A bZIP protein, Sisterless-a, collaborates with bHLH transcription factors early in Drosophila development to determine sex

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Sexual identity in Drosophila is determined by zygotic X-chromosome dose. Two potent indicators of X-chromosome dose are sisterless-a (sis-a) and sisterless-b (sis-b). Genetic analysis has shown that a diplo-X dose of these genes activates their regulatory target, the feminizing switch gene Sex-lethal (Sxl), whereas a haplo-X dose leaves Sxl inactive. sis-b encodes a transcriptional activator of the bHLH family that dimerizes with several other HLH proteins required for the proper assessment of X dose. Here, we report that sis-a encodes a bZIP protein homolog that functions in all somatic nuclei to activate Sxl transcription. In contrast with other elements of the sex-determination signal, the functioning of this transcription factor in somatic cells may be specific to X-chromosome counting. Using in situ hybridization, we determined the time course of sis-a, sis-b, and Sxl transcription during the first few hours after fertilization. The pattern of sis-a RNA accumulation is very similar to that for sis-b, with a peak in nuclear cycle 12 at about the time of onset of Sxl transcription. Considered in the context of other studies, these results suggest that the ability to distinguish one X from two is attributable to combinatorial interactions between bZIP and bHLH proteins and their target, Sxl, as well as to positive and negative interactions with maternally supplied and zygotically produced proteins.

[Key Words: Sex determination; X/A ratio; sisterless genes; Sex-lethal; bZIP proteins; bHLH proteins]

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In 1916 it was reported that genes are on chromosomes and that sex in Drosophila melanogaster is determined by the number of X chromosomes, with two X's signaling female development and one X signaling the male alternative (Bridges 1916). More than half a century later, the specific genetic elements of this quantitative developmental signal began to be identified (Cline 1988; Tortes and Sanchez 1989). Identification of these sex-determination signal elements awaited the discovery of their regulatory target, the X-linked gene Sex-lethal [Sxl] and the understanding that Sxl coordinates controls both sex determination and the vital process of X-chromosome dosage compensation (Cline 1979, 1983, 1984; Lucchesi and Skrisky 1981; Gergen 1987). Sxl controls all aspects of somatic sexual dimorphism through its effects on subordinate regulatory genes with more specialized functions (see McKeown et al. 1988; Gorman et al. 1993). Female differentiation is a consequence of Sxl being turned on in diplo-X individuals, whereas male differentiation takes place in haplo-X individuals because Sxl remains off. Genetic analysis showed that the sex-specific regulation of Sxl is already established by the blastoderm stage (Sanchez and Nöthiger 1983; Cline 1984; Gergen 1987), the point in Drosophila development when cells are first formed. Thereafter, Sxl activity is required continuously to maintain this early sexual pathway choice (Sanchez and Nöthiger 1982; Cline 1984; Maine et al. 1985). From the blastoderm stage through the rest of development, the on/off regulation of Sxl is at the level of RNA processing through the operation of a direct positive feedback loop involving the Sxl protein (Bell et al. 1988, 1991; Samuels et al. 1991). Sxl is transcribed in both sexes from a “maintenance” promoter, Pm (also called the late promoter), but only females splice the resulting transcripts into functional mRNAs, as only females have the Sxl protein that is required to remove a male-specific exon that would otherwise abort translation.

Although this RNA splicing control is an essential aspect of Sxl regulation throughout nearly all of development, the primary sex-determination signal itself does not act on Sxl RNA splicing but, rather, on Sxl transcription and does so before the cellular blastoderm stage (Keyes et al. 1992). This transcriptional effect involves a different Sxl promoter, Pe (for establishment or early), that is active only before cellularization, before the activation of SxlPm in both sexes. In contrast to transcripts derived from SxlPm transcripts derived from SxlPe are
spliced into functional mRNAs even in the absence of Sxl protein [Keyes et al. 1992]. The two X chromosomes in females activate Sxl<sub>pe</sub>, thereby generating a pulse of Sxl protein that will establish the productive Sxl RNA splicing mode once Sxl<sub>pe</sub> shuts down and Sxl<sub>pm</sub> becomes active. In contrast, males with their single X chromosome are unable to activate Sxl<sub>pe</sub>. As a consequence, when Sxl<sub>pm</sub> becomes active, no Sxl protein is present and transcripts are spliced nonproductively from that point on. The regulation of Sxl is summarized in Figure 1.

The fruit fly sex-determination signal has been viewed traditionally as an X/A ratio, since the dose of X chromosomes was shown to be counted with reference to the number of sets of autosomes [Bridges 1921, 1925]. With this model there would be female-determining "numerator" genes on the X chromosome acting as positive regulators of Sxl and corresponding male-determining "denominator" genes on the autosomes acting as negative regulators. It is now clear that the zygotic X chromosome dose is also measured with reference to a background of positive regulators of Sxl such as daughterless (da), and negative regulators of Sxl such as extramacrophaetae (emc), that are produced by the mother and supplied to the egg during oogenesis [Cline 1978, 1980; Younger-Shepherd et al. 1992].

Two strong numerator elements have been identified, sisterless-a and sisterless-b (sis-a and sis-b), whose genetic manipulation generates sex-specific lethal phenotypes attributable to misregulation of Sxl [Cline 1988; Torres and Sanchez 1989]. More recently, two weaker numerator elements have been discovered: runt [Duffy and Gergen 1991; Torres and Sanchez 1992] and sis-c [Cline 1993]. A gene called deadpan (dpn) acts as a negative regulator of Sxl and fits the criteria for a denominator element [Younger-Shepherd et al. 1992].

The first X/A element to be characterized at the molecular level was sis-b. sis-b function is provided by the protein product of the T4(scute) transcription unit of the Achaete-Scute Complex [Torres and Sanchez 1989, 1991; Parkhurst et al. 1990; Erickson and Cline 1991]. T4 encodes a member of the basic helix-loop-helix (bHLH) family [Villares and Cabrera 1987; Murre et al. 1989a]. bHLH proteins are transcriptional activators characterized by a HLH dimerization motif, a basic domain involved in DNA binding, and a promiscuous ability to form heterodimers [Murre et al. 1989a,b]. Remarkably, the two known maternal regulatory factors, da and emc, and the one autosomal zygotic factor, dpn, also encode HLH proteins [Murre et al. 1989a; Younger-Shepherd et al. 1992].

These discoveries led to the proposal that the X-chromosome counting process in Drosophila might involve subunit interactions between only positive and negative HLH proteins [Parkhurst et al. 1990]. The numerator element runt was an exception to this rule, as it encodes a transcriptional regulator of unknown biochemical function that is homologous to a proto-oncogene responsible for a subtype of acute myeloid leukemia [Kania et al. 1990; Daga et al. 1992]. However, runt is also different from sis-b in other ways: Its participation in the counting process is far weaker, and it only appears to participate in the sex signal over a spatially restricted domain of the young embryo [Duffy and Gergen 1991].

It is notable that none of these five genes is specific to sex determination and that all five are intimately involved in neuronal development or function. Because of this, it has been suggested that an entire neural regula-

![Figure 1. Model for the initiating events in sex determination.](genesdev.cshlp.org)
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tory network might have been co-opted during evolution for use as a primary sex-determination signal [Younger-Shepherd et al. 1992]. Curiously, even the closest homolog of $Sxl$ in the fly, $elav$ is involved in neural development (Robinow et al. 1988).

Is the other major numerator element of the sex-determination signal, $sis-a$, a protein-coding gene like $sis-b$, perhaps encoding yet another bHLH family member? Is $sis-a$ involved in vital processes unrelated to sex determination like so many other genes upstream of $Sxl$? Does $sis-a$ control $Sxl$ expression throughout the embryo, or only over a restricted spatial domain, like $runt$?

How is the timing of $sis-a$ expression related to that of other numerator elements, as well as that of the sex-determination target $Sxl$? How does the expression of $sis-a$ in the soma compare with that in the germ line? In this paper we present a molecular characterization of $sis-a$ that addresses these and other questions that are critical for understanding both the mechanistic details of sex determination and the evolutionary origin of the fruit fly sex determination signal.

**Results**

**Cloning and identification of $sis-a$ by P element-mediated transformation**

Extensive genetic mapping placed $sis-a$ at or near the distal breakpoint of $Df(1)N71$ [Cline 1986]. We used P element-mediated transformation to identify genomic clones from this region that were capable of complementing the female-specific lethal effect of the $sis-a^1$ mutation. Four different P element constructs (A1, A3, A4, A10, Fig. 2) were injected into Drosophila embryos, and multiple lines of each were tested for the ability to complement both the $sis-a^1$ mutation and $l(1)10Bb^7$, a lethal <0.01 cM away [locus 14 in Cline 1986]. We found that mutations in both genes were fully complemented by single copies of the four transgenes (Fig. 2, Table 1). Northern blots of early embryonic RNA from this region revealed two non-overlapping transcripts of ~1.1 and ~0.8 kb located close to the ends of the complementing region (Fig. 2). To determine the relationship of these transcripts to $sis-a$ and $l(1)10Bb$, we engineered frameshift mutations that altered the coding portion of each transcript (see Materials and methods) and introduced the mutant constructs into flies by P element-mediated transformation (Fig. 2, Table 1). Transgenic lines [$A10m1$], with the mutation affecting the 1.1-kb transcript, provided full $sis-a$ function but failed to complement $l(1)10Bb^7$. In contrast, transgenic lines with the mutation affecting the distal 0.8-kb transcription unit [$A10m2$] eliminated $sis-a$ function but complemented $l(1)10Bb^7$. Hence, the gene encoding the smaller transcript is $sis-a$. Moreover, it is distinct from the neighboring gene whose function is required for both sexes. Our analysis of this neighboring gene $l(1)10Bb$, which is a homolog of G10 of Xenopus [McGrew et al. 1989], will be presented elsewhere.

**sis-a appears to encode a transcription factor of the basic leucine zipper class**

We sequenced the 4.5-kb genomic region that contains $sis-a$ and $l(1)10Bb$, and sequenced a total of eight $sis-a$ cDNA clones from two early embryonic cDNA libraries. All eight cDNAs are collinear with the genomic DNA sequence: There are no introns (Fig. 3). Three independent $sis-a$ cDNAs were judged to be full length for the following reasons: First, the size [785 nucleotides without the poly[A] tail] is consistent with that predicted from Northern blots. Second, the 5' end of each cDNA begins 26 nucleotides downstream of a consensus TATAAA sequence and is preceded by a noncoded G residue [thought to indicate copying of the 5' cap by reverse transcriptase]. Finally, in RNase protection experiments, in vitro cDNA transcripts protected the same sized probe fragment as did in vivo mRNA (data not shown).

**Figure 2.** Genomic DNA tested for $sis-a$ function. A genomic restriction map of a 12-kb region around $sis-a$ is shown. The centromere distal end is to the left. HindIII [H], NheI [N], XhoI [X], SacII [S], MfuI [M], and NotI [Nt] sites are marked. The $Df(1)N71$ distal breakpoint is located between 1.1 and 3.7 kb from the left end of the map. The locations of the 0.8-kb $sis-a$ and the 1.1-kb $l(1)10Bb$ transcripts are shown. The genomic fragments used for P-element-mediated transformation are diagrammed below the map. The frameshift mutations in $P[A10m1(w^+)]$ and $P[A10m2(w^+)]$ are marked by X's.

| 0-2 hr transcripts | Genomic restriction map | Complementation: |
|---------------------|-------------------------|------------------|
| $P[aw^+]$ transgenes: | $H$ | $H$ | $H$ | $S$ | $MN$ | $MN$ | $Nt$ | $Nt$ |
| $P[A1a(w^+)]$ | | | | | | | | |
| $P[A3a(w^+)]$ | | | | | | | | |
| $P[A4a(w^+)]$ | | | | | | | | |
| $P[A10a(w^+)]$ | | | | | | | | |
| $P[A10m1a(w^+)]$ | | | | | | | | |
| $P[A10m2a(w^+)]$ | | | | | | | | |

| $sis-a$ | $l(1)10Bb$ |
|---------|------------|
| +       | +          |
| +       | +          |
| +       | +          |
| +       | +          |
| +       | +          |
| -       | -          |
| -       | +          |
| -       | +          |

[1] Y. Younger-Shepherd, M. Peer, and D. Wasserman, unpublished.
Table 1. Complementation of sis-a 1 mutant females by single copies of P-element transgenes

| Level of complementation | P[A1] | P[A3] | P[A4] | P[A10] | P[A10m1] | P[A10m2] | P[A10m3] |
|--------------------------|-------|-------|-------|--------|----------|----------|----------|
| Full                     | 21    | 5     | 4     | 5      | 7        | —        | 2        |
| Partial                  | —     | —     | —     | —      | —        | 3        | —        |
| None                     | —     | —     | —     | —      | 5        | —        | —        |

Female viability was 25%, 50% and 75% for the three partially complementing P[A10m3] lines and 70% for the defective P[A4] line. Crosses were of the form y w sis-a/Binskiys y x y w sis-a/1; P(w+, sis-a)/+ ∆∆ at 25°C. Complementation was scored as viability of y w sis-a/1 y w sis-a/1; P(w+, sis-a)/+ females relative to their y w sis-a/Binskiys; P(w+, sis-a)/+ sisters.

The predicted Sis-a protein begins at the first AUG in the mRNA and extends for 189 amino acids. There is a second potential start codon located 45 nucleotides downstream of the first. To determine whether the first AUG is the site of translation initiation, we changed this codon to CUG in the P[A10] vector, creating P[A10m3], and introduced this mutant construct into flies. Single copies of two of the five lines isolated fully complemented sis-a 1 mutant females, but the other three lines were reduced significantly in their ability to provide sis-a 1 function (Table 1). We believe that this reduction in sis-a complementation is a consequence of the mutation rather than the sites of insertion of the constructs, because of 36 independent nonmutated lines derived from the constructs shown in Figure 2, only one showed reduced rescue of sis-a 1 (70% rescue). Because of the loss of the first potential initiation codon reduced, but did not eliminate, sis-a 1 activity, we conclude that the 189-amino-acid form is the wild-type Sis-a protein but that its first 15 amino acids are not absolutely required for sis-a 1 function.

The predicted 21-kD Sis-a protein is highly charged and basic (predicted pl = 9.5). A striking run of six charged residues from 800-811.

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define dimerization specificities (O'Shea et al. 1992; Vinson et al. 1993). Sis-a is predicted to have only a limited ability to form such salt bridges. That fact, combined with the atypical sequence at the α positions, suggests that Sis-a may have a novel dimerization partner. In common with many transcriptional activators, the amino terminus of Sis-a is rich in acidic, Ser, Gln, and Pro residues.

sis-a activates Sxl in all parts of the embryo

Although all female somatic nuclei activate SxlP in response to the diplo-X dose of numerator elements, there are regional differences in the sensitivity of cells to perturbations in individual genes that contribute to the sex determination signal (Bopp et al. 1991; Keyes et al. 1992).

To determine whether the bZIP protein encoded by sis-a is necessary for the activation of SxlP in all or only in a subset of nuclei, we examined the effect of the sis-a1 mutation on the expression of β-galactosidase from a SxlP--lacZ fusion (Keyes et al. 1992). We stained 429 progeny of a cross between sis-a1/Y females and sis-a2/Y males, all homozygous for a P[SxlP--lacZ] reporter construct. Three staining classes of 3- to 5-hr-old embryos were observed in the following proportions: 26% had the strong, relatively uniform β-galactosidase-staining characteristic of wild-type females; 66% resembled wild-type males in failing to activate the SxlP--lacZ fusion in any cells; and 8% stained weakly, with β-galactosidase present only in the posterior of the embryos. Assuming that the male progeny, which never stain, accounted for 215 (50% of 429) of the 283 unstained embryos, we estimate that the sis-a1 mutation prevented the expression of the SxlP--lacZ fusion in two-thirds of the homozygous sis-a mutant female embryos and greatly reduced its expression in the remainder. Thus, like sis-b, but unlike runt, sis-a appears to be required in all somatic nuclei for the proper activation of Sxl.

The female dose of the sisterless genes causes male-specific lethality

A key aspect of the genetic characterization of the Drosophila sex-determination signal was the demonstration that increasing simultaneously the dose of sis-a+ and sis-b+ to the normal female value can kill chromosomal [haplo-X] males by causing them to activate Sxl. These studies were complicated because chromosomal rearrangements were used [duplications] that necessarily altered the dose of a large number of genes besides sis-a and sis-b. The fact that a null Sxl allele did not fully rescue males from this “double-sis-+–duplication” effect was attributed to a nonspecific effect of aneuploidy caused by the chromosomal duplications, rather than to the possible existence of additional vital targets of the sex-determination signal besides Sxl (Cline 1988). The availability of sis-+ transgenes now allows the manipulation of sis+ gene dose without changing simultaneously the dose of many other genes unrelated to sex determination; consequently, we can test whether a mere doubling of the dose of sis-a+ and sis-b+ in males can lead to lethality and whether such lethality is the result of ectopic activation of Sxl alone. Toward this end, we examined the effects of 10.5-kb P(sis-b+–) duplications generated earlier (Erickson and Cline 1991) in combination with the newly isolated 12-kb P(sis-a–) transgenes.

A double-sis-–duplication second chromosome carrying one sis-b+ and one sis-a+ transgene showed the dominant, male-specific, cold-sensitive lethality found previously for a second chromosome carrying enormously larger X-chromosomal duplications of these two genes. As in earlier experiments, male survival was influenced by the genetic background. It ranged from a low of <0.3 % in a cross to a y w stock to a high of 15% in a w1118 stock at 18°C [see Genetic analysis in Materials and methods]. Moreover, as had been the case with the chromosomal duplications, escaper males were generally sterile and often [33%] displayed a characteristic phenotype: missing external genitalia and analia. As expected, females were unaffected by the transgene-bearing second chromosome.

Data in Table 2 show that for the transgene duplications, the male-lethal effects of increased sis+ dose were suppressed completely by a Sxl null mutation, even when the sis+ dose was increased above the normal female value. In these crosses, an X-linked P(sis-b+) duplication was included in addition to a variety of different double-sis+-duplication second chromosomes;

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**Figure 4.** sis-a encodes a protein of the bZIP class of transcription factors. The sequence of the presumed bZIP motif of Sis-a is compared with a variety of bZIP proteins including six Drosophila sequences. Highly conserved amino acids among the bZIP proteins (including the invariant Asn and Arg residues) are shown against a black background. In the consensus sequence, B designates either Lys or Arg. The Lys residue that is changed to Glu by the mutation on the expression of β-galactosidase from a Sxl fusion (Keyes et al. 1992). Data in Table 2 show that for the transgene duplications, the male-lethal effects of increased sis+ dose were suppressed completely by a Sxl null mutation, even when the sis+ dose was increased above the normal female value. In these crosses, an X-linked P(sis-b+) duplication was included in addition to a variety of different double-sis+-duplication second chromosomes;
**Table 2. Sxl null mutation suppresses the male-specific lethality caused by multiple copies of sis-a+ and sis-b+ transgenes**

| P(sis+) duplications | Sxl\(^{+}\) P(sis-b\(^{+}\)) | Sxl\(^{-}\) P(sis-b\(^{+}\)) |
|-----------------------|-----------------------------|-----------------------------|
| PP/+/+/+/CyO          | PP/+/+/+/+/CyO              |
|                       |                             |
| P[Al(sis-a\(^{+}\)]5 + P[Al(sis-a\(^{+}\)]/2 | 0 | 124 | 124 |
| P[Al(sis-a\(^{+}\)]/89 + P[Al(sis-a\(^{+}\)]/I | 0 | 93  | 95  |
| P[Al(sis-a\(^{+}\)]/4 + P[BS(sis-b\(^{+}\)]/22 | 0 | 139 | 134 |
| P[Al(sis-a\(^{+}\)]/6 + P[BS(sis-b\(^{+}\)]/3 | 0 | 91  | 113 |

Crosses were at 18°C and of the form\(\Phi \Phi \Phi \Phi\) and of the form\(\Phi \Phi \Phi \Phi\) and of the form\(\Phi \Phi \Phi \Phi\). A 1:1:1:1 ratio of progeny is expected in the absence of lethal effects.

*aOnly males nonrecombinant for the cm-ct interval (1.1 cm) are recorded."

therefore, the experimental males carried either two extra doses of sis-a\(^{+}\) and one extra sis-b\(^{+}\) or one extra sis-a\(^{+}\) and two extra sis-b\(^{+}\). Even with these three extra doses of sis\(^{+}\), and even at low [least permissive] temperature, males with no functional Sxl gene were fully viable, in contrast with their Sxl\(^{+}\) brothers who invariably died. These results argue strongly that Sxl is the only significant target of the somatic sex-determination signal.

**Duplications of sis-a\(^{+}\) and sis-b\(^{+}\) are not sufficient to activate Sxl\(_{pe}\) to the female level in all tissues**

To what extent does doubling the dose of just the two strongest numerator elements, sis-a and sis-b, in animals with one X chromosome, mimic the effects on the Sxl establishment promoter of doubling the number of entire X chromosomes? To address this question we examined the progeny of a cross between females homozygous for the Sxl\(_{pe}\)-lacZ fusion and Sxl\(^{-}\) males carrying one copy of a double-sis\(^{-}\) duplication second chromosome. Under the conditions used, 12% of the double duplication males survived to adulthood (but were sterile). The abnormal pattern of \(\beta\)-galactosidase expression [Fig. 5B] easily distinguished sons carrying the two extra copies of sis\(^{+}\) from both of their sisters [Fig. 5C] and their nonduplication brothers [Fig. 5A]. These male embryos expressed little or no \(\beta\)-galactosidase anteriorly but produced levels of \(\beta\)-galactosidase that were often at, or near, the female level in the posterior. This posterior bias in the activation of the Sxl\(_{pe}\) reporter construct by extra copies of the sis genes in males is reminiscent of the posterior bias in residual expression of Sxl\(_{pe}\)-lacZ for females who are homozygous for sis-a\(^{+}\).

Like sis-b, sis-a is one of the first genes to be transcribed in the embryo; unlike sis-b, sis-a is not expressed in somatic cells at later stages.

The developmental profile of sis-a mRNA was determined using RNase protection assays [Fig. 6]. The other major numerator element, sis-b, is included for comparison. We found that sis-a and sis-b were expressed zygotically at the earliest time point examined, 0–2 hr after fertilization. The absence of these transcripts from adult females (and the results of in situ hybridizations, see below) established that these early transcripts were zy-
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Figure 6. Developmental profile of sis-a expression. RNase protection was used to monitor the amount of sis-a and sis-b mRNAs at the indicated stages. The sis-a, sis-b, and rp49 hybridizations each contained ~0.4, 0.8, and 0.05 μg poly(A)^+ RNA. Preadult RNA samples were from mixed sex populations.

gotic and not maternal in origin. Both mRNA species increased in amount during the 2- to 4-hr period and remained relatively constant through the first half of embryonic development. They disappeared ~10–12 hr after fertilization. scute (sis-b) mRNA reappeared in the third-instar and pupal collections, consistent with its proneural function in imaginal discs. In contrast, sis-a did not appear to be expressed after the first half of embryogenesis.

A much closer examination of sis gene expression was made by in situ RNA hybridization during the period of early embryogenesis when the sex-specific expression of Sxl is set by the X-chromosome dose. This study also included a parallel examination of expression from the sex-determination signal target Sxl. This detailed analysis showed that the differences in expression between sis-a and sis-b are far greater than had been suggested by RNA analysis on whole embryo homogenates. The early fly embryo is characterized by a series of rapid nuclear divisions that occur in the interior of the fertilized egg (Poulson 1950; Sonnenblick 1950; Foe and Alberts 1983). After the sixth nuclear division cycle, most of the nuclei begin a stepwise migration to the periphery to form the syncytial blastoderm 1 min into cycle 10. The first nuclei to reach the surface bud off and form the pole cells, which are the precursors to the germ cells of the fly. The somatic nuclei divide three more times at the surface before cellularization during cycle 14. The nuclei that remain behind in the center of the embryo cease to divide after cycle 10 and become polyplloid. These polyplloid yolk nuclei are believed to be involved in the utilization of the egg yolk and make no direct contribution to the structure or tissues of the embryo. Limited zygotic

Figure 7. In situ hybridization to sis-a transcripts in whole-mount embryos. Wild-type embryos were prepared and hybridized with a digoxigenin-labeled antisense RNA probe to detect sis-a mRNA. Embryos at nuclear cycles 5, 8, 10, 12, and 14 and during germ band extension (gbe) are shown. Precellular embryos were staged as described in Materials and methods. sis-a expression was first detected in cycle 8. The cycle 8 embryo is overstained relative to the others to emphasize the onset of sis-a transcription. The intense staining in later embryos represents expression from the polyploid yolk nuclei.
transcription is generally thought to begin as the nuclei reach the periphery, with the bulk of the zygotically expressed genes becoming active during cellularization (Lamb and Laird 1976; Zalokar 1976; Anderson and Lengyel 1979).

sis-a transcripts were first clearly detected at the end of the eighth nuclear cycle just before to the arrival of the pole cell nuclei at the surface of the embryo (Fig. 7). Initially, all of the embryonic nuclei expressed sis-a mRNA; however, as the pole buds formed during cycle 9, sis-a expression was extinguished in the prepole cell nuclei. The sis-a mRNA was not freely diffusible during these early cycles. It appeared to be associated closely with the somatic and yolk nuclei through nuclear cycle 10. By nuclear cycle 12, however, the sis-a transcripts were distributed relatively uniformly in the somatic (peripheral) portion of the embryo and were no longer associated with the nuclei. The sis-a mRNA reached its maximum level during late cycle 12 and early cycle 13 and decayed rapidly thereafter; therefore, by early cycle 14, when somatic cells first begin to form in the embryo, there was very little sis-a mRNA at the periphery. This condition persisted after cellularization, indicating that there is little or no expression of sis-a in either the somatic or the germ cells of the embryo.

In contrast with the somatic nuclei, which expressed little or no sis-a after cycle 14, the yolk nuclei accumulated high levels of sis-a mRNA from cycle 8 until they degenerated some 10–11 hr after fertilization. The mRNA produced from the yolk nuclei did not diffuse throughout the yolk but, instead, appeared to be associated with the “islands of cytoplasm” that have been reported to surround the yolk nuclei (Poulson 1950, Sonnenblick 1950).

The expression of sis-b in the peripheral portion of early embryos was very similar to that seen for sis-a; moreover, sis-b was also expressed only in somatic nuclei. We could first reliably detect sis-b mRNA at cycle 9. As with sis-a, the earliest sis-b transcripts were distributed nonuniformly, maintaining an association with the nuclei through cycles 9 and 10. The sis-b mRNA increased in amount and became uniformly distributed during cycle 11 and peaked abruptly in cycle 12 (Fig. 8). The peak was followed by a rapid decay; therefore, by early cycle 14 we could detect only traces of sis-b mRNA. At the end of cycle 14 and subsequently during gastrulation, the proneural scute pattern of expression developed. In contrast with sis-a there was no strong sis-b expression in the yolk nuclei.

The importance of this early sis-b expression was demonstrated by the remarkable coincidence between the time course of sis-b mRNA accumulation and the temperature-sensitive period of the allele sis-b sc3-I (Fig. 9). This allele is profoundly temperature sensitive for its sex-determination function but not for the proneural function required for adult bristle development, apparently as a consequence of a defect specifically affecting early expression (Parkhurst et al. 1993; see Genetic anal-

![Figure 8](https://example.com/figure8.jpg)
sis-b
transcript levels appear to be proportional
to gene dose

At what level does the signal amplification occur that is responsible for the remarkably nonlinear response of Sxl to the dose of the sisterless genes? Our in situ RNA hybridization results suggest that it must occur after sis gene transcription. We were able to discern small differences in staining intensity between embryos at the same stage of development throughout the period when the sis genes were expressed, but in most cases it was impossible to classify reliably the embryos as belonging to the lighter or darker staining classes. However, in late cycle 12, when the sis mRNA levels peaked, we could distinguish approximately equal numbers of lighter and darker stained embryos that appeared subjectively to differ by about twofold in staining intensity. Given the rapid changes in mRNA levels and the quantitative limitations of the technique, we cannot be certain that the lighter embryos were the one-dose males and the darker embryos the two-dose females, but the equal numbers in the two staining classes is suggestive.

The Sxl establishment promoter becomes active as sis expression peaks

The first description of early Sxl transcription indicated that Sxl\_pe-derived transcripts could be detected first at cycle 9, peaked in cycle 12, and declined to low levels by the end of cycle 14 (Keyes et al. 1992). We found it difficult to reconcile those results with our data for the timing of sis-a and sis-b expression and with the result that runt mRNA first appears around cycle 12 [M. Klingler and P. Gergen, pers. comm.]. For this reason, we re-examined the time-course of expression from Sxl\_pe using precisely the same conditions that we used to examine sisterless gene expression. In contrast with the earlier report, we detected Sxl expression first in cycle 12 embryos, the stage at which the sis-a and sis-b mRNAs were near their maximum levels. Sxl mRNA increased in abundance in female embryos during cycles 13 and 14, reaching peak levels early in cycle 14. By the end of cycle 14 the transcripts from Pe began to decay, leaving only low levels of Sxl\_pe mRNA that persisted into the early germ band-extended embryos. In the cycle 12 embryos much of the staining was apparent as two tiny dots in the somatic nuclei (only the female embryos stained with the Sxl\_pe probes). These nuclear dots are thought to represent nascent transcripts and have been observed for a number of genes (Shermoen and O’Farrell 1991). We did not observe such putative nascent transcripts for sis-a, perhaps because of the much smaller size of the primary transcript (0.8 vs. 8.5–16 kb). The Sxl transcripts were present both in the cytoplasm and as nuclear dots in cycle 13 embryos, indicating that the gene was actively transcribed at that time. The Sxl transcripts appeared to be entirely cytoplasmic by early cycle 14, indicating that Sxl\_pe was no longer active.

Discussion

One of the first things that a Drosophila embryo does is determine its sex by counting its X chromosomes. This urgency may reflect the importance of achieving X-linked gene dosage compensation as soon as zygotic gene expression commences in earnest at the cellular
blastoderm stage. The earliest manifestation of sex determination is the transcriptional activation of the female-specific Sex- lethal gene by the action of a diplo-X dose of X-linked numerator elements that include the sisterless genes. The molecular characterization of sis-a allows comparisons to be made between this X/A numerator element and sis-b, the other major numerator element whose molecular nature is known. These comparisons provide new insights into the general features of the Drosophila sex-determination signal and the mechanism by which it acts. Perhaps the most important simplifying result is that like sis-b, sis-a functions as part of the sex-determination signal by virtue of the protein product it encodes. Moreover, that protein product appears to be a member of a family of DNA-binding transcription factors, although not the same family in which the sis-b gene product belongs.

Major numerator element genes are expressed early, but only in somatic nuclei

In situ RNA hybridization results reported here show that the somatic expression of both sis-a and sis-b begins at nuclear cycles 8–9, before the point when cleavage stage nuclei have reached the periphery of the egg, and that expression peaks late in nuclear cycle 12. The time course of sis-a transcription during this early period is very similar to that of sis-b, suggesting that their gene products act directly on Sxl and not in a cascade with one sisterless gene acting on another. Analysis of sisterless gene transcription in sis null mutant embryos will be required to establish this point.

Our results for the onset of sis-b expression differ in some respects from those presented by Parkhurst et al. [1993]. They reported that sis-b expression begins in cycle 3 and that the early transcripts are uniformly distributed throughout the embryo. This would represent the earliest zygotic expression of any wild-type gene in Drosophila. In contrast, we found that embryos from cycles 1 to 7 were not distinguishable from one another in staining intensity. Moreover, we found that like the earliest sis-a transcripts, the earliest sis-b mRNAs were closely associated with the embryonic nuclei during cycles 9 and 10, a pattern of mRNA localization observed for many early expressed genes in Drosophila [Pignoni et al. 1992; D. Pritchard and G. Schubiger, pers. comm.]. The increase in sis-b mRNA concentration from cycle 3 to 12 reported by Parkhurst et al. [1993] is modest considering that the number of nuclei increase ~500-fold during this period. In contrast, we observed an abrupt and dramatic increase in sis-b mRNA levels during cycles 9–12, which appears more consistent with the exponential increase in nuclear number that occurs during this time.

Whatever the explanation for these discrepancies, the sharp temperature-sensitive period (TSP) for sis-b^pec3-1 suggests that regardless of when sis-b transcription is initiated, it only becomes important for sex determination after nuclear cycle 9. The sis-b^pec3-1 TSP begins in cycle 9 and ends by cycle 14, with the most sensitive period occurring during the time that both we and Parkhurst et al. [1993] observed maximum levels of sis-b mRNA. The onset of the TSP was particularly clear. Diplo-X embryos shifted down to the permissive temperature between 0 and 60 min after egg deposition were perfectly viable, and even those shifted at 60–90 min (cycles 8–11) were only slightly less viable than their control siblings. Still longer exposures at the nonpermissive temperature caused dramatic reductions in female viability. The near perfect coincidence between the sis-b TSP and expression results reported here established the functional importance of this early burst of sis-b expression. These results are consistent with an earlier TSP determination of what is surely a related dominant lethal effect of sis-b^pec3-1 in females that are heterozygous for Sxl^− [Torres and Sanchez 1991].

Expression of the sisterless genes preceded the expression of their target, Sxl, by about three cell cycles: We first detected Sxl transcripts at cycle 12 near the time that the sis mRNA levels peaked. This onset is somewhat later for Sxl transcription than was reported by Keyes et al. [1992]. The basis for this discrepancy is not clear. It could reflect differences between the fly stocks used or differences in the criteria used to stage early embryos. Our embryo preparations were stained with DAPI so that number and density of nuclei could be determined readily, whereas Keyes et al. [1992] relied on the appearance of pole cells in embryos that had been heavily proteolyzed. It seems unlikely that the discrepancies were caused by sensitivity differences, as both the onset of Sxl transcription and the peak of mRNA accumulation in our experiments were shifted relative to those reported by Keyes et al. [1992]. In any event, the present result is more consistent with expectations based on the study of the two sis genes that was done in parallel on the same collections of embryos.

It was reported earlier that Sxl^pe is never active in germ cells [Keyes et al. 1992]. The in situ RNA analysis that we report here suggests why this might be. The first nuclei to reach the cortex form the pole cells that establish the germ line of the organism. Although sis-a transcription begins in cleavage-stage nuclei before the nuclei have reached the egg cortex, sis-a expression is extinguished when they do arrive to bud off as pole cells. In contrast, when the rest of the nuclei reach the cortex at the beginning of cycle 10, they continue to express sis-a for some time. The same appears to be true for sis-b, but because its mRNA appears to be expressed slightly later and is not as localized as that from sis-a, the shutoff as pole cells form is not as striking. It remains to be determined whether it is the shutoff of numerator element expression per se that is responsible for the lack of activation of Sxl^pe in diplo-X pole cells and whether that shutoff is the active consequence of an inhibitor in the pole plasm or a passive consequence of the lack of an activator in this specialized cytoplast.

sis-a activates Sxl transcription in all somatic cells

Because all somatic cells determine their sex in response
to the dose of X chromosomes, one might have thought that numerator elements affect Sxl expression in all nuclei. Such an expectation was met by sis-b (Bopp et al. 1991). The results with the weaker numerator element, runt, however, gave a surprisingly different result (Duffy and Gergen 1991). Null mutations only affected Sxl expression over the central region of the embryo and, even then, did not invariably abolish Sx expression within this domain, consistent with this gene’s participation in the sex-determination process somewhat less central.

Using a Sxlpe-lacZ reporter construct, we show here that the strong numerator element sis-a behaves like the strong element sis-b in affecting Sxl expression throughout the embryo. Even with the non-null sis-a' allele, expression of Sxlpe was abolished in the majority of homozygous mutant female embryos.

Developmental specificity of sis-a and its potential implications for Sxl regulation

To understand how genetic elements that participate in the sex-determination signal work and how they were recruited during the course of evolution, it is important to determine the extent to which these elements participate in other aspects of development besides sex determination. It is striking that of the six elements of the sex signal that had been characterized previously at the molecular level, only Sxl is truly sex specific in its function. The molecular characterization of sis-a presented here suggests that the only essential function of sis-a may be its role in sex determination. The mutant lesion responsible for the female-lethal phenotype of sis-a is shown to be a missense change that affects a residue in the conserved DNA-binding domain of this bZIP protein. This is not the kind of lesion one would expect to differentially affect the functions of a multifunctional protein product if these functions all involve DNA binding. Unlike the other molecularly characterized elements of the sex signal, sis-a expression ceases before the nuclei of the young embryo become cellularized. The only sis-a expression that continues after the somatic activity state of Sxl has been determined is in the yolk nuclei. These nuclei are atypical: They never migrate to the egg cortex, and they degenerate midway through embryogenesis without becoming cellularized. It is not known whether they are necessary for development, or what their function might be. If sis-a has a non-sex-specific function, it is most likely to involve the yolk nuclei. Indeed it may be that sis-a was recruited as part of the sex-determination signal during evolution because it already had a very early function in yolk utilization.

The temporal and spatial specificity of sis-a expression may be responsible for the timely shutoff of Sxlpe that is critical for the proper operation of the sex-determination signal. The other known components of the sex signal are expressed at later stages and become dosage compensated in connection with their other functions in development. If Sxlpe were to remain responsive to the numerators after the onset of dosage compensation, at least some male cells would be in danger of inappropriately expressing Sxlpe. The fact that sis-a transcripts disappear just as Sxlpe shuts off raises the possibility that it is specifically the lack of Sis-a protein throughout the rest of development that prevents the reactivation of Sxlpe.

Implications of the sis-a study for the mechanism of X-chromosome counting

How is the twofold difference in sis gene dose amplified into an all-or-none, switch-like response by Sxlpe? The fact that sis transcripts levels are roughly proportional to sis gene dose suggests that amplification is not at the level of sis gene transcription. The timing of sis-a and sis-b expression suggests that they both act directly at Sxlpe. Considered in light of the fact that sis-a and sis-b encode different categories of transcription factors, this implies that amplification may occur as a consequence of there being qualitatively different sites for different transcriptional activators at Sxlpe. If each of these different classes of sites were also present in multiple copies, there would be additional opportunities for signal amplification. Herschlag and Johnson (1993) have recently reviewed the variety of ways that multiple transcription factor-binding sites at a regulated promoter can generate nonadditive effects on transcription that might be responsible for establishing sharp response thresholds for developmental signals. There are precedents in Drosophila for the nonlinear response of a developmentally regulated promoter being caused by repeated factor-binding sites, often for different classes of transcription factors (Driever et al. 1989; Struhl et al. 1989; Jiang et al. 1991; Ip et al. 1992a,b; Small et al. 1992; Jiang and Levine 1993).

Opportunities for signal amplification may also be provided by positive and negative interactions among the various subunits of these multimeric regulatory proteins before their interaction with Sxlpe. It seems likely that the bHLH proteins Sis-b and Da form heterodimers that bind to and activate Sxlpe since this protein heterodimer has been shown to bind DNA efficiently in vitro (Cabrera and Alonzo 1991; Van Doren et al. 1991). On the other hand, since the dose of da is the same in both sexes, this positive interaction by itself is unlikely to be involved in signal amplification (but see below). The sequence of the putative Sis-a leucine zipper frustrates attempts to predict what its possible dimerization partners might be (O’Shea et al. 1992; Vinson et al. 1993). Moreover, this sequence gives no indication that Sis-a is likely to form functional homodimers that might serve as an amplifying step.

Discrimination between the male and female sis gene doses is likely to involve negative interactions among their subunits before binding at Sxlpe. The two known negative signal elements, the HLH proteins Emc and Dpn, are believed to prevent or alter the DNA binding of Sis-b by dimerizing with it and preventing its association with Da protein (Van Doren et al. 1991). These or other inhibitory proteins could serve both to set the activation threshold for Sxlpe at an optimum for discrimination and to amplify the relative male/female difference in numer-
Materials and methods

DNA analysis and germ-line transformation

DNAs were isolated from each transformant and digested with restriction enzymes where indicated. EcoRI, EcoRI-SacII, SacII, and EcoRI-SacII digests were analyzed on 0.8-mm 1% agarose gels. Nonradioactive Southern blotting was performed using DIG-labeled probes generated by PCR. DIG-labeled probes were generated from a full-length eDNA template. The PCR products from each transformant and clone were sequenced using a 373 DNA sequencer. DNA sequences were analyzed using the program PHRED (Ewing et al. 1998). At least two independent PCR amplifications were sequenced, and the results were consistent. Analogous nonradioactive Southern blots and PCR results were obtained using an M13 forward primer 5'-GTAAAAACGACGGCCAGT-3' that was synthesized and digoxigenin labeled. PCR conditions were as follows: 30 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes. PCR products were analyzed on 1% agarose gels. 13-Galactosidase histochemistry was performed essentially as described (Kammen et al. 1984; Lindsley et al. 1988). Sequencing was done by standard methods (Sambrook et al. 1989). Cosmid (pWE15) clones containing Oregon-R DNA from the 10B4-6 region were generously provided by Richard Binari and Norbert Perrimon (Harvard University, Cambridge, MA). The fragments analyzed by gel-mine transformation were cloned into a derivative of the w+ P-element vector Casper (Pirrotta 1988) in which a NotI site replaced the BamHI site. Transformants were obtained by coinjection with the pTurbo transposable source (provided by R. Steward, Princeton University, NJ) using standard techniques (Spradling 1986). The genomic restriction fragments carried by each transformant are P[A1(w+ , sis-a+)] 12-kb NotI-Sau3A; P[A3(w+ , sis-a+)] 9.0-kb NotI–HindIII; P[A4(w+ , sis-a+)] 7.3-kb Sau3A–MluI; P[A10(w+ , sis-a+)] and its mutant derivatives P[A10m1], P[A10m2], and P[A10m3] 6.4-kb NheI–NheI. Mutations were created in plasmid subclones [pKS(+)] [Stratagene] and recloned into the larger P[A10] vector. The 4-bp insertion in P[A10m3] was made by filling in an XhoI site resulting in a frameshift after the amino acid codon 22 of l(1)T08Bb. P[A10m2] carried a 2-bp deletion that alters the sis-a reading frame after the amino acid 96 and was made by blunt ending the SacII site with T4 DNA polymerase. The mutation in P[A10m3] that changed the sis-a initiation codon to CUG was introduced using the method of Kunkel (1985) with a mutagenic primer AAAAATCACCCTGGAACGGA. cdNA clones were isolated from the ugt10 E6 library [3- to 12-hr embryo] of Poole et al. (1985) and a ugt10 cdNA library [0- to 4-hr embryo] of M. Noll (unpubl.). cdNA inserts were subcloned as EcoRI fragments into pKS(+) [Stratagene] for sequence analysis. For sequence analysis of the sis-a allele, a 1028-bp fragment was PCR amplified from a sis-a mutant male DNA. Clones from three independent amplifications were sequenced from position 11 to 766 with identical results. The only difference from the wild-type sequence in Figure 3 was the single nucleotide change at position 547.

RNase protection

Poly(A)+ RNA was prepared [Samuels et al. 1991] from Oregon-R adults, pupae, and staged embryos and larvae. RNase protection experiments were done as described [Sambrook et al. 1989]. The 750-nucleotide [EcoRI–SacII] genomic sis-a antisense probe protected the same 352-nucleotide 5' end fragment with both poly(A)+ RNA and RNA synthesized in vitro from a full-length cdNA template. The sis-b [Villares and Cabrera 1987] and rp49 [O’Connell and Rosbash 1984] probes protected internal fragments of 340 and ~350 nucleotides. The source of the two bands in the sis-b lanes in Figure 6 is not known. All experiments were confirmed to be in probe excess.

In situ analysis of transcription

The P[w+ , Sxlpe–lacZ+] transgene is similar to that described in Keyes et al. (1992). It carries a 3.7-kb Sall–BglII fragment that ends 44 nucleotides downstream of the start of Sxl exon E1 fused to a chimeric ADH–lacZ+ open reading frame. β-Galactosidase was assayed as described [Klämbt et al. 1991]. In situ hybridizations and digoxigenin labeling were done using modifications [Harland 1991; G. Panganiban (University of Wisconsin-Madison), pers. comm.] of the procedure of Tautz and Pfeifle (1989). Double-stranded DNA and single-stranded RNA probes containing the entire coding regions were used with similar results for sis-a and sis-b. Sxl expression was examined with RNA probes corresponding to either the embryo-specific first exon (specific for Sxlp–derived transcripts, Keyes et al. 1992) or to a region common to all Sxl cdNAS [exons 2, 4, 5, and 6; Samuels et al. 1991]. Similar results were obtained with both

A bZIP homolog in Drosophila sex determination

...atior products if they sequester proportionately more activator in males than in females [Parkhurst et al. 1990; Parkhurst and Ish-Horowitz 1992, Younger-Shepherd et al. 1992]. No analogous candidates for negative leucine zipper partners for Sis-a have been identified. Although such anti-Sis-a zippers may yet be found, it is possible that the HLH inhibitors Emc and Dpn function to partially sequester Sis-a [Bengal et al. 1992], or even that other "nonzippers" proteins sequester or otherwise inhibit Sis-a [Schule et al. 1990; Yang-Yen et al. 1990].

One interesting possibility raised by our analysis of sisterless and Sxl expression is that the negative factors, whatever their identity, may not function at the same time. If it is assumed that sis mRNA levels are predictive of Sis protein levels, our data suggest that Sxlpe+ becomes active after a threshold concentration of the Sis proteins is exceeded in females during cycle 12. Is this initial cycle 12 threshold of Sis protein reached by males as they proceed into cycle 13, and if so, how do they avoid activating Sxl? Curiously cycle 13 is the point when the denominator (negative) element dpn becomes active in the zygote [Bier et al. 1992, Younger-Shepherd et al. 1992]. Perhaps the specific role of zygotic dpn and other denominators is to raise the activation threshold for Sxlpe+ as the levels of Sis protein increase, keeping that threshold above a level that would activate Sxlpe+ in males. With such a model one need not postulate that undetectable levels of dpn expression influence the sex-determination signal early [Parkhurst and Ish-Horowitz 1992]. The initial threshold might be defined primarily by maternally supplied inhibitors such as Emc, with zygotic denominator elements assuming an increasingly important role as development proceeds.

As more information is gained on the nature and function of the maternal and zygotic factors that participate in the sex determination of Sxlpe+, the primary event of somatic sex determination, the textbook view of the sex signal as a rather static X/A ratio, may outlive its usefulness. The classic work that led to the concept of an X/A ratio did serve to exclude some possible mechanisms for X-chromosome counting, but it also may have caused more attention to be focused on zygotically expressed autosomal genes than will have been warranted. In essence, the sexual development of the fruit fly is determined through the operation of an X-chromosome counting mechanism that functions in a molecular milieu that includes both maternal and zygotic factors. The tools now available should enable rapid progress toward understanding how a simple twofold dose difference in a diverse assembly of X-linked genes that encode transcription factors can be transduced into such a profound difference in developmental fate. How this heterogeneous group of transcription factors came to be recruited for this task is a question of considerable evolutionary interest.
probes until late in cycle 14. For parallel analysis of sis-a, sis-b, and Sxl, Canton-S embryos were prepared (0-to 3.5-hr collections) and processed together until separated for the hybridization reactions. Subsequent steps were done in parallel under identical conditions. Staging of precellular embryos was based on nuclear number, location, and density (Foe and Alberts 1983). The nuclei were visualized by UV fluorescence after DAPI staining (0.5 mg/ml in PBS). Staging landmarks included the distance of the nuclei from the surface (cycles 7–9), the arrival of the first nuclei at the surface and formation of pole buds (cycle 9), pole cell formation (cycle 10), clearing of the cortical cytoplasm (late cycle 12, early cycle 13), and formation of membrane furrows (cycle 14). Embryos were judged to express RNA if staining was darker than observed with a control sense strand probe and if they could be distinguished from earlier (or later) embryos within the same preparation by their staining intensity.

Genetic analysis

Unless otherwise stated, mutations and chromosomes are described in Lindsley and Zimm (1992). The data on male-lethal effects of the double sis⁺ duplication presented in Results were generated from crosses involving y w cm Sxl¹⁰ c/Y, P[Al[w⁺, sis-a⁺]ds], P[BS(w⁻, sis-b⁻)]22/CyO males. Although the male-lethal data presented in the text and in Table 2 were generated with sis-a⁺ transgenes that also carried l(1)108Bb⁻, the later gene did not contribute to the effects on male viability. Equivalent male-specific lethal interactions were observed for the sis-a⁺ l(1)108Bb⁻ and sis-a⁺ l(1)108Bb⁺ transgenes in combination with the sis-b⁺ duplication carried by the Binsinscy balancer chromosome. Moreover, no such male-lethal effects were observed with the sis-a⁻, l(1)108Bb⁺ transgene.

The animals used to determine the sis-b TSP were generated from a cross of sis-b⁵⁺/⁵⁵/Binsinscy females to sis-b⁵⁻¹/Y males. Similar results were obtained with crosses of sis-b⁵⁻¹ homozygous females. Egg collections were for the periods indicated by the age intervals under the bars in Figure 9 (30, 60, or 120 min). Temperature shifts were made by transferring the containers from an air incubator to a temperature-controlled water bath at the times indicated. In contrast to the effect on sex determination, the pronuclear [bristle] aspect of this allelic's phenotype was found not to be temperature sensitive in any consistent fashion even when hemizygous in Sxl¹⁰ females.

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Note added in proof

The nucleotide sequence data reported in this paper have been submitted to the GenBank, EMBL, and DDBJ data libraries under accession number L22755.

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