The Drosophila melanogaster Sex Determination Gene sisA Is Required in Yolk Nuclei for Midgut Formation

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ABSTRACT

During sex determination, the sisterlessA (sisA) gene functions as one of four X:A numerator elements that set the alternative male or female regulatory states of the switch gene Sex-lethal. In somatic cells, sisA functions specifically in sex determination, but its expression pattern also hints at a role in the yolk cell, a syncytial structure believed to provide energy and nutrients to the developing embryo. Previous studies of sisA have been limited by the lack of a null allele, leaving open the possibility that sisA has additional functions. Here we report the isolation and molecular characterization of four new sisA alleles including two null mutations. Our findings highlight key aspects of sisA structure-function and reveal important qualitative differences between the effects of sisA and the other strong X:A numerator element, sisterlessB, on Sex-lethal expression. We use genetic, expression, clonal, and phenotypic analyses to demonstrate that sisA has an essential function in the yolk nuclei of both sexes. In the absence of sisA, endoderm migration and midgut formation are blocked, suggesting that the yolk cell may have a direct role in larval gut development. To our knowledge, this is the first report of a requirement for the yolk nuclei in Drosophila development.

In Drosophila melanogaster the genetic elements that signal chromosomal sex encode a diverse set of proteins that regulate the early transcription of Sex-lethal (Sxl), the regulatory switch gene that controls aspects of sex determination and dosage compensation. Central among the sex signal elements are the X-linked sisterlessA (sisA), sisterlessB (sisB), sisterlessC, and runt genes, which serve as the direct dose-sensitive determinants of X chromosome number (Cline 1988, 1993; Duffy and Cline 1991; Torres and Sanchez 1992; reviewed in Cline and Meyer 1996). In female embryos, the diplo-X dose of these four “X:A numerator” gene products, in conjunction with several maternally supplied “X:A signal transduction” gene products including Daughterless (Da), activate the Sxl establishment promoter Sxlp, initiating the female developmental program (Cline 1988; Kyes et al. 1992). In male embryos, the haplo-X dose of X:A numerator genes is insufficient to overcome the effects of maternal and autosomal inhibitors of Sxlp. Consequently, Sxl is left in its off, or male, state.

Although four genes function as X:A numerator elements, two, sisA and sisB (also called scute with reference to its proneural function), account for most of the dose sensitivity of the X counting process. They also are required to regulate Sxl expression throughout the embryo, unlike the weaker runt element (Bopp et al. 1991; Duffy and Gegen 1991; Erickson and Cline 1993; Cline and Meyer 1996). The sisB gene product is a basic helix-loop-helix (bHLH) transcription factor that dimerizes with maternally supplied Da to directly regulate Sxlp. The sisA gene encodes a divergent member of the basic leucine-zipper (bZIP) class of transcription factors, which is thought to bind Sxlp in conjunction with an as-yet-unidentified protein partner (Erickson and Cline 1993, 1998).

While sisB has been intensively studied genetically because of its role in neurogenesis, sisA is less completely characterized, because only a single sisA mutation exists (Cline 1986). This allele, sisA$, alters the DNA-binding region of the protein, rendering it defective in Sxlp activation and female-lethal when homozygous (Erickson and Cline 1993). While strongly female-lethal, sisA$ is without effect in males, raising the possibility that sisA could be unique among the sex signal elements in being a specific regulator of sex determination and dosage compensation.

An unusual feature of sisA is its high-level expression in the embryonic yolk nuclei or vitellophage (Erickson and Cline 1993, 1998). This expression pattern, which has been conserved for the $40$ million years separating D. melanogaster and D. virilis, indicates that if sisA has a second function, it likely involves the yolk nuclei. Although both yolk and yolk nuclei are found in the eggs of most insect species, little is known of the role the yolk nuclei play in development. As their alternative name implies, the vitellophage may participate in yolk...
utilization and Bownes et al. (1988) have proposed that they may be involved in the timed release of ecdysteroids stored in the yolk; but no direct data support either contention. Ultimately the yolk and yolk nuclei are enclosed within the developing midgut, where yolk protein degradation occurs (Bownes 1982b). Prior to the engulfment of the yolk in the midgut, however, yolk nuclei are found on the surface of the yolk sac in proximity to regions of morphogenetic movements, hinting that they could be involved in these processes (Bownes 1982a).

To further address the role of sisA in sex determination, to study its structure-function relationships, and to answer the question of alternative developmental functions, we undertook a genetic screen to isolate new sisA alleles. We obtained four sisA mutants, including two nulls and a temperature-sensitive allele. Analysis of these mutations reveals that sisA may have a lesser role in the expression of Sxl than does the other strong X:A numerator sisB, as considerable Sxl expression occurs in the absence of sisA protein. Our data also demonstrate that sisA has a second essential function, one that for the first time reveals a role for the yolk nuclei in the development of Drosophila.

MATERIALS AND METHODS

Drosophila: Flies were grown on a standard cornmeal, yeast, and molasses medium in uncrowded conditions. The criterion for viability was eclosion. Mutations and chromosomes are described at http://flybase.bio.indiana.edu:7083.

Isolation of sisterless mutations: Males of genotype y cm ct v/Y were fed 0.006 m or 0.012 m EMS in 1% sucrose (Grigliatti 1986) and crossed to attached-X females bearing a y chromosome duplication of the sisA region (v"Yy†). Single F1 y cm ct v/Y male progeny were mated with v sisA1 shi/* FM3, y 8 females. After 5-7 days at 25°, adults were removed and progeny shifted to 29° to kill shi males. As FM3 is male-lethal, only y cm ct v sisA1 shi/* and y cm ct v FM3 virgin female F2 progeny were recovered. Compensation was assessed by comparing the ratio of germaria (y cm ct v/* v sisA1 shi/* to yellow Bar (y cm ct v/* FM3, y 8) progeny. Ratios of <1:2 were taken as evidence of noncompensation and retested. Rather than count all progeny, phenotypic ratios were assessed visually through the sides of the vials. Vials with apparent distortions in the progeny ratio were examined in detail. We could reliably detect twofold deviations from unity scoring unanesthetized flies through the vial; however, some weak candidates may have been discarded. Candidate y cm ct v FM3 virgin females were retested and stocks created by crossing to y cm ct sisA1, scab, cm 5X1/ct, and FM7c males. This also served as an initial mapping strategy as homozygous sisA, sisB, or Sxl mutants are generally less viable than the transheterozygous combinations. The sisA+ and sisA- P element transgenes used in compensation experiments were P[A10m1, sisA-, [I(1)108Bb]] and P[A10m2, sisA-, [I(1)108Bb]] (Erickson and Cline 1993).

Sequencing of sisA alleles: Genomic DNA was prepared from sisA1 males and from sisA2, sisA3, and sisA5 males carrying transgenes with either the D. virilis sisA+ coding sequence (Erickson and Cline 1998) or a D. melanogaster sisA gene in which the first 15 sisA codons had been replaced by the HA epitope tag (unpublished). DNA from male-lethal mutants was PCR amplified using a 5’ primer specific for the endogenous sisA locus CTGTGATTTCACCATGGAACGGATC and nonspecific 3’ primer CTATGCTGACGATCTCGGGAT CCG. The entire sisA+ coding sequence was amplified using 5’ primer CCCATGACCGCCATTCTGAAAGTGAAACCGC and nonconsensus 3’ primer CGGTTATGTTGCGGAT TCAG and CGGATTACCATGGACGGATGTC. For each mutation products of three independent amplifications were sequenced using Sequenase 2.0 (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) with similar results.

Determination of the sisA+ TSP: Crosses were y cm ct v sisA1 FM7c females × FM7c/Y males. For downshifts, egg collection periods (and number of control females) were 0 (408), 0.5-1.5 (66), 1.5-2.5 (198), 2-3 (172), 2.5-3.5 (260), 3-4 (454), 3.5-4.5 (138), 4-5 (191), 4-6 (434), 5-7 (361), 6-8 (317), and 7-9 hr after oviposition (447). For upshifts, embryos were aged (number of control females) 0-4 (97), 3-5 (34), 4-6 (73), 5-7 (76), 6-8 (145), 7-9 (68), 8-10 (98), 9-11 (134), 10-12 (81), 12-16 (151), and 14-19 hr (186). Temperature shifts were made by moving vials from an air incubator to a water bath. After 48 hr, progeny were shifted to 25° to complete development.

Clonal analysis: Gynandromorphs were generated using the mitotically unstable ring-X chromosome In(1)wvC. In(1)wvC/y w females were crossed to y w v sisA1 v*wvC or y w sisA1/Y males. Scoring of cuticular structures was done on anesthetized adults. Due to the low viability and fecundity of our In(1)wvC/y w stock (now extinct), experimental and control gynandromorphs were generated over several months, during which time the ring-X stabilized. Consequently, not all data were obtained under identical conditions. This prevented a summation of overall viability; nonetheless, data from parallel crosses suggest that there was little difference between the survival of experimental and control gynandromorphs. The most comparable crosses produced 238 In(1)wvC/sisA1 females and 150 In(1)wvC/sisA1 females in sisA1 and 3 sisA1-gynandromorphs.

Immunocytochemistry and in situ hybridization: Embryos were processed, stained, and mounted according to Patel (1994). Mouse anti-SXL polyclonal (Bernst ein et al. 1995) and anti-p-galactosidase monoclonal (Promega, Madison, WI) antibodies were used at 1:1000 dilution. Embryos were fixed in heptane/PEM-4% formaldehyde for 20-40 min or in heptane/ PBS-10% formaldehyde for 50 min (in situ fixation). In situ hybridizations were as described (Erickson and Cline 1993, 1998).

RESULTS

Isolation of new sisterless mutations: We searched for new sisA alleles by screening for EMS-induced X-linked mutations that failed to complement the female-specific lethal allele sisA1. Although our goal was to isolate new sisA mutations, we also expected to recover sisB(sc) and Sxl alleles because these are male-viable and exhibit a dominant-lethal synergy in heterozygous sisA1 females (Cline 1988). An important feature of our screen was that it allowed the recovery of lethal sisA alleles, since the F1 males carried a duplication of the sisA+ region on their Y chromosomes (v"Yy†). From 17,034 fertile F1 males, we identified 11 mutations that failed to complement sisA1 upon restesting. Eight were male-viable. Five male-viable lines exhibited bristle defects character-
The sisA5 allele is a frameshift mutation that changes the reading frame after the 20th residue. sisA3 deletes amino acids 108–110 and replaces 111–118 with out-of-frame residues. sisA1 changes lys-128 to glu (Erickson and Cline 1993). sisA2 is an amber mutation that truncates the protein after ala-160. The sisA sequences from D. melanogaster, pseudoobscura, and virilis (mpv) are shown (Erickson and Cline 1998). The basic leucine zipper is labeled. (B) The sisA3 mutation. Nucleotide and amino acid sequences for the wild-type (top) and sisA3 (bottom) alleles. Identities are blocked. Deleted sequences are denoted by dots. (C) The sisA4 mutation is a 1.4-kb hob Element insertion flank ed by a 12-bp duplication that includes the sisA transcription start site.

Figure 1.—(A) Mutations affecting sisA. The sisA1 allele is a frameshift mutation that changes the reading frame after the 20th residue. sisA3 deletes amino acids 108–110 and replaces 111–118 with out-of-frame residues. sisA1 changes lys-128 to glu (Erickson and Cline 1993). sisA3 is an amber mutation that truncates the protein after ala-160. The sisA sequences from D. melanogaster, pseudoobscura, and virilis (mpv) are shown (Erickson and Cline 1998). The basic leucine zipper is labeled. (B) The sisA3 mutation. Nucleotide and amino acid sequences for the wild-type (top) and sisA3 (bottom) alleles. Identities are blocked. Deleted sequences are denoted by dots. (C) The sisA4 mutation is a 1.4-kb hob element insertion flanked by a 12-bp duplication that includes the sisA transcription start site.

Molecular characterization of sisA alleles: The putative sisA2, sisA3, and sisA5 mutations were cloned by PCR amplification. Genomic DNA was prepared from sisA mutant males that had been rescued by P-element transgenes containing a functional sisA+ gene indicating that these were new sisA alleles and revealing the existence of a novel lethal sisA phenotype. The male-lethal alleles were designated as sisA2, sisA3, and sisA5, and the partially male-viable line as sisA4.

Molecular characterization of sisB alleles: The putative sisB4, sisB5, and sisB31 mutations were cloned by PCR amplification. Genomic DNA was prepared from sisB31 mutant males that had been rescued by P-element transgenes carrying a modified sisB+ gene using a 5' primer specific for the endogenous sisB locus, we amplified sequences extending from the sixth codon into the 3' untranslated region of sisB and sequenced the products.

The sisA2 mutation has a C-to-T nucleotide substitution that changes codon 161 (Gln) into a TAG stop signal. Although the sisA2 change occurs late in the 189-amino-acid coding sequence, the mutant protein is likely to be nonfunctional because it is truncated within the leucine zipper dimerization domain (Figure 1A). The sisA3 allele contained a single-base deletion (TAC sisB31-1), but failed to complement the null Sxlf1 allele. They were confirmed as Sxl alleles by genetic mapping (GCA → TACCA) that creates a frameshift after the 20th codon, suggesting that it also produces a nonfunctional polypeptide (Figure 1A).

The sisA3 allele is complex, consisting of a base substitution, an 8-bp frameshifting deletion, and a single-base deletion that restores the normal reading frame (Figure 1B). The alteration occurs just after a run of six gly-ser repeats and replaces 11 amino acids with 8 novel residues prior to the restoration of the normal reading frame. The altered amino acids occur in an evolutionarily conserved N-terminal extension of the basic DNA-binding domain (Figure 1A).

Males carrying the sisA4 allele were partially viable and the entire sisA4 coding region was PCR amplified from genomic DNA. The sisA4 coding sequence was wild type. Initial PCR and Southern analysis suggested an insertion mutation upstream of sisA and this was confirmed using PCR primers located upstream and downstream of the predicted insertion site. The DNA sequence revealed a 1.4-kb hob Element transposable element flanked by a 12-bp target site duplication that includes the sisA transcription start site (Figure 1C).
sisA has a vital function in embryos of both sexes: The most striking phenotype of the four new sisA alleles is male-lethality, indicating that sisA has a second vital function in Drosophila. The putative null alleles, sisA1 and sisA2, are invariably male-lethal. No sisA3 or sisA4 males have been observed in controlled experiments (Table 1) or in balanced stocks at any temperature. The complex sisA5 allele is also strongly male-lethal; however, escapers are occasionally observed, suggesting that the sisA1 protein retains residual function (Table 1). A small number of sisA6 males survive to adulthood, indicating that the hobo insertion does not eliminate all transcription of sisA (Table 1). The surviving sisA3 and sisA5 males were fertile and of normal morphology. The male-lethal effects of all four sisA alleles were completely rescued by sisA-fl, but not sisA- transgenes (Erickson and Cline 1993), confirming that the male-lethality was due solely to the sisA gene (data not shown).

Previously, the only known effect of loss of sisA function was female-specific lethality, due to the failure to activate Sxl during the initial stage of sex determination (Cline 1986, 1988; Erickson and Cline 1993). The four sisA alleles reported here are also female-lethal; however, the lethality cannot simply be ascribed to defects in Sxl activation if females also require sisA's second vital function. To determine if sisA has a second function in females, we asked if the strong constitutive SxlK+ allele could rescue the viability of sisA1 females. SxlK+ bypasses the requirement for the X-counting elements in sex determination by activating the female mode of Sxl splicing independent of transcription from Sxl0 (Bernstein et al. 1995). We found that SxlK+ was incapable of rescuing sisA1 females even though it efficiently suppressed the lethal sex determination defect in sisA1/sisA2 females (Table 2), suggesting that sisA's second function is needed in both sexes.

To determine the developmental stage at which sisA acts we examined the lethal period for sisA1 males. Embryos were collected from crosses between w v sisA1/; y w + + females and wild-type (Oregon-R) males and monitored for viability at different developmental stages. Hemizygous sisA1 males accounted for one-fourth of the progeny in these crosses with the remaining animals expected to be fully viable. We examined 954 fertilized eggs and found that 245 (25.7%) failed to hatch, suggesting that sisA1 mutant males die as embryos. Very little lethality was observed at later stages (93% of collected first-instar larvae emerged as adults with a 2:1 ratio of females to males), showing that the lethal phase is limited to the embryonic period.

**Effects of sisA on sex determination:** The sisA1 mutation is fully male-viable but strongly female-lethal. The viability of homozygous sisA1 females varies from 0.1 to 3%, depending on culture conditions and undefined aspects of genetic background, but is always greater than that of sisA1 hemizygous females, suggesting that sisA1 retains some sex determination function (Cline 1986, 1988). This inference, however, is only valid if a single nondosage-compensated copy of sisA1 provides females with a sufficient amount of sisA1's second function. Otherwise, the greater lethality of sisA1 hemizygotes could be explained as the additive effect of two developmental defects. Data in Table 2 (lines 2-4) show that the majority of females hemizygous for sisA1 are viable if they also

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**TABLE 1**

New sisA mutations are male-lethal and defective in sex determination

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Male viability at 25°</th>
<th>Female viability at 18°</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>Males/ reference</td>
</tr>
<tr>
<td>sisA1</td>
<td>100</td>
<td>240/ 234</td>
</tr>
<tr>
<td>sisA2</td>
<td>&lt;0.03</td>
<td>0/ 3413</td>
</tr>
<tr>
<td>sisA3</td>
<td>0.2</td>
<td>2/ 1028</td>
</tr>
<tr>
<td>sisA4</td>
<td>5.8</td>
<td>12/ 206</td>
</tr>
<tr>
<td>sisA5</td>
<td>&lt;0.07</td>
<td>0/ 1461</td>
</tr>
<tr>
<td>Df(sisA1)</td>
<td>sisA1</td>
<td>0.6</td>
</tr>
</tbody>
</table>

**TABLE 2**

Experimental genotype of sisA is required in females

<table>
<thead>
<tr>
<th>Experimental genotype</th>
<th>Viability (%)</th>
<th>Viability reference (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SxlK+ sisA1/sisA2</td>
<td>&lt;0.24</td>
<td>420</td>
</tr>
<tr>
<td>SxlK+ sisA1/sisA2</td>
<td>81.0</td>
<td>137</td>
</tr>
<tr>
<td>SxlK+ sisA1/sisA3</td>
<td>89.4</td>
<td>303</td>
</tr>
<tr>
<td>SxlK+ sisA1/Df(sisA1)</td>
<td>88.4</td>
<td>294</td>
</tr>
</tbody>
</table>

**Crosses at 25°:** y cm SxlK+ v sisA1/sisA2/FM7c Y, where x is y cm ct v sisA1 and y w v sisA2, y cm ct v sisA1 f, y cm ct v sisA2, or y cm ct v sisA1 and w v sisA2. Female viability: y pn v sisA1/FM7c Y, y pn v sisA2 f, y cm ct v sisA1 f /v"Yy", y cm ct v sisA2 f /v"Yy", y cm ct v sisA1 Y, y w v sisA1 f /v"Yy", y w Df(1)N71, sisA1 / v"Yy" males.
To carry the Sxl\textsuperscript{N4} allele (also shown by Bernstein et al. 1995). This confirms that one copy of sisA\textsuperscript{1} is sufficient to provide the non-sex-specific functions to females and thus confirms the previous inference that sisA\textsuperscript{1} retains partial sex determination function.

Because sisA\textsuperscript{1} is partially functional, the relative viability of sisA\textsuperscript{1} transheterozygotes can be used as a sensitive measure of any residual sex determination function in the new sisA\textsuperscript{1} alleles. By this criterion sisA\textsuperscript{2}, sisA\textsuperscript{3}, and sisA\textsuperscript{5} are sex determination null mutations because the viability of these alleles, heterozygous with sisA\textsuperscript{1}, is indistinguishable from a deletion of the sisA region under the most permissive conditions (Table 1). In contrast, the hobo insertion mutation sisA\textsuperscript{4} partially complements sisA\textsuperscript{1}, demonstrating that sisA\textsuperscript{4} possesses residual sex determination activity. Although sisA\textsuperscript{4} has nearly the same sex determination function as the sisA\textsuperscript{1} allele, we have not observed any homozygous sisA\textsuperscript{4} females, indicating that the combination of the two partial functional defects is invariably lethal to females.

To determine whether sisA is absolutely required for Sxl expression, we examined sisA\textsuperscript{2} and sisA\textsuperscript{3} mutant embryos using anti-SXL antibodies and in situ hybridization. The results indicate that while sisA is necessary for proper Sxl regulation, Sxl\textsubscript{nu} can be expressed in some nuclei even in the complete absence of sisA (Figure 2). Most homozygous sisA\textsuperscript{5} embryos exhibited residual SXL staining in the anterior thoracic and mid-ventral regions with the phenotypes ranging from no expression to patchy high-level SXL staining. In contrast, severe reductions in the other strong numerator element sisB have been reported to eliminate expression of Sxl protein in all parts of the embryo (cited in Bopp et al. 1991), a result we confirmed using the Achaete-scute complex deletion Df[1]sc\textsuperscript{19} (data not shown).

To obtain a more direct measure of the effect of the loss of sisA on Sxl\textsubscript{nu}, we examined transcription from Sxl during nuclear cycles 12–14 using in situ hybridization. Although we could not distinguish nonstaining sisA\textsuperscript{5} females from males in precellular embryos, we identified a number of diplo-X embryos that expressed low levels of Sxl\textsubscript{nu} transcript in a patchy mosaic pattern reminiscent of the later Sxl protein pattern (data not shown), confirming that the SXL staining accurately reflects the residual activity of Sxl\textsubscript{nu} in sisA null embryos.

After cellularization sisA is expressed exclusively in the yolk nuclei: The earliest sisA expression has been described in detail for both D. melanogaster and D. virilis (Erickson and Cline 1993, 1998). There are no maternal sisA messages. Zygotic expression begins during the cleavage stage and continues until the beginning of cellularization in nuclear cycle 14. At that time expression in periphery stops, and there is an abrupt increase in expression from the yolk nuclei or vitellophages. Thereafter, sisA appears to be expressed exclusively in the yolk, being maintained at high levels through late germ-band retraction, until ceasing during stages 13 or 14 (Figure 3). This temporal progression suggests either that the non-sex-specific vital function of sisA acts prior to cellularization, perhaps coincident with sisA's sex-determining function, or that it involves the yolk nuclei.

The sisA temperature-sensitive period occurs early in embryogenesis: The hobo insertion mutation sisA\textsuperscript{4} is temperature sensitive for male viability. This allowed us to ask when the non-sex-specific function of sisA is required by monitoring the viability of sisA\textsuperscript{4} males after timed temperature shifts. We found that the non-sex-specific sisA\textsuperscript{4} temperature-sensitive period (TSP) begins at about 2.75 hr after fertilization, a time corresponding to the end of the syncytial blastoderm phase, and ends \(<\)2 hr later (normalized to 25°C) during extension of the germ band (Figure 4). Since sisA\textsuperscript{4} retains residual function at the restrictive temperature, it is formally possible that this TSP represents only the most sensitive period and that sisA is required at other times as well. Nevertheless, the data indicate that males require sisA from a time just after sex is determined (1–2.5 hr after fertilization;
The yolk nuclei originate from peripheral nuclei fuse to form the 6000 cells of the nuclei mutant for sisA. These nuclei originate from all regions of the embryo and none appear to possess a predetermined yolk fate. When the membranes surrounding the peripheral nuclei fuse to form the 6000 cells of the cellular blastoderm, they enclose 175–225 polyploid yolk nuclei within a large syncytial yolk cell. Each yolk nucleus has an associated cytoplasm that fuses with that of other yolk nuclei and with a thin layer of cytoplasm on the surface of the yolk mass. As gastrulation and germ-band extension proceed, the yolk nuclei migrate to form an interconnected network on the inner surface of the yolk cell (Poulson 1950). The yolk nuclei persist until after the yolk sac is engulfed by the developing midgut, where they eventually degenerate without producing any larval or adult structures (Campos-Ortega and Hartenstein 1997).

The development and structure of the yolk cell suggests that sisA should function nonautonomously within this syncytial cell. In other words, a sisA defect should be tolerated in mosaic animals, provided a sufficient number of functional yolk nuclei are present within the yolk cell. To test this idea, we generated sisA null mosaics using the mitotically unstable ring-X chromosome In(1)wvc. The In(1)wvc chromosome is lost at high frequency in the earliest nuclear divisions to generate gynandromorphs containing large clones of male (haplo-X) and female (diplo-X) tissue (Zalokar et al. 1980). In the absence of lethal effects male and female clones survive equally well, with the percentage of male adult tissue determined by the divisions in which the ring-X was lost and by the position of the clones with respect to the blastoderm fate map. If the second function of sisA is required exclusively in the yolk, it should be possible to isolate large-patch gynandromorphs in which any surface structure is mutant, because nearly all should be yolk nuclear mosaics (Zalokar et al. 1980).

In contrast, if low-level expression elsewhere were responsible for sisA’s vital function, one would expect the recovery of sisA1-gynandromorphs to be reduced, and the distribution of surface clones to be biased against those structures originating closest to the site of sisA action (Bryant and Zornetzer 1973; see also Zusman and Wieschaus 1985).

We analyzed the tissue and size distribution of sisA mutant clones in 57 sisA1-gynandromorphs and 21 control sisA1-gynandromorphs. sisA1 null mutant clones were recovered in every adult structure with similar frequency (Table 3), indicating that there is no single region of the blastoderm surface that must be sisA+ for embryonic viability. The average “maleness” index for the cuticular structures was 43% for the sisA1 mosaics and 34% for the sisA1 controls, both within the range published for nonlethal controls, and much greater than that seen for most lethal alleles (Bryant and Zornetzer 1973; Zusman and Wieschaus 1985; Cline 1988). The average size of the sisA null mutant patches was quite large. Many (24/57) sisA1 mosaics had more than half male tissue, and four were almost entirely male (97%), suggesting that animals are viable even if they develop from the first few divisions with most of their nuclei mutant for sisA. The recovery of these large-patch gynandromorphs also demonstrates that sisA function is not needed later during imaginal disc development, in accordance with the sisA1 TSP determination (Figure 3) and larval/pupal expression analysis (Erickson and Cline 1993).
### TABLE 3
Analysis of *sisA* mutant gynandromorphs

<table>
<thead>
<tr>
<th>No. of structures</th>
<th>Total structures scored</th>
<th>%</th>
<th>No. of structures</th>
<th>Total structures scored</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experimental sisA&lt;sup&gt;2&lt;/sup&gt; crosses</strong></td>
<td></td>
<td></td>
<td><strong>Control sisA&lt;sup&gt;1&lt;/sup&gt; crosses</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>δ clones</strong></td>
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<td></td>
<td><strong>δ clones</strong></td>
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<tr>
<td>Eye/antenna</td>
<td>55</td>
<td>114</td>
<td>0.48</td>
<td>13</td>
<td>42</td>
</tr>
<tr>
<td>Proboscis</td>
<td>53.5</td>
<td>114</td>
<td>0.47</td>
<td>12.5</td>
<td>42</td>
</tr>
<tr>
<td>Humerus</td>
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<td>114</td>
<td>0.49</td>
<td>11.5</td>
<td>42</td>
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<tr>
<td>Wing/thorax</td>
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<td>Foreleg</td>
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<td>Midleg</td>
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Listed imaginal disc and histoblast derivatives were scored in each gynandromorph. Except for genitalia, left and right halves were scored separately. Mosaic discs counted as 50% male. Patterned after Cline (1988).

Apart from sexual dimorphisms, no structures were missing from any gynandromorph.

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**The embryonic phenotypes of *sisA* null mutants:** To characterize the phenotypes of the *sisA* alleles without complications due to altered dosage compensation, we examined male *sisA* null mutant embryos for developmental defects. Mutant males were generated from attached-X females (X<sup>-</sup>X/Y) that had been mated with *sisA* males carrying a Y chromosome duplication of *sisA<sup>±</sup>*. The *sisA* male embryos could be distinguished from their 2X and 3X sisters because they failed to express the female-specific Sxl protein and from nullo-X embryos by their postblastoderm development.

The earliest obvious defect in fixed *sisA* null embryos was the presence of a space, or gap, between the yolk cell and the adjacent soma. The gap was first evident in stage 8 embryos and the separation between the yolk and surrounding layers persisted in later stages (Figure 5). The gap was most clearly seen in embryos that had been fixed for <30 min, but could also be observed in more heavily fixed preparations. The aberrant junction between the yolk and the surrounding tissue appears to be an area of structural weakness as many *sisA* null mutant embryos break there when placed under a coverslip (Figure 5, C and E).

Examination of lethally arrested *sisA* null embryos revealed a variety of terminal phenotypes. Some arrested late in germ-band retraction, while others developed a wide range of cuticular and internal structures occasionally nearly completing development (Figure 5, G–I). The only identifiable common feature was a variably positioned yolk mass that often exhibited large globular yolk droplets. Similar ectopic accumulations of unenclosed yolk have been described in mutants that fail to form endoderm or midgut (Bronner et al. 1994), suggesting that *sisA* mutants may be defective in the ability to form the endoderm-derived midgut.

*sisA* null mutations prevent endoderm migration and midgut formation: The Drosophila larval midgut originates from two spatially distinct groups of cells, the anterior midgut (AMG) and posterior midgut (PMG) primordia (Campos-Ortega and Hartenstein 1997). The physical separation of the primordia requires that the AMG and PMG migrate over the visceral mesoderm and around the yolk before they fuse and form the midgut. Late in germ-band extension, the primordia form lobed structures that extend toward each other as they move along the visceral mesoderm and over the yolk cell (Reuter et al. 1993; Tepass and Hartenstein 1994). By the end of germ-band retraction the AMG and PMG have joined laterally on both sides of the yolk to form an epithelium that will then wrap around the yolk, enclosing it within the newly formed midgut.

To determine if *sisA* null mutants are defective in
endoderm specification, migration, or other aspects of midgut formation, we examined the development of the endoderm and midgut in sisA\(^5\) male embryos using the lacZ-expressing enhancer trap A490.2M3 (Reuter et al. 1993; Tepass and Hartenstein 1994) to mark the cells that contribute to the midgut. We found that the midgut primordia formed normally and that the AMG began to adopt its characteristic two-lobed structure dur-

**Figure 5.**—Phenotypes of sisA null mutant embryos are suggestive of defects in the yolk cell. (A) Normal female expressing SXL at stage 8 or 9. (B±I) sisA mutant male embryos. Yellow arrows point to gaps between the yolk mass and surrounding cells. Black arrows to unenclosed or ectopic yolk. (B) Stage 8, when structural defects are first evident. Note pole cells in proctodeum. (C) Stage 9 or 10 embryo broken at gap between yolk and somatic tissue. (D) Stage 10: pole cells have migrated out of the proctodeum, which has twisted laterally. (E) Stage 12. (F) Stages 13±15. Note normal fore- and hindgut structures and unenclosed yolk. (G±I) Terminally arrested sisA embryos. (G) Exemplifies the earliest arrest and (I) the most developed. Fixation was for 20 min (A±D, I) or 40 min (E±H).

**Figure 6.**—Endoderm precursors form but their migration is blocked in sisA null mutants. Anterior (AMG) and posterior midgut (PMG) primordia were identified using the lacZ-expressing enhancer trap A490.2M3. The wild-type β-gal expression pattern is shown in germ-band extension, stage 10 (A), early germ-band retraction, stage 12 (B), and in two embryos at the completion of germ-band shortening, stage 13 (C and D). In sisA\(^5\) males, the AMG and PMG primordia form and express the lacZ marker at stage 10 (E), but migration is prevented during and after germ-band retraction when development becomes abnormal (F±H). Embryos were fixed as for in situ hybridization.
ing stage 10 in sis\(^A\) \(^1\) mutants, but that subsequent development of the AMG and PMG was abnormal (Figure 6). In particular, there was little extension of the AMG lobes and no migration of the AMG or PMG precursors in sis\(^A\) \(^1\) mutants. Consequently, the yolk was left as an unenclosed mass that moved to various ectopic locations as other aspects of development continued (Figure 5).

**DISCUSSION**

**A screen for sisterless mutations:** Previous attempts to identify new sisterless mutations have failed to generate new sis\(^A\) alleles, although sis\(^B\), Sxl, and sis\(^C\) mutations were recovered (Cline 1993; Timmer 1995; Cline and Meyer 1996). Here we used a noncomplementation screen to identify mutations that interacted with sis\(^A\) \(^1\) to produce a female-lethal phenotype. The key feature of our screen was that it allowed the recovery of lethal sis\(^A\) alleles, the importance of this being demonstrated by the male-lethal phenotypes of all four new sis\(^A\) mutations. That, combined with the fact that no sis\(^A\) alleles were recovered from large-scale screens where lethals would not have survived (Timmer 1995), suggests that it may be difficult to mutate the 600-bp sis\(^A\) gene in a way that creates sex determination-specific alleles such as sis\(^A\) \(^1\).

A notable sidelight of the screen was the efficient recovery of sis\(^B\) or sis\(^C\) alleles. Although the Achaete-scute complex has been extensively studied genetically, few point mutations exist. A recent screen for bristle defects, for example, produced one EMS-induced sis\(^B\) point mutation from >28,000 F\(_1\) chromosomes screened (Gomez-Skarmeta et al. 1995). Here we recovered five EMS-induced sis\(^C\) alleles, each possessing a different bristle phenotype, suggesting that the most effective means of isolating novel sis\(^B\) point mutations may be to search for new sis\(^B\) alleles.

**Sequence and function of the new sis\(^A\) alleles:** Two of our mutations, sis\(^A\) \(^2\) and sis\(^A\) \(^3\), are nulls by molecular and genetic criteria. While the sis\(^A\) \(^5\) frameshift occurs early in the coding sequence and reveals no structural information about the protein, the null character of the late nonsense mutation sis\(^A\) \(^2\) further supports the importance of the proposed leucine zipper domain for SISA function. A third strong mutation, sis\(^A\) \(^3\), affects eight residues of a conserved N-terminal extension of the proposed DNA-binding region, demonstrating the functional importance of this segment of the sis\(^A\) protein.

The sis\(^A\) \(^3\) mutation is complex, consisting of a base substitution and two frameshifting deletions. The changes are located downstream of a highly diverged region that separates the conserved N- and C-terminal halves of the protein. This structural characteristic of SISA, conserved sequence blocks separated by randomized segments of varying length, is commonly observed among homologous proteins in Drosophila species, but the mecha-

isms that generate such randomized segments are obscure. In this regard, the sis\(^A\) \(^1\) allele, although itself nonfunctional, may be illustrative of how compensating frameshifts can produce randomized segments and yet maintain conserved sequence blocks on either side.

The sis\(^A\) \(^4\) mutation was caused by the insertion of a hobo element in the sis\(^A\) promoter region. This partial loss-of-function allele is temperature sensitive (ts) both for yolk function and sex determination, suggesting that it may have a ts effect on sis\(^A\) expression. While ts mutations generally affect protein structure, there are precedents for cis-acting ts alleles in Caenorhabditis elegans (Perry et al. 1994) and for the Drosophila sex determination elements da and sis\(^B\) (Cronmiller et al. 1988; Erickson and Cline 1991, 1993; Parkhurst et al. 1993). Alternatively, the ts nature of sis\(^A\) \(^4\) might reflect an underlying temperature sensitivity in the two developmental processes. The sex-determining X-counting mechanism is inherently sensitive to temperature regardless of the particular alleles employed (Cline 1988; Torres and Sanchez 1991). Whether a similar, natural heat sensitivity applies to the sis\(^A\) yolk function remains to be determined.

**sis\(^A\) is needed for proper Sxl expression but is not essential for Sxl\(_{pe}\) promoter activity in all cells:** By genetic criteria sis\(^A\) and sis\(^B\) are the most potent zygotic determinants of X chromosome dose (Cline and Meyer 1996) and both are needed for the proper activation of Sxl\(_{pe}\) in all regions of the embryo (Bopp et al. 1991; Torres and Sanchez 1991; Erickson and Cline 1993). However, previous studies left open the question of what happens to Sxl expression in the complete absence of sis\(^A\) activity. Here we show that a surprisingly high level of Sxl expression remains. While the effects of the loss of sis\(^A\) are qualitatively different from the effect of the loss of sis\(^B\), sis\(^A\) is nonetheless critical for proper Sxl activation throughout the embryo. A number of sis\(^A\) null females express trace levels of SXL, while only a small fraction express wild-type levels of SXL in more than a few cells.

Curiously, the residual Sxl expression in sis\(^A\) null embryos is not obviously different from that seen with the partial loss-of-function sis\(^A\) \(^1\) allele (Erickson and Cline 1993). While careful quantitation might reveal the expected difference, the implication is that, while not null, sis\(^A\) \(^1\) is severely defective for sex determination.

Given the strong effect of sis\(^A\) \(^1\) on sex determination, and the expectation that the lys-to-glu change in the basic region should seriously hinder SISA’s DNA binding, why is the sis\(^A\) \(^1\) allele male-viable? One possibility is that the sis\(^A\) yolk function might not depend on DNA binding. If SISA were to function negatively by sequestering other proteins in an inactive complex, the mutant sis\(^A\) \(^1\) protein might be able to perform this function without binding DNA, in a manner analogous to the Id proteins in muscle development (Murre and Baltimore 1992). An alternative is that the polyplloid yolk
nuclei produce an excess of sisA protein and that the viability of sisA males is explained by high-level expression of a partially functional protein.

Pleiotropy and the evolution of the sex-signal elements: Primary sex-determining mechanisms are among the most rapidly evolving developmental processes. Sxl, for example, appears to have been recruited to sex determination relatively late in the Dipteran lineage (see Saccone et al. 1998). If sisA, -B, -C, and runt coevolved with Sxl as signal elements, it was likely due to their X-linkage and because they possessed preexisting expression patterns and functions that could be easily modified or directly coopted for use in sex determination. This would account for their pleiotropy, why their primordial functions were unrelated to sex, and why each is expressed in a fairly broad pattern coincident with, or slightly after, the early burst of Sxl expression. While nearly all the maternal and zygotic sex signal elements have alternative functions in the nervous system, we suspect that their acquisition as signal elements may be more directly related to other, earlier-acting functions. runt, for example, functions as a broadly expressed gap gene just after sex is determined (Duffy and Gergen 1991), while sisA and maternal da participate in dorsal-ventral axis specification during the period when Sxl is most active (Gonzalez-Crespo and Levine 1993). Our discovery that sisA has a vital developmental function in the yolk nuclei suggests that sisA was likely chosen as a signal element by virtue of its early uniform yolk expression. Whether that required the acquisition of new regulatory information or simply exploited an underlying prepattern necessary for yolk-specific expression remains to be determined.

sisA has a vital function in the yolk nuclei: Taken together, the expression and TSP analyses strongly implicate the yolk nuclei as the site of sisA function. While we cannot exclude the possibility that undetected low-level sisA expression occurs outside the yolk, our in situ hybridization data were particularly clear during gastrulation and germ-band extension, minimizing the chances that low-level expression was missed or obscured by the strong yolk signal during these critical periods (Figure 3).

On the basis of the obvious diffusion of sisA mRNA (Figure 3) and the extensive cytoplasmic connections (Poulson 1950) between the yolk nuclei, we suspected that sisA would act nonautonomously within the yolk cell. If sisA's second vital function is restricted to the yolk cell, one would predict that gynandromorphs carrying large patches of sisA null mutant tissue could be easily isolated, as these animals should be yolk nuclear mosaics, and thus viable, regardless of their cuticle phenotype. The 57 sisA gynandromorphs perfectly matched these predictions. There was no apparent difference in the viability of control sisA and null sisA gynandromorphs (materials and methods) and little or no regional bias as to where sisA function could be lost (Table 3). Although the gynandromorph data are most simply explained by a nonautonomous function of sisA within the yolk cell, we cannot exclude certain alternative explanations for these results. The most important limitation was that because we scored only the adult cuticle phenotype in the mosaic flies, we were able to directly infer the genotype of only a fraction of the blastoderm surface. This leaves open the possibility that sisA is required in other regions of the embryo that are not represented in the adult cuticle (Zusman and Wieschaus 1985).

Perhaps the strongest support for the hypothesis that sisA functions in the yolk nuclei is the phenotype of sisA null embryos. The alteration in yolk cell structure, or in its attachment to the surrounding tissues, which is visible shortly after the beginning of the sisA TSP (Figures 2 and 5), links the mutant phenotype directly to the cell in which sisA is expressed and to the stage when it is active. The subsequent failure of the AMG and PMG primordia to migrate over the yolk cell surface to create the midgut (Figure 6) further strengthens the connection between gene expression and phenotype.

Yolk nuclei have a developmental role in embryogenesis: The role of the yolk has been one of the more neglected areas of study in Drosophila development. Early transplantation experiments suggested that the yolk played no inductive role in development (Hadorn 1974), a conclusion supported by the absence of any previous reports of developmental mutations acting in the yolk. While sisA has an essential function in the yolk cell, its role does not appear to be inductive, as both the endodermal and mesodermal primordia of the midgut, the organ most directly affected by the loss of sisA, form in sisA null mutants.

The yolk nuclei are commonly known as vitellophages for their presumed function in digesting yolk. Given that yolk protein metabolism does not begin until after the yolk has been engulfed by the midgut and continues long after the yolk nuclei have been degraded (Bownes 1982b), we suspect that their primary function is developmental. Considering the ultimate fate of the yolk—to be devoured within the midgut—it is reasonable to think that the yolk nuclei might play a role in gut formation. The idea is not new. In his studies on embryonic development, Poulson (1950) hypothesized that the close contact between the midgut rudiments and the yolk nuclei was essential for gut formation. Although Poulson's claim that some yolk nuclei are incorporated directly into the midgut epithelium has not been borne out by subsequent investigation (Campos-Ortega and Hartenstein 1997), the implication of the present work is that the yolk nuclei are indeed important for midgut development.

The failure of endoderm migration in sisA mutants suggests that there must be proper contact between the endoderm and the yolk sac for migration to occur. A particularly intriguing idea is that the yolk cell, like the
visceral mesoderm, serves as a track, or substratum, for the migrating endoderm (Azpiazu and Frasch 1993; Reuter et al. 1993; Tepass and Hartenstein 1994). siSA could be involved directly by regulating the expression of cell adhesion proteins within the yolk cell. Alternatively, siSA might be required to maintain the structural integrity of the yolk cell, perhaps by regulating the expression of cytoskeletal components. In that case, the structural collapse of the yolk sac might prevent normal recognition of the yolk membrane by the AMG and PMG primordia in siSA mutants.

Although the failure to form a midgut is the most prominent early developmental defect observed, siSA mutants exhibit other abnormalities, such as mislocalized yolk, a twisting of the proctodeum, and abnormal germ-band retraction (Figures 2, 5, and 6), which hint that other morphological movements might depend on proper contact between the yolk cell and the surrounding viscera and soma. The presence of dense plaques and gap junctions between cells in the extended germ-band and the yolk sac (Rickoll and Counce 1980) is consistent with the idea that there may be interactions between these tissues.

siSA null mutant embryos exhibit a wide range of terminal phenotypes: some are missing head components and some lack posterior structures, while others form nearly normal larvae (Figure 5, G-I). We suspect that most such late defects are a secondary consequence of the earlier failure of endoderm migration. In the absence of the enveloping midgut, yolk is distributed to a variety of locations, and the ectopic yolk masses likely interfere with many structures by simple displacement. This phenotypic variation may explain why siSA was not identified in saturation screens for X-linked mutations affecting embryonic development (Wieschaus et al. 1984; Eberl and Hilliker 1988), although low mutability may also have played a part.

Our results clearly implicate siSA and the yolk nuclei in the normal development of the larval midgut. Interestingly, several other genes required for midgut specification, maintenance, or migration are also expressed in the yolk nuclei, including serpent, hunchback, and fork head (Reuter 1994; Rehor et al. 1996). Little is known about the roles of these genes in the yolk in part because their complex expression patterns and pleiotropy make it difficult to identify a specific yolk function, but primarily because there has been little indication that the yolk nuclei have any important function. Now that the yolk nuclei have been shown to be important for development, further analysis of the regulation and function of these and other genes in the yolk should lead to a greater understanding of the yolk cell's function in midgut formation and may even reveal additional roles of the yolk nuclei in Drosophila. Whether they will uncover unexpected similarities between the developmental roles of the yolk cells of insects and the visceral yolk sacs, yolk cells, or yolk syncytial layers of vertebrates is a question of considerable interest.

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