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; Torres 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* coordinately controls both sex determination and the vital process of X-chromosome dosage compensation (Cline 1979, 1983, 1984; Lucchesi and Skripsky 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* develop-

ment 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 *Sxl_{Pm}* in both sexes. In contrast to transcripts derived from *Sxl_{Pm}*, transcripts derived from *Sxl_{Pe}* are

spliced into functional mRNAs even in the absence of Sxl protein (Keyes et al. 1992). The two X chromosomes in females activate *Sxl_{Pe}*, thereby generating a pulse of Sxl protein that will establish the productive *Sxl* RNA splicing mode once *Sxl_{Pe}* shuts down and *Sxl_{Pm}* becomes active. In contrast, males with their single X chromosome are unable to activate *Sxl_{Pe}*. As a consequence, when *Sxl_{Pm}* 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 *extramacrochaetae* (*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 neg-

ative 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, *dnp*, 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.

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 *run*? 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*¹, 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, demonstrating that the two genetic functions were located in the 4.5 kb common to the four constructs (Fig. 2; Table 1). North-

ern 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*¹. 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*¹. 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. *Hind*III (H), *Nhe*I (N), *Xho*I (X), *Sac*II (S), *Mlu*I (M), and *Not*I (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.

Table 1. Complementation of *sis-a*¹ mutant females by single copies of *P*-element transgenes

Level of complementation	Number of transgenic <i>P</i> (<i>w</i> ⁺ , <i>sis-a</i>) lines complementing <i>sis-a</i> ¹ / <i>sis-a</i> ¹ females						
	<i>P</i> [A1]	<i>P</i> [A3]	<i>P</i> [A4]	<i>P</i> [A10]	<i>P</i> [A10m1] <i>l</i> (1)10Bb(<i>fs</i>)	<i>P</i> [A10m2] <i>sis-a</i> (<i>fs</i>)	<i>P</i> [A10m3] ATG→CTG
Full	21	5	4	5	7	—	2
Partial	—	—	1	—	—	—	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*/Binsinscy ♀ × *y w sis-a*/Y; *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*/Binsinscy; *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* mutant females, but the other three lines were reduced significantly in their ability to provide *sis-a*⁺ 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* (70% rescue). Because the loss of the first potential initiation codon reduced, but did not eliminate, *sis-a*⁺ 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*⁺ function.

The predicted 21-kD Sis-a protein is highly charged and basic (predicted pI = 9.5). A striking run of six consecutive Gly-Ser repeats near the middle of the protein

might serve as a structural hinge. The carboxy-terminal portion of the sequence is similar to the basic leucine zipper (bZIP) class of transcription factors (Vinson et al. 1989) [Fig. 4]. bZIP proteins are defined by a characteristic basic DNA-binding motif and a heptad repeat of leucine (or other hydrophobic) residues that form a helical coiled-coil structure mediating dimerization (Landshulz et al. 1989; O'Shea et al. 1991; Ellenberger et al. 1992). The predicted basic DNA-binding region of Sis-a protein shares the invariant asparagine and arginine residues but lacks the characteristic pair of alanines. The mutation in the defective allele, *sis-a*¹, that greatly reduces but does not eliminate function, changes Lys to Glu in a moderately conserved position of the DNA-binding motif (Figs. 3, 4). The putative leucine zipper of Sis-a contains a tetraheptad repeat with three leucines and one isoleucine at the "d" position of the helix. It differs from a canonical coiled-coil sequence at the "a" position, which is occupied by small or hydrophilic residues rather than large hydrophobic ones. Nevertheless, these residues are compatible with a helical structure, and a number of coiled-coil proteins do contain small or charged residues in the a position (Lupas et al. 1991). The "e" and "g" positions of bZIP proteins often form interhelical salt bridges that

bp

1 CCATTCTATGAGGTAGAACCGGAATAACCCATCCAGTGTCCAATGCGAAGTACCTCGGAAATGCTATAAAACGGGGCTGCATATT

+1 M E R S H

91 GCCTAAGGCGCATTAGCACACGAAATCTCGACGAGAGCAGTCACAGACCACTGCACACATCGAAAAAATCACCATGGAAACGGAGTCAT

L Y L P T L S Y A A M G H V Y A P Y R G S S S P A L S T A S

181 CTTTACTTGGCCACTCTGAGCTACGCGGCGATGGGTACGTATACGACCGTATCGGGAAGCAGTTACCCGCACTATCGACAGCATCG

S T S S K P E Q I E E L V S Q Q L H H L K M H Y A D E E Q R

271 TCAACATCATCGAAGCCGAGCAGATCGAGGAGCTGGTGTCCAGCAGCTGCATCATCTCAAAATGCATACGCCGACGAGGAGCAACGC

Y V D Q M L L E N P I V V E R R A P P P L K T E L A M D C R

361 TACGTAGACAGATGCTCTGGAGAAATCCATTGTGTGGAGCGCGTGCACCGCCCGCTGAAACGGAGCTTGCTATGGATTGCGCGC

G S G S G S G S G S G S D V K D A Q R Q R A E S C R K S R Y

451 GGTATCGGGTTCGGATCAGGTTCCGGTTCGGTTCGGATGTCAGGATGCCAGCGTCAGAGGGCGAGTCTGCGGCAAAATCCCGCTAC

N N I K K A K L R F R H K F V S G Q L K K S A V M L D T M

541 AACAAACAGATCAAGAGGCAAGCTGCGCTTCCGGCACAAATTCGTAGCGGGCAGCTGAAGAAGAGCGCCGTCATGTTGGACACAGATG

R D V A Q A E R Q L L E R G Y P A A T L D R M R A T F G L

631 CGGGATGTGATTGCCAGGCGGAGGAGGAGCTGCTCGAGCGGGGATATCCCGCCGCACTCGATCGGATGCGGGCCACTTTCGGCTTA

E M E Q (189)

721 GAAATGGAGCAGTGATATCATAGTGTAGTATGTGTGCGAGGACGGATCCCTGACTAGTCAGCATTAGCCATACCCTTTTTTTTTTTT

811 TCTTGTGACTATATCTAGTATTTATCTAAGCATATTAATAATTTTAAATAAGATTAAGAATCATATTGAAACGAATGCTTTCTTAT

901 TCAATATTTTGTATATGTTTCGACGATCAGATGGAGTCAAGGATAACTGTTAATTTCACTTTACAAGAAATTTCCCATTA (980)

aa

Figure 3. Sequence of the *sis-a* gene. The nucleotide and predicted amino acid sequences of *sis-a* are shown. The TATA box, mRNA initiation site, and poly(A) addition signals are shown against a black background. The cDNAs extended from the +1 position at nucleotide 100 through the poly(A) sequence that begins after the G residue at nucleotide 881. The predicted molecular mass of the Sis-a protein is 21,275 daltons. The four hydrophobic repeat residues of the putative leucine zipper motif are circled. The location of the *sis-a*¹ mutation is underlined, and the affected amino acid is shown white on black. Identified sequence polymorphisms are underlined, as are the affected amino acids. The

*sis-a*¹ mutation is an A → G (K → E) change at nucleotide 547. cDNA polymorphisms in the M. Noll library are T → G (D → E) at 686, A → G (silent) at 720, T → A at 798, and a 3-bp (CTT) deletion at 812–814. cDNAs from both libraries contained 11 instead of 12 T residues from 800–811.

Table 2. *Sxl* null mutation suppresses the male-specific lethality caused by multiple copies of *sis-a*⁺ and *sis-b*⁺ transgenes

<i>P(sis</i> ⁺) duplications (=PP)	Number of males recovered ^a			
	<i>Sxl</i> ⁺ <i>P(sis-b</i> ⁺)		<i>Sxl</i> ⁻ <i>P(sis-b</i> ⁺)	
	<i>PP/+ +</i>	<i>+ +/CyO</i>	<i>PP/+ +</i>	<i>+ +/CyO</i>
<i>P[A1(sis-a</i> ⁺) <i>]/5 + P[A1(sis-a</i> ⁺) <i>]/2</i>	0	124	124	144
<i>P[A1(sis-a</i> ⁺) <i>]/89 + P[A1(sis-a</i> ⁺) <i>]/1</i>	0	93	95	93
<i>P[A1(sis-a</i> ⁺) <i>]/4 + P[B5(sis-b</i> ⁺) <i>]/22</i>	0	139	134	131
<i>P[A1(sis-a</i> ⁺) <i>]/6 + P[B5(sis-b</i> ⁺) <i>]/3</i>	0	91	113	97

Crosses were at 18°C and of the form ♀♀ *y w cm Sxl*^{f1} *ct sn P[B5(w*⁺, *sis-b*⁺)*]/28/y w + + + + P[B5(w*⁺, *sis-b*⁺)*]/28* × ♂♂ *y w cm Sxl*^{f1} *ct/Y; P(w*⁺, *sis*⁺) *P(w*⁺, *sis*⁺)*/CyO*. 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 β-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 β-galactosidase anteriorly but produced levels of β-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*¹.

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-

Figure 5. Duplication of *sis-a*⁺ and *sis-b*⁺ causes ectopic activation of *Sxl*_{pe} in X/Y embryos. Embryos with one copy of a *Sxl*_{pe}-*lacZ* fusion were fixed and stained with X-gal. (A) Normal X/Y male embryo. (B) X/Y embryos with a *sis-a*⁺, *sis-b*⁺ double duplication. (C) Female X/X embryo. Embryos were the progeny of a cross between *y w / y w* females homozygous for *Sxl*_{pe}-*lacZ*, and *y w cm Sxl*^{f1} *ct/Y; P[A1(w*⁺, *sis-a*⁺)*]/4, P[B5(w*⁺, *sis-b*⁺)*]/22/CyO* males. Of 465 embryos scored, 122 were unstained, 110 exhibited nonuniform staining similar to that in B, and 233 stained relatively uniformly as in C.

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 μ g 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 polyploid. These polyploid 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 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^{sc3-1}* (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. Comparative time course of *sis-b* and *Sxl* expression in early embryos. Wild-type embryos at nuclear cycles 5, 11, 12, 13, and 14 (early and late) are shown. Embryos were collected and prepared together and hybridized separately with digoxigenin-labeled RNA probes specific for *sis-b* or for the embryonic E1 exon of *Sxl*. *sis-b* expression was first apparent at cycle 9 (not shown). The striped staining in the late cycle 14 embryo is the *scute* pattern of expression and is unrelated to sex determination. The *sis-b*-stained embryos represent the darkest staining class at each cycle and are presumed to be female. Only female embryos stained with the *Sxl* probe.

Figure 9. A brief, very early temperature-sensitive period for *sis-b^{sc3-1}* females. The viability of *sis-b^{sc3-1}* homozygous females was compared with that of their fully viable, hemizygous *sis-b^{sc3-1}/Y* brothers as a function of temperature. The age of the embryos at the moment of the temperature shift is indicated in hours after oviposition. The abscissa scale has been adjusted for the fact that development is approximately twice as fast at 29°C as at 18°C. Number of males scored for the time points are 29 → 18°C: 521, 174, 167, 467, 516, 356, 1060; 18 → 29°C: 792, 427, 130, 181, 275, 375, 194 (for details, see Materials and methods).

ysis in Materials and methods). It behaves as a strong female-specific lethal at the nonpermissive temperature (29°C) and has no effect on viability at 18°C (Erickson and Cline 1991; Torres and Sanchez 1991). The experiment of Figure 9 shows that *sis-b* expression is important for female development only between 1 and 2.5 hr after fertilization (corresponding to nuclear cycles 9 through early 14), with the most sensitive period being between 1.5 and 2 hr postfertilization (cycles 12 and 13).

sisterless 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* gene 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 *run* 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 (Shermoe 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^{sc3-1}* suggests that regardless of when *sis-b* transcription is initiated, it only becomes important for sex determination after nuclear cycle 9. The *sis-b^{sc3-1}* 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 establish 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^{sc3-1}* 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 cytoplasm.

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 *Sxl* expression within this domain, consistent with this gene's participation in the sex-determination process somewhat less central.

Using a *Sxl_{pe}-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-a¹* allele, expression of *Sxl_{pe}* 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 *Sxl_{pe}* 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 *Sxl_{pe}* 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 *Sxl_{pe}*. The fact that *sis-a* transcripts disappear just as *Sxl_{pe}* 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 *Sxl_{pe}*.

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 *Sxl_{pe}*? The fact that *sis* transcript 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 *Sxl_{pe}*. Considered in light of the fact that *sis-a* and *sis-b* encode two different categories of transcription factor, this implies that amplification may occur as a consequence of there being qualitatively different sites for different transcriptional activators at *Sxl_{pe}*. 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 *Sxl_{pe}*. It seems likely that the bHLH proteins Sis-b and Da form heterodimers that bind to and activate *Sxl_{pe}*, 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 *Sxl_{pe}*. 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 *Sxl_{pe}* at an optimum for discrimination and to amplify the relative male/female difference in numer-

ator 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 "nonzipper" 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 *Sxl_{pe}* 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 12 is the point when the denominator (negative) element *dnp* becomes active in the zygote (Bier et al. 1992; Younger-Shepherd et al. 1992). Perhaps the specific role of zygotic *dnp* and other denominators is to raise the activation threshold for *Sxl_{pe}* as the levels of *Sis* protein increase, keeping that threshold above a level that would activate *Sxl_{pe}* in males. With such a model one need not postulate that undetectable levels of *dnp* 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 regulation of *Sxl_{pe}*, 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.

Materials and methods

DNA analysis and germ-line transformation

DNA manipulations, Southern blots, and dideoxy DNA se-

quencing were 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 germ-line 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 transposase 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[A10m1]* was made by filling in an *XhoI* site resulting in a frameshift after the amino acid 22 of *l(1)10Bb*. *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 λ gt10 E6 library (3- to 12-hr embryo) of Poole et al. (1985) and a λ gt10 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-a¹* allele, a 1028-bp fragment was PCR amplified from *sis-a¹* 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⁺, Sxl_{pe}-lacZ⁺)* transgene is similar to that described in Keyes et al. (1992). It carries a 3.7-kb *SalI*-*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 (Klambt 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 *Sxl_{pe}*-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

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 Sxl*^{l1} *ct/Y*; *P*[*A1*(*w*⁺, *sis-a*⁺)]/4, *P*[*B5*(*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)10Bb*⁺, 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)10Bb*[−] and *sis-a*⁺ *l(1)10Bb*⁺ 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)10Bb*⁺ transgene.

The animals used to determine the *sis-b* TSP were generated from a cross of *sis-b*^{sc3-1}/*Binsinscy* females to *sis-b*^{sc3-1}/*Y* males. Similar results were obtained with crosses of *sis-b*^{sc3-1} 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 proneural (bristle) aspect of this allele's phenotype was found not to be temperature sensitive in any consistent fashion even when hemizygous in *Sxl*^M 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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