcripts also begins in exon 2, the inclusion of an additional male-specific exon, exon 3, interrupts the ORF. It extends for only 126 or 144 bp (depending on which of the two acceptor sites of exon 3 is used; see Bell et al. 1988). Thus, instead of encoding a ~350-amino-acid protein with two RNP domains, the male RNAs are predicted to produce a short peptide of 42 or 48 amino acids, which terminates upstream of the RNP domains.

To test this prediction, we generated monoclonal antibodies against a *lacZ-Sxl* fusion protein that is encoded by sequences located downstream of the male-specific termination codons in exon 3. These antibodies should recognize epitopes present in female proteins, while the epitopes should not be present in the truncated male peptides. When screening a hybridoma bank prepared from mice immunized with the *lacZ-Sxl* fusion protein, we obtained 30 lines that detected *Sxl* epitopes in a $\phi 10$ -*Sxl* fusion protein constructed from the same *Sxl* cDNA fragment (Fig. 1B). Several of these lines were used to examine *Sxl* proteins in *D. melanogaster*, and they gave essentially the same results.

In the experiment shown in Figure 1C, an immunoblot of protein extracts from female and male animals at different stages of development was probed with one of the anti-*Sxl* monoclonal antibodies. The antibody detected two major cross-reacting protein species of 36 and 38 kD. These proteins were present in female animals at all stages tested. In contrast, the corresponding male stages did not have detectable levels of immunoreactive antigens. These findings are consistent with the general predictions of our model for the late on/off regulation of the *Sxl* gene.

Tissue-specific distribution of Sxl protein in adult females

The differential processing of nascent *Sxl* transcripts in adult females generates multiple spliced products that exhibit some tissue specificity (Salz et al. 1989). Hence, it was of interest to examine *Sxl* proteins in different tissues from adult females.

The species of *Sxl* protein in adult female ovaries, abdomens (lacking the ovaries), thoraces, and heads are shown in Figure 2A. As expected from genetic studies and clonal analysis (Cline 1979; Sanchez and Nöthiger 1982), *Sxl* proteins were present in all of the female tis-

Figure 1. Full-length proteins of *Sxl* are only expressed in females. (A) Sex-specific differences in the structure of “late” *Sxl* transcripts. (a) The structure of a female-specific RNA that was deduced from the female cDNA clone cF1; (b) structure of the male cDNA clone cM1 [see Bell et al. 1988]. The ORFs are shown as solid boxes. (B) The putative proteins encoded by cF1 and cM1 are shown in a and b, respectively. The RNP homologous domains are indicated by open boxes. Fusion proteins were obtained by inserting sequences encoding the carboxy-terminal 255 residues of cF1 into *lacZ*- (c) and $\phi 10$ - (d) coding sequences [see Materials and methods]. Both fusion proteins lack the first 99 residues of the cF1 ORF but retain the two RNP homologous domains. (C) Total proteins extracts were prepared from female and male animals at different developmental stages and separated on a 10% SDS-polyacrylamide gel. Equal amounts of protein were loaded in the corresponding female and male lanes and probed with monoclonal antibodies raised against the 255 carboxy-terminal residues of *Sxl* protein. (1L) First-instar larvae; (2L) second-instar larvae; (3L) third-instar larvae. The spot in the male adult lane is an artifact.

sues examined. However, the different forms of the protein appeared to be preferentially distributed in one tissue or another. The two major cross-reactive antigens (36 and 38 kD) were equally abundant in extracts prepared from both ovaries and heads (see Fig. 2A). In contrast, in extracts from ovary-depleted abdomens the level of the 36 kD protein was low, while the 38-kD antigen was the

predominant species. In extracts from thoraces, the only antigen detected was the 38-kD protein (see Fig. 2A,B). In addition to the two major species, there appeared to be several minor forms of *Sxl* protein. In ovaries we detected two larger *Sxl* proteins of ~40 and 42 kD. These protein species were not evident in other adult female tissue (Fig. 2A). In heads, the two major bands consis-

Figure 2. Detection of *Sxl* protein in adult extracts. (A) Distribution of *Sxl* protein variants in different tissues of adult females. Protein extracts were prepared from dissected body parts of female *Oregon-R* flies. Each lane contains tissue from two individuals probed with anti-*Sxl* monoclonal antibody. The cross-reactive band in thoraces appears to migrate slightly faster than the major 38-kD antigen observed in other tissues. However, this apparent difference seems to be an artifact caused by the large amount of myosin present in the thoracic extracts. When thoracic extracts are mixed with extracts from other tissues, the *Sxl* species migrate together. (B) Immunoblot analysis of different tissues from male and female *Oregon-R* flies and from *Sxl*^{lP3G2/Y} males (*Df Sxl* males) carrying a deletion of the *Sxl* gene [Salz et al. 1987]. Protein extracts prepared from four heads were each loaded in the three lanes to the left, and extracts prepared from two thoraces were each loaded in the three lanes to the right. Cross-reactive material in the male lanes is indicated with arrowheads.

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tently appeared broader (see Fig. 2B) than in other female tissues, indicating that they may actually be composed of several nonresolved antigens.

The electrophoretic mobilities of the detected antigens (36–42 kD) are in good agreement with the range of molecular masses predicted from the different ORFs found in *Sxl* cDNAs (37–41 kD; M. Samuels et al., in prep.). Hence, much of the diversity in *Sxl* proteins observed in various tissues of adult females could arise from the alternate and cell-type-specific processing of *Sxl* transcripts rather than from post-translational modifications of the protein. In support of this possibility, the cF1 *Sxl* cDNA described by Bell et al. (1988) was found to encode a protein of the same size both in vitro in a reticulocyte translation system (not shown) and in vivo in transformed flies (Bell et al. 1991). Moreover, the protein encoded by this cDNA comigrates with one of the major female-specific species (the 38 kD) observed in wild-type flies.

Sxl protein in adult males

As a control for the experiments described in the previous section, we also used the *Sxl* antibody to probe extracts prepared from different male tissues. While *Sxl* cross-reacting antigens were not detected in blots of whole male extracts (see Fig. 1C), we were surprised to discover two very weakly stained bands in extracts prepared from adult male heads and thoraces (Fig. 2B). The two bands recognized by the *Sxl* antibody in male extracts were smaller (35 and 33 kD) than the two major female *Sxl* proteins (36 and 38 kD). They were more prominent in extracts from the male head than from the thorax, while neither was found in the male abdomen (not shown). Though these two bands were present at levels estimated to be between 20 and 40 times lower than the major female-specific *Sxl* bands, they were reproducibly observed in extracts prepared from heads and thoraces. Interestingly, weakly stained bands of approximately the same electrophoretic mobility were also evident in heads and thoraces of adult females.

Two lines of evidence suggest that the *Sxl* gene encodes the 33- and 35-kD protein species in males. First, these bands were detectable with several different monoclonal lines that presumably recognize different *Sxl* epitopes. Second, the bands were absent in males carrying deletions of the *Sxl* gene (Fig. 2B). Both of the cross-reacting bands were absent in head and thorax extracts from *Sxl*^{IP3G2} males (Salz et al. 1987). The same result was obtained when examining head and thorax extracts from males carrying another *Sxl* deficiency, *Sxl*^{IP7BO} (Salz et al. 1987). Although we suspect that the amino acid sequence of these minor proteins is colinear with most of the sequence for the female-specific *Sxl* proteins (see Discussion), their smaller size indicates that they must have some differences in structure. Moreover, they apparently do not function like female-specific *Sxl* proteins as they seem to be unable to impose the female mode of *Sxl* splicing in males (as evident here from the lack of proteins of female size in the male extract).

Sxl proteins in embryogenesis

Our previous analysis of the developmental profile of *Sxl* RNAs suggested that there are three distinct phases during embryogenesis (Salz et al. 1989). The first phase is immediately after fertilization when the developing embryo contains relatively high levels of maternally derived female *Sxl* RNAs that have been deposited in the egg during oogenesis. In the second phase, the maternal RNAs disappear and a new set of embryonic RNAs are expressed. These embryonic RNAs are thought to be involved in the initial selection of the sexual development pathway, and they represent the first products of zygotic *Sxl* expression. In the third phase, the embryonic RNAs disappear and are replaced by the late female and male transcripts. This change, which occurs around the time of cellular–blastoderm formation, is thought to represent the transition from early regulation of *Sxl* during pathway initiation to late post-transcriptional regulation of *Sxl*.

It was of interest to compare the different phases of *Sxl* RNA accumulation during embryogenesis with the profile of *Sxl* proteins products. In Figure 3, protein extracts prepared from embryos at different stages were probed with anti-*Sxl* monoclonal antibodies. Maternal *Sxl* RNA is abundant in unfertilized eggs and early cleavage stages (Salz et al. 1989). However, we detected no maternal *Sxl* protein in extracts from unfertilized eggs (not shown) and in the earliest cleavage stages (0–1.5 hr in Fig. 3). A low level of *Sxl* protein can first be detected in the next stage, 1.5–3 hr; however, the yield of *Sxl* protein is very

Figure 3. Developmental profile of *Sxl* proteins in embryonic extracts. Proteins of staged *Oregon-R* embryos were separated by SDS-PAGE and transferred to nitrocellulose. The immunoblot was incubated with monoclonal anti-*Sxl* antibodies. Ages of the embryos are indicated at the top in hours after egg deposition (25°C). Cross-reactive materials are numbered 1–5; bands 1 and 2 comigrate with the abundant 36- and 38-kD antigens, respectively, observed in adult extracts. The nonlabeled bands of lower molecular mass that appear in all lanes are due to cross-reactivity of the secondary antibody used in these immunoblots, as judged from controls processed without the primary antibody (not shown). A different secondary antibody is used for whole-mount immunostaining.

low and somewhat variable at this stage, probably reflecting the difficulties in accurate staging in mass collections. The abundance of *Sxl* proteins increased dramatically in the next stage, the 3- to 4.5-hr period. At this point we observed two major antigens of 36 and 38 kD (band 1 and 2), as well as at least three larger, minor antigens (bands 3–5). Since maternal *Sxl* RNAs disappear at ~2 hr, well before *Sxl* proteins become abundant, it seems likely that these *Sxl* proteins are the products of *Sxl* expression in the zygote. The *Sxl* embryonic RNAs are present between 2 and 4 hr (Salz et al. 1989), and we assume that the *Sxl* proteins detected in the 1.5- to 3-hr interval are translated primarily from these messages. The late female-specific RNAs first appear between 3 and 4 hr. Thus, *Sxl* proteins detected in the 3- to 4.5-hr interval are likely to be a mixture of embryonic and late species. During the remainder of embryogenesis, the two major bands increase in abundance, reaching a plateau at

mid-embryogenesis. In contrast, the relative amounts of the minor larger *Sxl* proteins are highest in 3- to 6-hr embryos, and these levels start to decline after mid-embryogenesis.

Female-specific *Sxl* expression during embryogenesis

During embryogenesis, as is the case at other stages in the life cycle, we would expect to find *Sxl* proteins in females but not in males. To test this prediction, we stained whole mounts of embryos with anti-*Sxl* antibody. As illustrated in Figure 4, A and B, two different classes of wild-type embryos were observed: One class gave uniform staining with the anti-*Sxl* antibody, whereas the other class was completely devoid of staining. We can first unambiguously distinguish stained and unstained embryos during the last nuclear cycle prior to cellularization when the stained embryo has accumu-

Figure 4. Female-specific expression of *Sxl* protein in embryos. Whole-mount embryos were stained with monoclonal anti-*Sxl* antibody. *Sxl* expression in *Oregon-R* embryos is shown in A (at the blastoderm stage) and B (at the germ-band extension stage). Progeny from *C(1)DX, y f/Y* mothers and *y f^{36a} fog/y⁺ Y mal¹²⁶* fathers are distinguished by three different phenotypes: wild-type, female embryo (C); *fog*, male embryo (D); and nullo-X embryo (E). (F–I) Mosaic *Sxl* expression in gynandromorph embryos that derive from *R(1)w^{vC}/y w f^{36a}* females crossed to *y w f/y⁺ N⁺ Y* males: cellular blastoderm (F); germ-band extension (G); and late stages after germ-band retraction (H and I). Embryos shown in C–F and H are oriented anterior to the left and dorsal at the top; in G and I the embryo is rotated slightly laterally to view the dorsal side.

lated a substantial amount of *Sxl* protein. No staining was seen in the progenitors of the germ line, the pole cells (see Figs. 4A and 5B).

The existence of two classes of embryos would be consistent with our expectation that *Sxl* is expressed only in females. Female-specific expression of *Sxl* at this stage was demonstrated by three different lines of evidence.

The first is based on analysis of the number of stained and unstained embryos in wild-type and *Sxl* mutant backgrounds. As expected from the sex ratio in a wild-type population, 50% of the wild-type embryos were uniformly stained, while 50% gave no staining (see Table 1). The number of stained embryos was reduced to ~25% when the progeny were from mothers and fathers that were heterozygous and hemizygous, respectively, for *Sxl* deficiencies [*Sxl*^{fP7BO} or *Sxl*^{fP3G2}; see Table 1]. These percentages correlate with the number of female progeny expected to carry a functional copy of the *Sxl* gene. Similar numbers were obtained for a null allele, *Sxl*^{f1} (Table 1). These results demonstrate that antibody staining in embryos depends on the presence of a functional *Sxl* gene and thus confirm the specificity of the monoclonal antibodies for *Sxl* protein.

The second line of evidence came from analysis of *Sxl* protein distribution in gynandromorph embryos. Such embryos are mosaics for male (XO) and female (XX) cells

Table 1. Anti-*Sxl* staining of embryos as a function of *Sxl*⁺ dose

Parents	Uniform staining [no. (%)]	No staining [no. (%)]
+/+ × +/Y	240 (50)	238 (50)
Df(<i>Sxl</i>) fP7BO/+ × +/Y	234 (49)	241 (51)
Df(<i>Sxl</i>) fP7BO/+ × Df(<i>Sxl</i>) fP7BO/Y ^a	101 (25)	295 (75)
Df(<i>Sxl</i>) fP3G2/+ × Df(<i>Sxl</i>) fP3G2/Y ^b	18 (23)	61 (77)
<i>Sxl</i> ^{f1} /+ × <i>Sxl</i> ^{f1} /Y ^c	278 (28)	716 (72)

^a*y cm Sxl*^{fP7BO}/Binsinscy × *y cm Sxl*^{fP7BO}/Y.

^b*dx Sxl*^{fP3G2} f/Binsinscy × *dx Sxl*^{fP3G2} f/Y.

^c*y w cm Sxl*^{f1} *ct*⁶ *sn*³/+ × *y w cm Sxl*^{f1} *ct*⁶ *sn*³/Y.

due to loss of an unstable ring X chromosome [*R* (1)*w*^{vc}] in early cleavage stages (Hall et al. 1976). As shown in Figure 4, F–I, a small fraction of embryos from this cross gave a nonuniform, mosaic staining pattern. The nonstained regions in these embryos varied greatly in size, consistent with size variations of XO patches (30–70%) found in surviving mosaic flies (Zusman and Wieschaus 1987). Mosaic distribution of the protein was already detectable at the blastoderm stage when *Sxl* is activated (Fig. 4F).

Figure 5. Nuclear localization of *Sxl* protein in embryonic and larval tissue. (A) Syncytial blastoderm embryo during the last metasynchronous nuclear divisions. Mitotic front of anaphase nuclei is located in a region corresponding to 20–30% EL (anterior to the left). (B) Posterior region of a blastoderm embryo during cellularization. (sn) Somatic nucleus; (pc) pole cell. (C) Region surrounding the cephalic furrow (cf) in a gastrulating embryo displaying mitotically active domains in close proximity to the furrow. (D) Distribution of *Sxl* proteins in female salivary glands from late third-instar larvae. Arrowheads indicate regions where *Sxl* proteins accumulate.

The third line of evidence came from analysis of *Sxl* antibody staining pattern in embryos whose sex could be determined on the basis of their phenotype. For this purpose we took advantage of the the X-linked gene *folded gastrulation* (*fog*) (Zusman and Wieschaus 1987). Embryos that are *fog*⁻ have a distinct morphology that can be detected shortly after the onset of gastrulation. We crossed attached-X females (XX/Y) to *fog*/Y males carrying a duplication of *fog*⁺ on the Y chromosome. All male embryos derived from this cross were expected to be hemizygous for *fog* and, hence, exhibited the folded gastrulation phenotype; whereas female embryos should be phenotypically *fog*⁺ (either XX/*fog* or XX/Y). Analysis of the progeny from this cross revealed that all wild-type embryos gave uniform staining, with the *Sxl* antibodies indicating that female embryos express *Sxl* (Fig. 4C). Most of the *fog* embryos (92%) were completely devoid of staining (Fig. 4D); however, 8% stained uniformly with the antibody. We do not understand the reason for this staining at present. It could be due to recombination or stability problems of the attached X or caused by misregulation of *Sxl* in males carrying the *fog* chromosome. Further studies are required to distinguish between these possibilities. One quarter of the siblings in this cross had a distinctive phenotype that resulted from the lack of X chromosomes (null-X embryos); and, as expected, these did not stain with anti-*Sxl* antibodies (Fig. 4E).

Nuclear localization of *Sxl* proteins

In wild-type embryos, *Sxl* protein was first detected around the beginning of nuclear cycle 12. The staining was localized in all somatic syncytial nuclei at this stage (Fig. 5A). Moreover, *Sxl* staining remained associated with the condensed chromosomes during the metaphase-anaphase nuclear divisions. The level of *Sxl* protein continued to increase in all somatic cells through cellularization. While high levels of protein were found in the somatic nuclei, no staining was observed in the pole cells (Fig. 5B). The *Sxl* protein remained preferentially localized in the nucleus during the subsequent stages of embryogenesis. As can be seen in the gastrulating embryo shown in Figure 5C, the interphase nucleus typically displayed dots of heavy staining superimposed on a more diffuse staining in the nucleoplasm. It is likely that the dots correspond to regions of the nucleus where high levels of *Sxl* protein accumulate. The photomicrograph

in Figure 5C also demonstrates association of *Sxl* protein with condensed chromosomes in cells undergoing mitosis.

Nuclear localization was also found at other stages of *Drosophila* development in all somatic tissue examined. For example, in third-instar larvae strong *Sxl* staining was observed in the polytene nuclei of female salivary glands cells (Fig. 5D). The punctate pattern of *Sxl* staining was particularly evident in these polytenized nuclei. These observations suggest that *Sxl* proteins may be associated with some defined subnuclear structures both in polytenized and diploid nuclei.

Sxl expression depends on *da* and *sis* activities

Genetic studies indicated that initiation of *Sxl* activity in diplo-X embryos requires the maternally inherited product of the *da* gene (Cline 1978, 1983a). To determine what effects *da* has on the expression of *Sxl* proteins, we examined *Sxl* antibody staining pattern in embryos from homozygous *da*¹ mothers that were kept at the non-permissive temperature of 25°C. Under these conditions the number of females that eclosed was <1% (Table 2). As can be seen in the photographs of embryos shown in Figure 6, A–D, the *da* mutation caused a striking perturbation in the pattern of *Sxl* staining. Instead of the essentially uniform distribution of *Sxl* protein observed in female progeny of wild-type mothers, *Sxl* staining in embryos from *da*¹ mothers was typically restricted to small patches of cells. As shown in Table 2, slightly less than half the embryos (41%) gave partial staining, and these were presumably females. The remaining embryos (59%) gave no detectable staining (see Fig. 6D). This class probably consisted of the males plus a few percent of female embryos. No embryos were found that gave a pattern of staining that was uniform. Since female viability was extremely low, these findings would suggest that the spatially restricted expression of *Sxl* protein observed in many of the embryos does not allow female survival.

The nonuniform distribution of *Sxl* proteins in *da*¹ progeny was already evident at the earliest stage at which *Sxl* staining could be detected, the syncytial blastoderm (see Fig. 6A). The typical staining pattern consisted of an anterior domain that corresponds to the presumptive anterior midgut and posterior head segments and/or a posterior domain that corresponds to the presumptive posterior midgut. These domains of *Sxl* expres-

Table 2. *Sxl* expression depends on *da* and *sis* activity

Parents		Uniform expression ^a	Spatially restricted expression [no. (%)]	No expression [no. (%)]	Female viability [no. (%)]
<i>da</i> ¹ / <i>da</i> ¹ ; +/+ × +/Y ^b	25°C	0	104 (41)	150 (59)	<1
<i>sc</i> ³⁻¹ / <i>sc</i> ³⁻¹ × <i>sc</i> ³⁻¹ /Y ^c	18°C	109 (49%)	4 (2)	111 (49)	100
	25°C	13 (11%)	43 (37)	60 (52)	11

^a Includes animals with variable but detectable levels.

^b *da*¹ chromosome was marked with *cl*, *cn*, and *bw*.

^c *sc*³⁻¹ (*sisb*^{*sc*3-1}) was marked with *w*.

Figure 6 Anti-*Sxl* staining of whole-mount embryos from *da¹/da¹* mothers that were grown at 25°C. (A) Syncytial blastoderm. (pc) Pole cells. (B–D) Germ-band extension (mx) maxillary head segment; (amg) anterior midgut invagination; (pmg) posterior midgut invagination. The embryos in B and C display nonuniform staining, while D shows an embryo of the same cross that does not express *Sxl* protein. The variability in staining intensities that appear in B and C is due to superimposing different focal planes. *Sxl* expression in embryos that derives from a homozygous *sis-b^{sc3-1}* strain is shown in E–J. At 18°C (E, G, I, and J), embryos exhibit uniform staining or no staining (cf. embryos in I). (J) A higher magnification of the two germ-band-extended embryos in I. The broad arrowhead in J (top) indicates cells in the upper embryo that do not express *Sxl*, while different levels of nuclear staining are indicated with narrow arrowheads in the embryo below. (F and H) Nonuniform *Sxl* expression in embryos grown at 25°C. All embryos are oriented with anterior to the left and dorsal to the top.

sion varied considerably in size and differed from embryo to embryo (see Fig. 6B,C); however, even in the more extreme cases, there was usually some residual staining in the maxillary head segment. Cells within the stained domains appeared to express wild-type levels of *Sxl* protein; no intermediate levels were observed. Since the restricted patterns of *Sxl* expression persisted throughout embryogenesis, it seems likely that once *Sxl* is activated, it remains on.

Sxl activation in female embryos requires at least two different zygotically active loci in addition to the mater-

nal *da* gene product: *sis-a* and *sis-b* (Cline 1986, 1988a). To determine what effects these elements have on the expression of *Sxl* proteins, we have examined the antibody staining pattern in embryos mutant in the *sis-b* element (Fig. 6E–H). For this purpose, we used the *sis-b* allele, *scute³⁻¹* (*sis-b^{sc3-1}*; Cline 1988a). The viability of homozygous *sis-b^{sc3-1}* females ranges from 100% at 18°C to <1% at 29°C, with a very well-defined temperature-sensitive period that begins at nuclear cycle 9 and ends at the beginning of nuclear cycle 14 (T.W. Cline, unpubl.).

sis-b^{sc3-1} progeny were collected at two different temperatures, 18 and 25°C, and stained with anti-*Sxl* antibody (Table 2). At the permissive temperature (18°C) the staining pattern resembled that observed in collections of wild-type animals: half of the animals were stained, and *Sxl* protein appeared to be expressed in all somatic cells of these embryos (Fig. 6E,G). Unlike the wild-type animals, however, the level of staining in the majority of mutant animals was not the same throughout the embryo. As illustrated by the early gastrulating embryo in Figure 6E, lower levels of staining were consistently observed in cells located in the medial region, while higher levels of staining were found in cells near the anterior and posterior ends of the embryo. Surprisingly, these regional differences in the level of *Sxl* protein persisted through germ-band extension (see Fig. 6G,I) and could be detected even after germ-band retraction. Since homozygous *sis-b^{sc3-1}* females were 100% viable at 18°C, the variable levels of *Sxl* protein in different cells of the embryo, as shown in Figure 6J, appeared to be tolerated by the animal and were sufficient to support apparently normal female development.

The picture was dramatically changed at 25°C, a temperature at which only 11% of the homozygous females survived (Table 2). There was a large reduction in the number of embryos showing staining in all cells and an increase in the number of embryos with regions in which there was no detectable *Sxl* protein. The spatially restricted patterns of *Sxl* expression were first observed at the syncytial blastoderm stage and persisted throughout the remainder of embryogenesis. Moreover, the altered patterns were reminiscent of those observed in embryos with insufficient levels of maternal *da* activity. As illustrated in the cellular blastoderm shown in Figure 6F and the germ-band-extended embryo in Figure 6H, most of the embryos showed a band of staining near the anterior and posterior ends, with little or no staining in the medial region. The domains tended to be smaller and the levels gradually decreased under more restrictive conditions. The regions of *Sxl* expression least likely to be disrupted were located around the anterior and posterior midgut invaginations. At 29°C, when female viability was almost zero, <1% of the embryos showed any anti-*Sxl* staining (J. Hager, pers. comm.)

Discussion

We have tested the post-transcriptional model for on/off regulation of *Sxl* using antibodies directed against *Sxl*

epitopes that are encoded by mRNA sequences downstream of the male-specific exon. Our results support this model: In females, where *Sxl* function is required, our antibodies detect a set of protein species of the expected molecular weight. In contrast, these proteins are not detected in male animals where *Sxl* function must be turned off. The female-specific proteins can first be detected on immunoblots early in embryogenesis, and they persist through the remainder of the life cycle. Consistent with expectations from earlier genetic studies (Cline 1979; Sanchez and Nöthiger 1982, 1983), antibody staining of embryo whole mounts and dissected tissues from later stages indicates that the *Sxl* protein is present in all female tissue.

The findings that *Sxl* proteins are preferentially localized in the nucleus where RNA splicing takes place and the punctate *Sxl* staining pattern in the nucleus resembles that observed for known components of the RNA splicing machinery (see Fu and Maniatis 1990) contribute to the growing body of evidence that *Sxl* proteins may function directly to control RNA processing. First, the targets of *Sxl* activities include the genes *transformer* and *Sxl* itself, both of which are regulated at the level of RNA splicing (Boggs et al. 1987; Bell et al. 1988; Salz et al. 1989; Sosnowski et al. 1989). Second, the predicted sequence of proteins encoded by female *Sxl* mRNAs has two domains that share homology with a family of RNA-binding proteins (Bell et al. 1988). Third, *Sxl* protein has been shown to bind to RNA in vitro in a sequence-specific manner (Inoue et al. 1990). Fourth, ectopic expression of female *Sxl* protein in transformed male flies imposes the female-specific splicing of *Sxl* exon 3 (Bell et al. 1991).

Sxl protein in males

Consistent with the conclusions drawn from genetic analysis (Cline 1978, 1983a, 1984; Maine et al. 1985), no full-length *Sxl* proteins are detectable in males at any stage in the life cycle. However, we were surprised to find that protein extracts from adult males contain several very low-abundance proteins of ~33–35 kD, which cross-react with our anti-*Sxl* antibodies. Although these proteins are clearly smaller than the female-specific *Sxl* proteins (whether detected by antibody or predicted from known ORFs of cDNAs: M. Samuels et al., in prep.), they nevertheless appear to be derived from the *Sxl* gene since they are not found in males carrying *Sxl* deletions. In addition, since our anti-*Sxl* antibodies were generated against protein sequences encoded by exons downstream of the terminating male-specific exon (see Fig. 1), the 33- to 35-kD species must also share epitopes with the female-specific *Sxl* proteins. In males, these small proteins were found in the adult head and thorax but were not observed in any other tissue or stage in the life cycle. Interestingly, similar low-abundance *Sxl* proteins can be detected in females. These proteins comigrate with the species found in males and, as in males, are only detected in the head and thorax of adult animals.

What is the origin of these low-abundance *Sxl* pro-

teins? Based on their similar size and tissue distribution, it seems likely that the proteins observed in adult males and females are the same. Since they are smaller than the major female-specific proteins, one obvious explanation is that they arise by proteolytic cleavage of the full-length species. This is not an attractive possibility, at least for the proteins in males, since there is no apparent source of larger “precursor” female *Sxl* protein. Furthermore, experiments with a female *Sxl* cDNA expressed in adult males show that full-length *Sxl* protein can be synthesized in this sex and that such a protein is reasonably stable (Bell et al. 1991).

A more likely possibility is that these small proteins are encoded by an ORF that is somewhat shorter than but closely related to the ORF encoding the female-specific proteins. Such an ORF could be present in a very minor population of *Sxl* mRNAs that have structures different from the major male and female transcripts. Alternatively, these smaller proteins could be translated from an initiation codon located downstream of the normal start site in exon 2. Two in-frame AUGs are present in exon 4, which is downstream of the male-specific exon (Bell et al. 1988). The first of these is in a context that has a poor match to the fly consensus initiation region, while the context of the second AUG has a much better match to the consensus. Proteins initiating from the first codon would lack the first 38 amino acids of the full-length *Sxl* protein, while proteins initiated from the second would lack the first 48 amino acids. In either case, the predicted molecular mass of the translation products would closely correspond to that of the smaller low-abundance *Sxl* proteins.

Reinitiation of translation at an AUG downstream of a small ORF is not uncommon and has been documented for a number of eukaryotic mRNAs (for review, see Kozak 1989). However, since these minor immunoreactive species were also detected in females where *Sxl* RNAs lack this small upstream ORF, we consider it more likely that leaky scanning rather than reinitiation of translation causes the production of smaller products. Consistent with this possibility, the AUG in exon 2, which is the likely start site for the female-specific *Sxl* proteins, is in a context that has a poor match to consensus for initiation and, hence, might occasionally be skipped in favor of one of the downstream AUGs.

While these minor products are restricted in their tissue distribution to the heads and thoraxes of adult animals, this apparent tissue specificity need not signify a functional role for the proteins. Rather, it may reflect some tissue-specific features of the translational machinery that enhance leaky scanning. Whatever role these proteins might have, they are clearly unable to turn on the *Sxl* gene in the female mode. If they could, we would expect to observe female-specific mRNAs and proteins in male heads; however, we do not. The failure of these proteins to activate the female mode of splicing in males could be due to the fact that they are present at such low levels. Alternatively, as a consequence of their truncated structure, they may have no regulatory activity.

Sxl is not expressed in the embryonic germ line

Genetic studies have shown that the regulation and functioning of *Sxl* in the germ line differs in important ways from that in the soma (for review, see Pauli and Mahowald 1990). The results reported here also indicate a remarkable difference in the behavior of *Sxl* in these fundamentally distinct tissue types. The germ line is set aside early in *Drosophila* embryogenesis when the cleavage nuclei migrate to the periphery of the embryo. Nuclei migrating to the posterior end of the embryo form a special cluster of cells, the pole cells, while the remaining somatic nuclei undergo several more nuclear divisions before cellularization takes place. The decision to activate *Sxl* appears to be made shortly after the somatic nuclei first reach the periphery of the embryo since newly synthesized *Sxl* proteins can be detected by nuclear cycle 12. In contrast, *Sxl* is not activated at this time in the germ line, and no nuclear (or cytoplasmic) *Sxl* protein can be detected in the pole cells. The pole cells remain unstained through the cellular blastoderm stage and into gastrulation when they disappear from view by invaginating into the surrounding somatic tissue. These findings indicate that *Sxl* activation in the germ line must occur later in development than in the soma and support the notion that *Sxl* activation in the germ line must involve mechanisms that are different from those used in the soma.

Differential regulation of early Sxl expression

In spite of the presence of high levels of maternally derived *Sxl* mRNA in unfertilized eggs (Salz et al. 1989), our results indicate that little, if any, *Sxl* protein is deposited into the egg during the course of oogenesis. Moreover, since the maternal *Sxl* mRNAs were found to turn over in the first hours of embryogenesis (Salz et al. 1989) prior to the time when we first detected *Sxl* proteins, it seems likely that these maternal *Sxl* messages are not translated in the early embryo. These findings would resolve the issue of a *Sxl* maternal effect (Cline 1980; Oliver et al. 1988) and support the view that the initial response of the zygotic *Sxl* gene to the X/A ratio does not require maternally supplied *Sxl* activity. What mechanisms prevent the deposition of *Sxl* protein in the developing egg or the translation of the maternal *Sxl* mRNAs in the embryo remain to be determined.

The initial selection of the sexual development pathway in response to the X/A ratio of the zygote involves regulatory mechanisms that are distinct from those that maintain the determined state and control sexual differentiation. Genetic studies have implicated several loci in the activation process. These include the maternal-effect gene *da* and the zygotically acting numerator element *sis-b* (*sis-b* appears to correspond in large part to the T4 transcriptional unit of the *acheate-scute* complex; Torres and Sanchez 1989; Parkhurst et al. 1990; Erickson and Cline 1991). Our analysis of *Sxl* protein accumulation in early embryos provides molecular evidence that these loci play an important role in the early expression

of the *Sxl* gene. In progeny of *da* mothers *Sxl* protein accumulation is abnormal; instead of uniform staining of all somatic nuclei found in wild-type embryos, we observed a spatially variable staining pattern with many nuclei having no detectable *Sxl* protein. Similar results were obtained with a temperature-sensitive allele of *sis-b* (*sis-b^{sc3-1}*); in this case, the perturbations in *Sxl* expression were enhanced as the temperature was increased, and no protein could be detected under the most restrictive conditions. Consistent with a role for the *da* and *sis-b* gene products in the initial activation of *Sxl*, the abnormalities in *Sxl* antibody staining were evident as soon as protein was first detected in the syncytial blastoderm.

Although the results presented here show only that *Sxl* protein accumulation is affected by *da* and *sis-b* mutations, it is likely that *da* and *sis-b* gene products regulate transcription of the early *Sxl* gene. In previous studies we have identified a distinct set of embryonic *Sxl* mRNAs (Salz et al. 1989). They are transcribed from a female-specific promoter that is active for a brief period in the early embryo and encode protein products that are largely colinear with the late female-specific proteins (L. Keyes et al., in prep.). The *Sxl* proteins that are first detected in early embryos are probably the translation products of these embryonic RNAs. The early transcripts are initially expressed just prior to the time when *Sxl* antibody staining is first observed, whereas the late transcripts are expressed after *Sxl* antibody staining is already evident (L. Keyes et al., in prep.).

Genetic experiments have shown that sensitivity of *Sxl* activation to the dose of X/A numerator elements like *sis-b* is a feature that distinguishes this category of genetic elements from X/A signal transduction elements such as *da* that also control *Sxl* expression (Cronmiller and Cline 1986; Cline 1988a). *sis-b*, along with *sis-a* and perhaps other loci, are X-linked genes that function in the zygote to communicate the number of X chromosomes to *Sxl*. The counting process seems to be based on the specific dose of the gene products from each locus. In contrast, the maternally derived *da* gene product should be present at the same level in both male and female zygotes. It is not one of the variable components of the counting system but, rather, functions as a cofactor in the *Sxl* activation process. Our analysis here of the molecular effects of leaky mutations in *sis-b* and *da* on the early expression of *Sxl* suggest that these two different categories of elements may participate in rather different steps in the activation of *Sxl*. Reductions in the level of maternal *da* activity appear to affect the level of zygotic *Sxl* protein produced in an all-or-none fashion at the level of individual embryonic cells, generating a patchy *Sxl* staining pattern. In contrast, intermediate levels of *Sxl* protein are observed at the cellular level as *sis-b* activity is reduced. Since only a single allele each of *da* and *sis-b* was examined, neither of which is likely to generate an altered protein product (Cronmiller et al. 1988; T.W. Cline, unpubl.), it is possible that this difference reflects a peculiarity of one of these mutant alleles; alternatively, it could represent a fundamental difference

in how the *sis-b* and *da* products function in the activation of *Sxl* expression.

Sxl expression in some regions of the embryo appears to be far more sensitive to reductions in the level of *da* or *sis-b* activity than expression in other regions. In view of the difference between *da* and *sis-b* just mentioned above, it is curious that the pattern of sensitivity across the embryo is so similar for mutations in both genes. The fact that the expression of *scute-alpha* (*sis-b*) during the temperature-sensitive period for its effects on *Sxl* activation is spatially uniform (Carbrera et al. 1987; Romani et al. 1987), coupled with the fact that the nonuniform staining patterns are so similar for the two mutants, would argue against these regional differences arising from nonuniform distribution of functional *da* and *sis-b* products. Instead, it seems more likely that they reflect regional differences in the threshold of *da* and *sis-b* activity required for *Sxl* expression. Regional differences among cells in their sensitivity to the X/A balance have been suggested ever since the pioneering studies on the X/A signal by Dobshansky and Schultz (1934). It remains to be seen whether these differences have important functional implications for the operation of the sex determination system or are simply fortuitous consequences of the ways in which other regulatory and metabolic networks impinge on the sex determination system under genetically abnormal conditions.

Materials and methods

Strains and culturing

Flies were grown on a standard medium (Cline 1978) at 25°C unless otherwise indicated. All *Sxl* alleles (with their current, slightly modified designations), marker mutations, and balancers are described in Lindsley and Zimm (1985, 1986, 1987, 1990). The allele originally designated as *scute*^{3.1} is referred to here as *sis-b*^{sc3.1}, as discussed in Cline (1988a), as well as for the additional reasons (T.W. Cline, unpubl.) that hemizygous mutant females (viable as a consequence of a gain-of-function *Sxl* allele) display only a relatively weak neural phenotype that has combined *acheate* and *scute-alpha* character and is not temperature sensitive in any consistent fashion. The Bithorax Complex (Peifer et al. 1987) provides precedent for using different gene names to distinguish among genetically and developmentally separable regulatory elements that control the same transcription unit, as seems to be the case with *scute-alpha* and *sis-b* (Erickson and Cline 1991).

DNA techniques

The *lacZ-Sxl* fusion gene was constructed from a female-specific cDNA cF1 (Bell et al. 1988) by the following strategy. The original cDNA clone was cut internally with *BalI* at position 763 (see Bell et al. 1988) and in the 3' polylinker with *PstI*. The isolated 1.3-kb fragment was then inserted into the *HincII* and *PstI* cloning sites of Bluescript and recovered as a *XhoI-PstI* fragment that was cloned into the *SalI* and *PstII* sites of the pTRB expression vector (Bürglin and De Robertis 1987). This construct fuses *lacZ*-coding sequences in-frame with the 255 carboxy-terminal residues of *Sxl* linked by two artificially introduced residues. In addition, a 1.3-kb filled-in *BalI-EcoRI* frag-

ment of the same cDNA clone was inserted into the blunt-ended *BamHI* cloning site of pAR3039 (Studier and Moffat 1986) to obtain a $\phi 10$ -*Sxl* fusion gene. On induction, this gene produces a chimeric $\phi 10$ -*Sxl* protein fusing the 255 carboxy-terminal residues of *Sxl* downstream of the first 10 residues of the viral T7 gene $\phi 10$.

Generation of monoclonal antibodies

The *lacZ-Sxl* antigen was prepared by SDS gel purification from induced JM101 cultures. Female BALB/c mice were injected intraperitoneally with 100 μ g of this antigen in RIBI adjuvant (RIBI ImmunoChem. Research, Inc.). Spleen cells from these mice were isolated 3 days after the third boost and fused to SP2/0 myeloma cells in the presence of PEG (Boehringer Mannheim). Parental hybridoma lines were screened with partially purified $\phi 10$ -*Sxl* fusion protein using a fast procedure described by Hawkes et al. (1982). The resultant positives were confirmed by testing on immunoblots loaded with bacterial extracts containing induced $\phi 10$ -*Sxl* antigens and subcloned by limiting dilution.

Western blot analysis

Staged embryos were washed from food plates, dechorionated in 50% bleach, and washed again in phosphate-buffered saline prior to total protein extraction. Adult tissue was dissected in *Drosophila* Ringer's medium. Protein extracts of these tissues were essentially prepared as described in Driever and Nüsslein-Volhard (1988). They were frozen in liquid nitrogen and, while thawing, homogenized in 2 $\times$ loading buffer, containing 8 M urea. After incubation for 5 min at 95°C, insoluble material was sedimented and supernatant was sonicated in an ultrasonic bath for 30 min to solubilize the viscous DNA content. Subsequently, 10–15 μ l was applied to each slot on a 10% SDS-polyacrylamide gel. After electrophoresis, protein was transferred to nitrocellulose paper in Tris-glycine buffer for 2 hr at constant 250 mA. To assess the amount of total protein loaded in each lane, the blot was stained with Ponceau S red. After blocking with 5% low fat dry milk powder in TBS/0.05% Tween-20 (Sigma), the blot was incubated overnight with the first antibody (1 : 10 dilution of hybridoma supernatant in TBS, 0.05% Tween-20) at 4°C. For subsequent detection of antigen-antibody complexes on the blot, we used the alkaline phosphatase-conjugated anti-mouse kit from Promega.

Immunolocalization in whole-mount embryo

Embryos were dechorionized for 2–3 min in full-strength bleach, washed thoroughly in PBS, and incubated in heptane-saturated fixative (4% paraformaldehyde, Polysciences EM grade, in PBS) for 30 min at room temperature. The fixative phase was then removed and replaced with 90% methanol, 10% 0.5 M EGTA. After several vigorous shakes, the devitellinized embryos that sank to the bottom were collected and rinsed several times in the methanol-EGTA solution followed by rinses in plain methanol. For staining, embryos were gradually rehydrated in PBS and incubated with 1% BSA, 1% Triton X-100, in PBS, for 3–4 hr at room temperature. First antibody was applied as a 1 : 10 dilution of hybridoma supernatant (0.3 mg/ml of total protein) in PBS, 0.1% BSA, and 0.1% Triton X-100, and incubated overnight at 4°C. After several washes in the same buffer, embryos were treated with biotinylated second antibody for 2 hr at room temperature and subsequently with biotinylated HRP-avidin complexes according to the Vectastain protocol (Vector Laboratories). The bound complexes were visualized with a

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DAB detection kit from the same company. Photomicrographs were taken on Ektar 125 (Kodak) with a Zeiss photomicroscope.

Immunostaining of larval tissue

Salivary glands of third-instar larvae were dissected in *Drosophila* Ringer's medium and fixed in 4% paraformaldehyde, PBS, for 20 min at room temperature. Tissues were permeabilized and blocked by incubating in 1% BSA, 1% Triton X-100, and PBS for 4 hr at room temperature. Peroxidase staining proceeded as described for embryos (see above).

Acknowledgments

We are indebted to Marty Marlow for her excellent technical assistance in preparing monoclonal antibodies. We thank Eric Wieschaus for many stimulating discussions and for providing us with the *fog* and *R(1)w^{cV}* fly strains. Our thanks also go to all of our colleagues in the Schedl and Cline laboratories, particularly to Jeff Hager, Jamila Horabin, Linda Keyes, and Mark Samuels for sharing unpublished results and critical reading of the manuscript. This work was supported by grants from the National Institutes of Health, American Cancer Society, and March of Dimes to T.W.C. and P.S. D.B. received an EMBO postdoctoral fellowship.

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