Molecular and Cellular Biology, Aug. 1995, p. 4430–4440 Vol. 15, No. 8
0270-7306/95/$04.00+0
Copyright 1995, American Society for Microbiology

scute (*sis-b*) Function in *Drosophila* Sex Determination

GIRISH DESHPANDE,[1] JOSEPH STUKEY,[2] AND PAUL SCHEDL[1]*

Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544,[1] and Department of Food Science, Cook College, Rutgers University, New Brunswick, New Jersey 08902[2]

Received 26 October 1994/Returned for modification 29 December 1994/Accepted 2 May 1995

The primary sex determination signal, the X chromosome-to-autosome (X/A) ratio, controls the choice of sexual identity in the *Drosophila melanogaster* embryo by regulating the activity of the early promoter of the *Sex-lethal* gene, *Sxl-P$_e$*. This promoter is activated in females (2X/2A), while it remains off in males (1X/2A). Promoter activation in females is dependent upon X-linked numerator genes. One of these genes, *sisterless-b* (*sis-b*), corresponds to the *scute* (*sc*) locus of the *achaete-scute* complex, and it encodes a helix-loop-helix transcription factor. In the studies reported here we have used monoclonal antibodies to study the expression and functioning of the *sc*(*sis-b*) protein. Sc is first detected at nuclear cycle 6 to 7, well before *Sxl-P$_e$* is first active. At this stage, the protein is in the cytoplasm, not the nucleus. Only after the formation of the syncytial blastoderm, at nuclear cycle 10 to 11, does a substantial fraction of the protein enter the nucleus, and this nuclear import closely coincides with the initial activation of *Sxl-P$_e$*. Consistent with the idea that the dose of *sc*(*sis-b*) is critical for its function as an X-chromosome counting element, wild-type syncytial blastoderm embryos could be grouped into two classes based on the level of protein. Western blot (immunoblot) analysis demonstrates that this difference in protein level correlates directly with the activity state of the *Sxl* gene. Finally, we provide the first direct evidence that Sc forms heteromeric complexes in vivo in early embryos with the maternally derived helix-loop-helix protein Daughterless. This in vivo complex is likely to be critical for Sc function in *Sxl-P$_e$* activation.

The choice of sexual identity in the fruit fly *Drosophila melanogaster* is determined by the X chromosome-to-autosome (X/A) ratio (1, 16, 20, 48, 49). A ratio of 1 (2X/2A) signals the initiation of the female developmental pathway, while a ratio of 1/2 (1X/2A) signals male development. The system that measures the X/A ratio operates only transiently during early embryonic development, and it functions to set the activity state of the binary-switch gene *Sex-lethal* (*Sxl*) (17). In 2X/2A animals, the X/A signaling system turns on the *Sxl* gene, while the gene is not activated in 1X/2A animals. In the on state, the *Sxl* gene directs female development by controlling several regulatory cascades that function in different aspects of somatic sexual development (14, 32, 41, 44, 50). These regulatory cascades include the *transformer-doublesex* somatic sexual differentiation pathway and the dosage compensation pathway (2, 8, 44). The *Sxl* gene also provides the mechanism for remembering the female determined state (15). This is accomplished through an autoregulatory feedback loop in which the Sxl protein directs its own expression by promoting the female-specific splicing of primary transcripts produced from the *Sxl* late or maintenance promoter, *Sxl-P$_m$* (3, 4, 36, 37). Similarly, the male determined state is maintained by the default splicing machinery, which splices primary transcripts from *Sxl-P$_m$* in the nonproductive, male mode. In 1X/2A embryos, in which the *Sxl* gene remains off, the different regulatory cascades operate in the default mode, leading to male development.

While the on-off regulation of the *Sxl* gene from early embryogenesis through to the adult stage is determined by the alternate splicing of *Sxl* transcripts from *Sxl-P$_m$*, the initial choice of sexual identity is made at the level of transcription (38). In 2X/2A embryos an early *Sxl* promoter, *Sxl-P$_e$*, located in the first intron of the maintenance transcription unit, is activated shortly after nuclear migration. The mRNAs expressed from this promoter encode an embryonic form of the *Sxl* protein (7, 38). This embryonic protein sets the autoregulatory feedback loop in motion by directing the female-specific splicing of the first transcripts produced from the late promoter *Sxl-P$_m$*, which initially turns on just prior to cellularization. In 1X/2A animals the *Sxl-P$_e$* promoter is not activated and in the absence of embryonic Sxl protein, the first transcripts from *Sxl-P$_m$* are spliced in the default, or male, mode.

The *Sxl* early promoter, *Sxl-P$_e$*, appears to be the direct target for the system that signals the X/A ratio. Genetic studies have identified several different components of this signaling system. The first component is the X-linked numerators, the genes *sisterless-a* (*sis-a*), *sisterless-b* (*sis-b*) (or *scute* [*sc*]), *runt*, and *sisterless-c* (*sis-c*), which function to promote the choice of the female pathway (17, 18, 27–29, 45, 53, 54). In 2X/2A animals loss-of-function mutations in these genes can block the activation of the *Sxl* gene, causing sex transformations and lethality. The defect in initiating the *Sxl* autoregulatory feedback loop has been shown to be due to a failure to properly activate *Sxl-P$_e$* (38). The second component of this signaling system is the genes which function as autosomal denominators, promoting the male pathway. Thus far, only one zygotic denominator, *deadpan* (*dpn*), has been identified (5, 58). In 1X/2A animals, mutations in *dpn* can lead to sex transformations and lethality by initiating the *Sxl* autoregulatory feedback loop. This defect in the choice of sexual identity is due to the inappropriate activation of *Sxl-P$_e$* in male embryos (30). The third component of the signaling system is genes whose products are contributed by the mother during oogenesis. One of these, *daughterless* (*da*), is required for the choice of the female pathway and facilitates the activation of *Sxl-P$_e$* in 2X/2A animals by the products of the X-linked numerator genes (11, 13, 21, 22, 24). The other maternal gene is *extramacrochaete* (*emc*), and it appears to function much like the zygotic autosomal gene *dpn* in keeping *Sxl* off in 1X/2A animals (31, 55).

Consistent with their roles in regulating the *Sxl-P$_e$* promoter, molecular analysis of the different components of the X/A

* Corresponding author. Phone: (609) 258-5003. Fax: (609) 258-5323.

signaling system indicates that they resemble known transcription factors. Sis-a is a member of the bZIP family of transcription factors (29), while *runt* is homologous to a proto-oncogene responsible for acute myeloid leukemia (25). The four other components of the signaling system, Da, Emc, Sc, and Dpn, are all members of the helix-loop-helix (bHLH) family of proteins. This class of proteins can form both homo- and heteromeric complexes, and they function as transcriptional regulators. Moreover, their regulatory effects on transcription have been found to depend upon the type of heteromeric complex that is formed; some complexes function to activate transcription, while others either are unable to activate transcription or may actually block activation (6, 42, 43). In addition to the involvement of these bHLH genes in sex determination, they also function at later points in development in neurogenesis (19, 35, 52–54).

With the aim of learning more about how the X/A ratio signals the activation of *Sxl-P*$_e$, we have initiated molecular studies on the product of the *sc* gene. Molecular and genetic experiments have shown that the *sc* gene corresponds to the proneural *sc* gene from the *achaete-scute* gene complex (AS-C) (9, 10, 57). The AS-C consists of four different genes: *achaete*, *scute*, *lethal of scute*, and *asense*. All four encode bHLH proteins. They function in the establishment of neuroblast identity and are required during embryogenesis and later during the larval and pupal stages for proper neural development. In the work reported here we have used monoclonal antibodies to examine the time of appearance and subcellular distribution of Sc in the early pre-cellular blastoderm embryo. Consistent with the postulated function of *sc* as a numerator element in the X/A signaling system, it is expressed at different levels in male and female embryos just prior to the activation of *Sxl-P*$_e$. In addition, we show that Sc forms a heteromeric complex with the bHLH *da* protein in vivo.

MATERIALS AND METHODS

Strains and culturing. Flies were grown on a standard medium (12) at 25°C unless otherwise indicated. All alleles, marker mutations, and balancers are described by Lindsley and Zimm (40).

DNA techniques. Several *sc* (*T4*) gene fusions were constructed to provide antigen for the production and screening of antibodies. The following strategy was used to generate a stable *malE-T4-lacZα* tripartite fusion gene product by using *T4* cDNA sequences (57). Plasmid pMALc (New England BioLabs) was first digested with *Eco*RI and *Sal*I, Klenow fragment treated to create blunt ends, and then religated. This modification of pMALc placed the *lacZα* fragment out of frame with respect to *malE* coding sequences. A 335-bp cDNA fragment of the *T4* gene was isolated by digestion with the restriction enzymes *Ssp*I and *Xmn*I. This fragment encodes the predicted internal amino acid residues 175 through 286 but does not include the bHLH motif. Insertion of this fragment in the correct orientation into the *Stu*I site of the above-described modified pMALc vector placed the *malE* gene in frame with *T4* codons, which in turn were in frame with the *lacZα* sequences. Insertion of the *T4* fragment in the opposite and incorrect orientation introduces a translation stop codon just 3 positions downstream of the *malE-T4* fusion point. A glutathione *S*-transferase (GST)-based tripartite fusion analogous to *malE-T4-lacZα* was assembled by switching the GST domain from vector pGEX2 (51) for *malE* sequences of the constructed fusion gene. A *trpE-T4* fusion gene was assembled by ligation of an *Ssp*I-derived 808-bp *T4* cDNA fragment into the blunt-ended *Bam*HI site of expression vector pATH3 (39). This *T4* fragment encodes the C-terminal 171 residues of the *Drosophila* protein, including the region used in both tripartite fusions.

Generation of monoclonal antibodies. The *malE-T4-lacZα* fusion gene product was purified from induced DH5α cultures by affinity chromatography according to the protocol of the manufacturer (New England BioLabs). Female BALB/c mice were injected intraperitoneally with 50 to 100 μg of purified antigen in Freund's adjuvant. Following the second boost, mouse sera were collected and screened for the presence of *T4*-specific antibodies by using *Escherichia coli* whole-cell lysates of DH5α that were induced for the expression of a *trpE-T4* fusion product. The selected mouse was given a final boost with the GST gene-*T4-lacZα* antigen that had been purified from induced DH5α cultures according to the manufacturer's protocol. The spleen cells from this mouse were isolated 3 days after the final boost and fused to myeloma cells in the presence of polyethylene glycol (Boehringer Mannheim). Approximately 600 parental hybridoma lines were screened with purified GST gene-*T4-lacZα* fusion protein by the rapid procedure described by Hawkes et al. (34). A total of 88 potential positives were rescreened against both *trpE* and *trpE-T4* antigens in Western blot (immunoblot) analyses using induced DH5α whole-cell lysates. Those parental hybridoma lines producing antibodies that specifically recognize the *trpE-T4* fusion protein but not the *trpE* control antigen were selected for subcloning. Although supernatants from four different hybridoma lines were tested initially, line 5A10 was used for all experiments reported here.

Western blot analysis. Staged embryos were washed from food plates, dechorionated in 50% bleach, and washed again in phosphate-buffered saline (PBS) prior to total protein extraction. Protein extracts were prepared essentially as described by Driever and Nusslein-Volhard (26). Embryos were frozen in liquid nitrogen and then removed for thawing and homogenization in 2× protein loading buffer. After incubation for 10 min at 95°C, insoluble material was sedimented. Subsequently, 10 to 15 μl was applied to each slot on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel. After electrophoresis, protein was transferred to nitrocellulose paper in Tris-glycine buffer for 2 h at constant 250 mA. To assess the amount of total protein loaded and transferred for each lane, the blot was stained with Ponceau S red. After blocking with 5% low-fat dry milk powder in PBS–0.1% Tween 20, the blot was incubated overnight with the first antibody (1:10 dilution of hybridoma supernatant in PBS–0.1% Tween 20 at 4°C). A horseradish peroxidase-conjugated anti-mouse antibody kit from Promega was used for subsequent detection of antigen-antibody complexes on the blot.

Immunolocalization in whole-mount embryos. Embryos were dechorionated for 2 to 3 min in 50% bleach, washed thoroughly in PBS, and incubated in heptane-saturated fixative (4% paraformaldehyde, Polysciences electron microscopy grade, in PBS) for 30 min at room temperature. The fixative phase was then removed and replaced with 90% methanol–10% 0.5 M ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). After several vigorous shakes, the devitellinized embryos that sank to the bottom were collected and rinsed several times in the methanol-EGTA solution and then in plain methanol. For staining, embryos were gradually rehydrated in PBS and incubated with 1% bovine serum albumin (BSA)–1% Triton X-100 in PBS for 3 to 4 h at room temperature. The first antibody was applied as a 1:10 dilution of hybridoma supernatant in PBS–0.1% BSA–0.1% Triton X-100 and incubated overnight at 4°C. After several washes in the same buffer, embryos were treated with biotinylated secondary antibody for 2 h at room temperature and subsequently with biotinylated horseradish peroxidase-avidin complexes according to the Vecstastain protocol (Vector Laboratories). The bound complexes were visualized with an immunohistochemical detection kit from the same company. Photomicrographs were taken on Ektar 125 film (Kodak) with a Zeiss photomicroscope. For confocal microscopy, anti-mouse secondary antibody coupled to the fluorescent probe, rhodamine red, was used in place of the biotinylated secondary antibody. Nucleic acids were stained with YoPro dye (Molecular Probes). Samples were washed as described above and were mounted in Aquamount.

Immunoprecipitations. Immunoprecipitations were performed essentially as described previously (33). Monoclonal antibodies against Da and Dpn proteins were obtained from Claire Cronmiller and Harold Vassein (5, 23).

RESULTS

Monoclonal antibodies raised against the C-terminal domain of the *sc*(*T4*) gene product recognize a 42-kDa protein from total embryonic extract. The numerator element *sis-b* corresponds to the proneural *scute* (*sc*) gene in the AS-C and is encoded by the *T4* transcription unit. Conceptual translation of the *T4* mRNA gives a predicted Sc of ~39 kDa. Located in

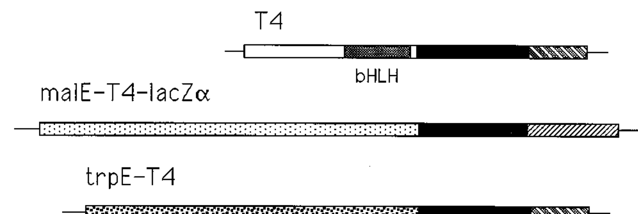


FIG. 1. Schematic representation of *sc* expression constructs used to generate monoclonal antibodies. The top schematic represents the *sc* (*T4*) protein. In order to generate antibodies specific to Sc, the well-conserved bHLH domain and the very C-terminal region (diagonal lines) were excluded. As indicated in the schematic showing the *malE-T4-lacZα* sandwich construct, antibodies were raised against the *T4* peptide sequences extending from amino acid residues 175 through 286 (solid box). Monoclonal antibody-producing lines recognizing *T4* peptide sequences were identified by using the fusion protein expressed from the *trpE-T4* construct.

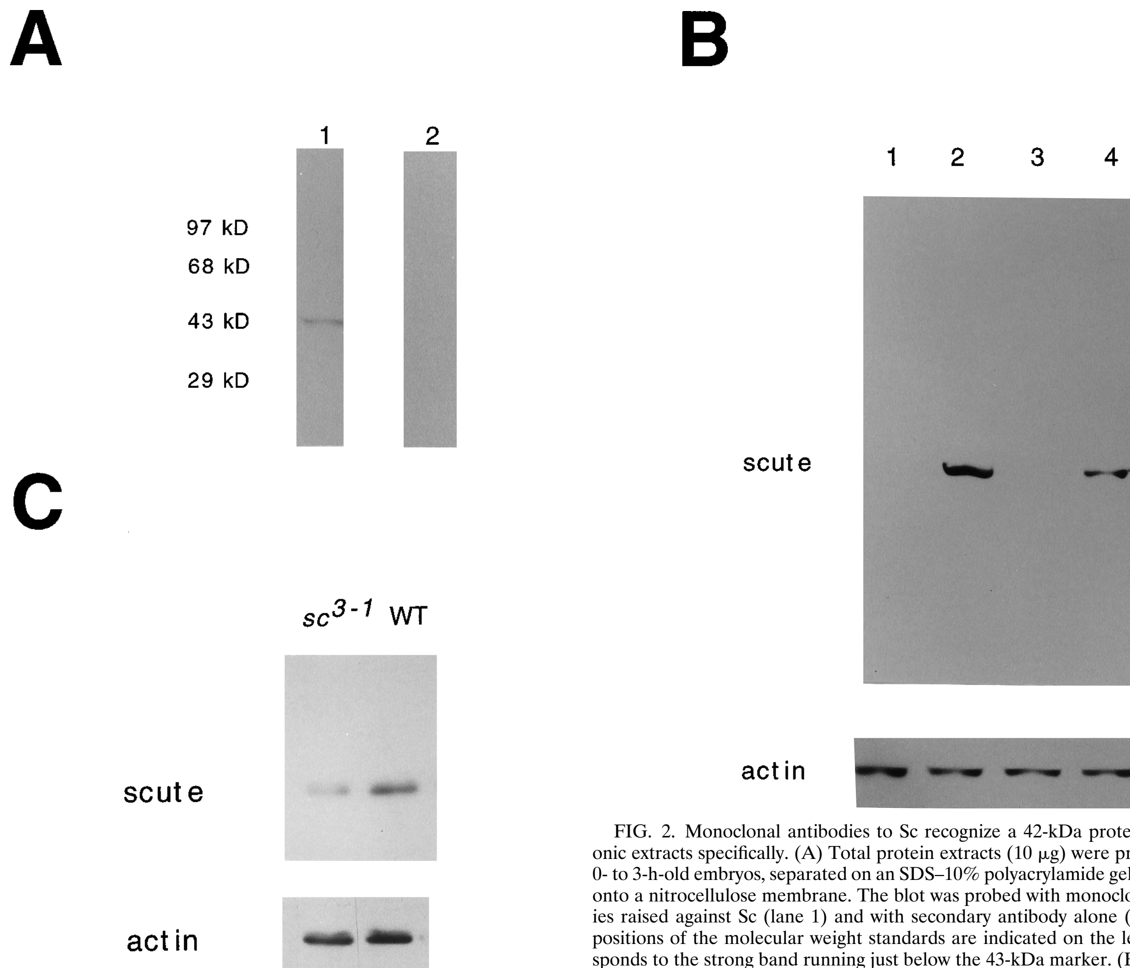


FIG. 2. Monoclonal antibodies to Sc recognize a 42-kDa protein in embryonic extracts specifically. (A) Total protein extracts (10 μg) were prepared from 0- to 3-h-old embryos, separated on an SDS–10% polyacrylamide gel, and blotted onto a nitrocellulose membrane. The blot was probed with monoclonal antibodies raised against Sc (lane 1) and with secondary antibody alone (lane 2). The positions of the molecular weight standards are indicated on the left. Sc corresponds to the strong band running just below the 43-kDa marker. (B) Truncated Sc produced in sc^{10-1} deficiency males is not recognized by the Sc monoclonal antibody. Total protein extracts were prepared from sc^{10-1} deficiency males and used in a Western blot with antibodies to Sc. Extracts from balancer males as well as nuclear extracts were used as positive controls. Lane 1, sc^{10-1} deficiency male; lane 2, nuclear extract; lane 3, sc^{10-1} deficiency male; lane 4, balancer male. Antibodies to actin protein were used as a loading control. (C) Sc levels are altered in the sc^{3-1} mutant. Wild-type (WT) and sc^{3-1} flies were reared at 22°C. Extracts were prepared from embryos collected between 0 and 2.5 h. Proteins were separated on an SDS–10% polyacrylamide gel, blotted onto a nitrocellulose membrane, and probed with Sc monoclonal antibodies or affinity-purified anti-actin antibodies (control). Relative levels of Sc in sc^{3-1} and wild-type lanes were determined by scanning the X-ray films of several different Western blots of mutant and wild-type extracts.

the N-terminal half of the protein is a 60-amino-acid bHLH domain. This bHLH domain shows a high degree of sequence similarity to the bHLH domains found in the proteins encoded by the other AS-C genes *lethal-of-scute* (*T3*), *achaete* (*T5*), and *asense* (*T8*) and, to a lesser extent, to other members of the bHLH family of transcription factors (47, 57). The C-terminal half of Sc, with the exception of the very C terminus (which has an amino acid sequence similar to those of the C termini of the *lethal-of-scute* [*T3*] and *achaete* [*T5*] proteins), appears to be unique. In order to obtain monoclonal antibodies which would specifically recognize Sc but not the other proteins from the AS-C, we used a part of the C-terminal domain that roughly corresponds to this unique region (amino acids 175 to 286) as an antigen. Fusion proteins containing only this truncated C-terminal domain were unstable, and a sandwich construct (Fig. 1) was required to obtain sufficient quantities of the Sc antigen for immunization (see Materials and Methods). We then used a *trpE-T4* fusion protein to monitor the immune response and identify potential monoclonal lines that express antibody directed against Sc.

We recovered several lines that recognize the *trpE-T4* fusion protein, and these were analyzed further with Western blots of proteins from embryo extracts. If the monoclonal antibodies recognize Sc in embryo extracts, we expect to observe a protein species of approximately 39 kDa in the Western blots. The other bHLH genes from the AS-C should produce proteins that are significantly larger or smaller than Sc. *asense* (*T8*) is expected to encode a larger protein of about 50 kDa, while *lethal-of-scute* (*T3*) and *achaete* (*T5*) are predicted to encode smaller proteins of 29 and 22 kDa, respectively. Hence, if our monoclonal antibodies also recognized any of the other members of the AS-C, it should be possible to resolve these proteins species from Sc. As illustrated by a Western blot with the monoclonal antibody line 5A-10 (Fig. 2A, lane 1), we observed one very prominent band with an apparent molecular size of 42 kDa. This is very close to the predicted size of the protein encoded by the *sc* (*T4*) gene. We did not, however, observe bands corresponding to the sizes predicted for the other bHLH proteins from the AS-C. Similar results were obtained with other monoclonal lines; in each case we also observed only one prominent band of the size predicted for Sc. An identical blot was probed with secondary antibody alone. As shown in Fig. 2A, lane 2, no cross-reactivity was observed.

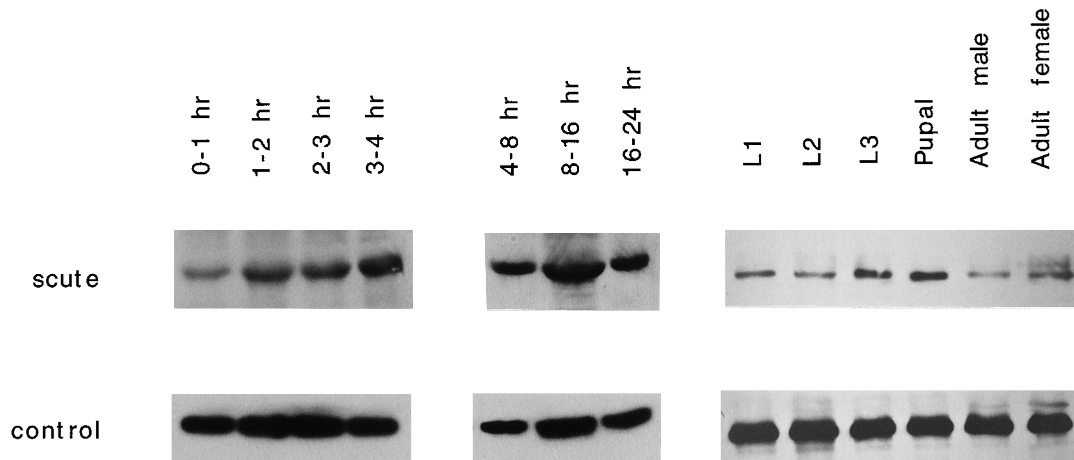


FIG. 3. Developmental expression profile of Sc. Total protein extracts were prepared from flies at different developmental stages, separated on an SDS–10% polyacrylamide gel, and blotted onto a nitrocellulose membrane. The blot was probed with monoclonal antibodies raised against Sc. Antibodies to actin (0- to 24-h embryonic extract blot) and tubulin (L1 to adult) were used as loading controls. L1, first-instar larvae; L2, second-instar larvae; L3, third-instar larvae.

To provide further evidence for the specificity of our Sc monoclonal antibodies, we used an *sc* mutant allele, $sc^{10\text{-}1}$, which is predicted to encode a truncated version of Sc. DNA sequence analysis of $sc^{10\text{-}1}$ suggests that the mutation is due to a C-to-T transition at nucleotide 1147. This nucleotide change produces an in-frame translation stop codon after amino acid position 162 in the Sc sequence (57). Since the translation of the $sc^{10\text{-}1}$ protein is expected to stop after position 162, the protein produced by this mutant should lack the C-terminal segment (residues 175 to 286) that we used to generate the Sc monoclonal antibody. Protein extracts were prepared from $sc^{10\text{-}1}$ adult males and used in a Western blot. As expected, the $sc^{10\text{-}1}$ protein is not detected by the monoclonal antibody (5A-10), while it is present in wild-type males.

Temperature-dependent reduction in the level of Sc with the temperature-sensitive allele $sc^{3\text{-}1}$. To further test the specificity of the monoclonal antibodies we took advantage of the *sc* mutant allele $sc^{3\text{-}1}$. Previous studies by Parkhurst et al. (47) have shown that this mutation affects the regulation of the *sc* gene in early embryos, and the expression of *sc* transcripts in $sc^{3\text{-}1}$ appears to be delayed and is markedly reduced relative to that of the wild type. This delay and the reduction in the level of *sc* transcripts are associated with a temperature-sensitive defect in female sex determination that is due to a failure to properly activate $Sxl\text{-}P_e$ (29, 30). The defects in $Sxl\text{-}P_e$ activity in $sc^{3\text{-}1}$ female embryos are evident even at permissive temperatures (18 and 22°C) and become more pronounced at nonpermissive temperatures (25 and 29°C) (30). If our antibody specifically recognizes Sc, we should observe a reduction in the level of the 42-kDa protein species in Western blots of $sc^{3\text{-}1}$ embryos. Shown in Fig. 2C is a Western blot of extracts prepared from 0- to 3-h wild-type and $sc^{3\text{-}1}$ embryos (grown at 22°C) probed with the Sc antibody or antibody directed against actin. As expected, we find that the level of Sc in $sc^{3\text{-}1}$ mutant embryos is reduced relative to that in the wild type. In other experiments (not shown) we have found that the level of Sc (relative to that of actin) in mutant embryos is further reduced at higher temperatures.

Developmental expression pattern of Sc. Previous Northern (RNA) analysis has shown that the *sc* transcripts begin to accumulate in very early (0- to 2-h) embryos. The level of *sc* mRNA continues to increase between 2 and 4 h and then remains constant through the first half of embryonic development. Message accumulation then appears to cease midway through embryogenesis, and *sc* RNA cannot be detected in 12- to 24-h embryos or in first- and second-instar larvae. *sc* mRNA reappears during disc development in third-instar larvae and pupae, while it cannot be detected in adults (29).

As shown in Fig. 3, the pattern of accumulation of Sc is somewhat different from that of the mRNA. In Western blots we can first detect Sc in 0- to 1-h embryo collections. In this context it is interesting to note that there is some disagreement regarding the earliest point of *sc* mRNA expression (29, 47). Parkhurst et al. (47) have reported that *sc* transcripts can be detected as early as nuclear division cycle 3, while Erickson and Cline (29) found that expression was not evident until around nuclear cycle 9. Since most of the embryos in our 0- to 1-h collections are pre-nuclear-cycle 9, it seems likely that *sc* mRNA is first expressed somewhat earlier than the ninth cycle. This possibility is supported by results of whole-mount staining experiments presented below.

Only relatively low levels of Sc are evident in 0- to 1-h embryos. (Although it is low, we believe that the signal in early embryos is real and zygotically derived, as unfertilized eggs which are often found in 0- to 3-h embryo collections never show staining above background.) The level of protein increases in the interval between 1 and 3 h and reaches a maximum in 3- to 4-h embryos. This high level of protein appears to be sustained in the interval between 4 h and the end of embryogenesis at 24 h. By way of contrast, no mRNA is detected during the last 12 h of embryogenesis (29). Moreover, though no *sc* mRNA is found in the first and second larval stages (29), readily detectable levels of protein are observed. These findings suggest that Sc synthesized during the first 12 h of embryogenesis is stable for several days after the *sc* mRNA has apparently turned over and is no longer detectable. In third-instar larvae and in pupae the level of Sc increases, presumably as a consequence of the de novo mRNA accumulation observed in these stages (29). Finally, though little or no *sc* mRNA is evident in adults, protein is readily detected. As was the case earlier in development, the protein found in adults is likely to be derived from the translation of *sc* mRNA during the third-instar larval or the pupal stage.

***Df(1)sc19* embryos do not make Sc.** To provide evidence for the specificity of our monoclonal antibodies in whole mounts, we examined the staining pattern in embryos lacking the *sc*

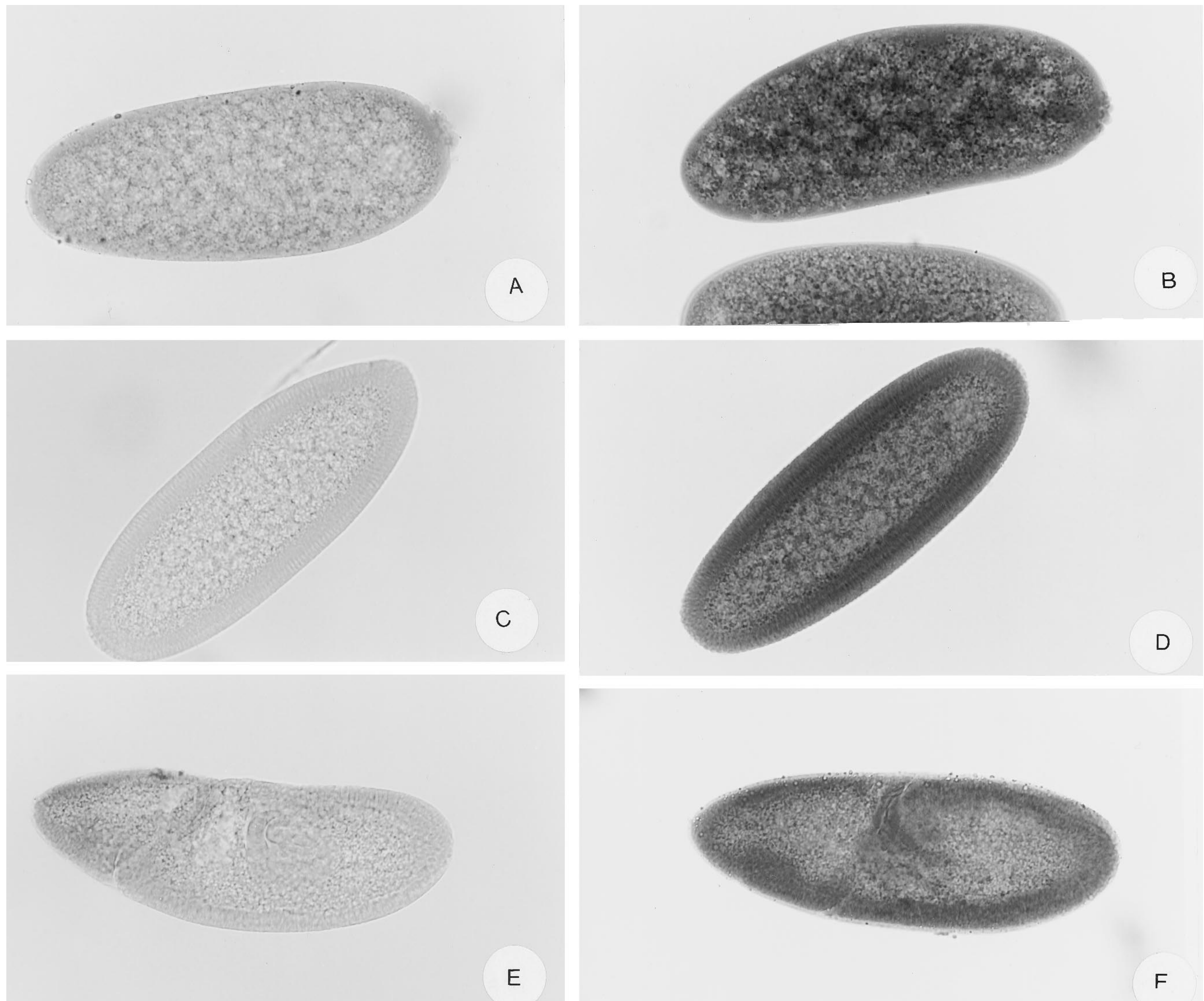


FIG. 4. Specificity of the monoclonal antibodies to Sc. Whole-mount wild-type (B, D, and F) and *Df(1)sc19* (A, C, and E) embryos were stained with monoclonal antibodies to Sc and visualized by immunohistochemical detection. (A and B) Pre-blastoderm stage; (C and D) cellular blastoderm stage; (E and F) germ band-extended stage.

gene. For this purpose we used a deficiency chromosome, *Df(1)sc19*, which deletes the *sc*, *achaete*, and *lethal-of-scute* genes of the AS-C. The X-linked deficiency *Df(1)sc19* was balanced against the *Binsinscy* (*Bins*) chromosome, which carries a duplication of the AS-C. Three classes of embryos, which differ in their dose of *sc*, are expected to be produced from this *Df(1)sc19*; *Bins* stock. In the first class, which should comprise a quarter of the population, would be male embryos that carry the *Df(1)sc19* chromosome and consequently lack the *sc* gene. In the second class would be *Bins* male embryos and *Df(1)sc19/Bins* female embryos that have two copies of *sc*. This class should represent one half of the population. Finally, the third class (the remaining quarter) would consist of *Bins/Bins* female embryos which have four copies of *sc*.

As anticipated, approximately one-fourth of the embryos showed little or no staining with the Sc monoclonal antibody, while staining was clearly evident in the remaining three-fourths. Representative embryos at three different stages of development—nuclear cycle 10 (panels A and B), cellular blastoderm (panels C and D), and germ band extended (panels E and F)—are presented in Fig. 4. As can be seen from comparing the negative and positive embryos at equivalent stages of development, little or no staining can be detected in the putative *sc* deletion mutant animals. The lack of cross-reacting material in approximately a quarter of the embryos (coupled with our Western analysis) argues that the antibodies produced by this monoclonal line specifically recognize Sc. Similar results were obtained with the other monoclonal antibodies and with another mutant chromosome, *In(1)sc8Lsc4R*, which lacks the *sc* gene.

Interestingly, among the positive embryos there appeared to be two classes: approximately two-thirds of the positive embryos showed moderate staining, while the remaining one-third showed more intense staining (data not shown). We presume that this reflects the fact that one-half of the total embryos should have two copies of *sc* while one-fourth should have four copies.

Monoclonal antibodies to Sc can detect differences in the levels of protein in early embryos. Sex determination in the

Drosophila soma is dependent upon the relative gene dose of the zygotically active X-linked numerator elements and the maternal or zygotic denominators that function as autosomal counting elements (18, 54). It is hypothesized that the twofold difference between male and female embryos in the copy number of numerator genes like *sc* translates directly into a twofold difference in the level of the corresponding gene product (18, 54). This dose dependence in the level of X-linked gene expression would be unique to early embryogenesis and would reflect the fact that the dosage compensation system either is inoperative at this stage of development or functions in both sexes until it is turned off in females when the *Sxl* protein is first produced. In either case, a prediction of this model is that there should be two populations of early embryos, corresponding to 1X and 2X animals, which differ in the level of Sc.

To test this hypothesis we stained early *white*[1] embryo collections with our Sc monoclonal antibodies. Although we can detect differences in the level of Sc in pre-cycle 9 embryos (see also below), it is not possible to reliably classify them into two distinct groups on the basis of the intensity of antibody staining—some show stronger staining, some show weaker staining, but there is also a fairly large group of embryos which show intermediate levels of staining. The embryo population can, however, be readily divided into two groups after syncytial blastoderm formation when the amount of Sc has increased. The differences in the level of Sc staining between the two groups is illustrated by the two cycle 12 embryos shown in Fig. 5A. We analyzed 389 blastoderm embryos that were between cycles 11 and 13. To eliminate cycle-dependent differences in protein levels, these embryos were first grouped by nuclear density and were then scored for the relative intensity of Sc antibody staining. Of these syncytial blastoderm embryos, 189 showed dark staining while 200 stained more lightly, which is near the expected ratio of 1X to 2X animals. These findings suggest that the level of Sc is proportional to the gene dose during the syncytial blastoderm stage and perhaps even earlier. This corresponds closely to the time when the X/A ratio is thought to be measured and the *Sxl* gene first activated (7, 28).

The presence of two groups of embryos which differ in the level of Sc seems to persist until the onset of gastrulation, at which point all of the similarly staged embryos begin to show essentially the same staining intensity and spatial distribution. Since the *sc* transcripts expressed in the initial burst disappear at the beginning of nuclear cycle 14 (29, 47), the differences in protein level evident during the early stages of gastrulation are likely to reflect the perdurance of Sc synthesized (in 1X and 2X animals) prior to cellularization. On the other hand, the equalization of protein levels in the slightly older embryos suggests that the dosage compensation system must be functioning in males and turned off in females at or before the time when the *sc* gene is reactivated and the proneural pattern of expression develops.

Weakly stained embryos are male, while female embryos stain more darkly. In the experiments described in the previous section we could divide the pre-cellular blastoderm embryos into two groups on the basis of the level of Sc antibody staining. Our interpretation of these results is that the group showing lighter staining corresponds to male embryos which have only single *sc* gene, while the group showing stronger staining corresponds to female embryos which have two *sc* genes. If this interpretation is correct, then the staining intensity of the embryos should be correlated with the activity of the regulatory target for Sc, the *Sxl* gene. The *Sxl* gene should be in the off state in the more lightly staining male embryos, while

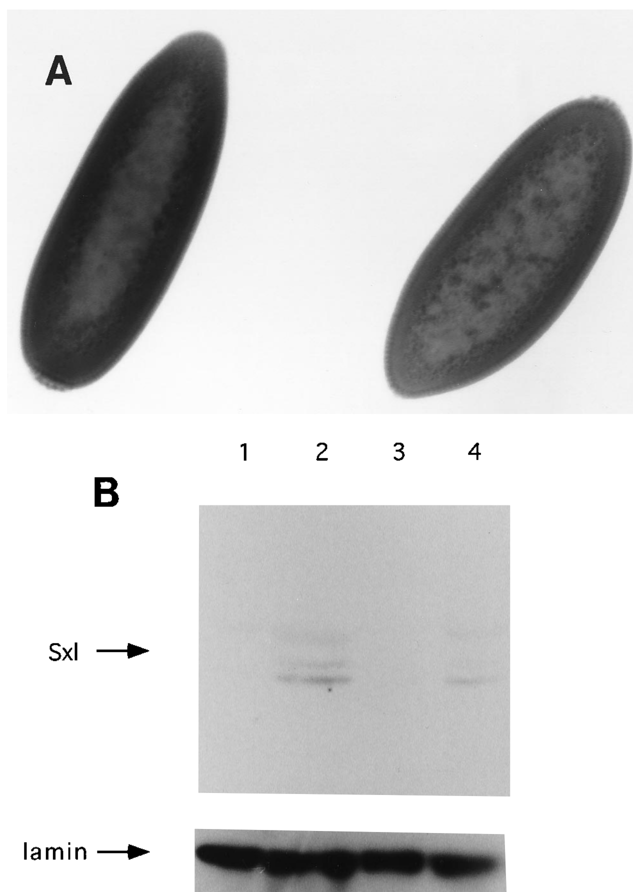
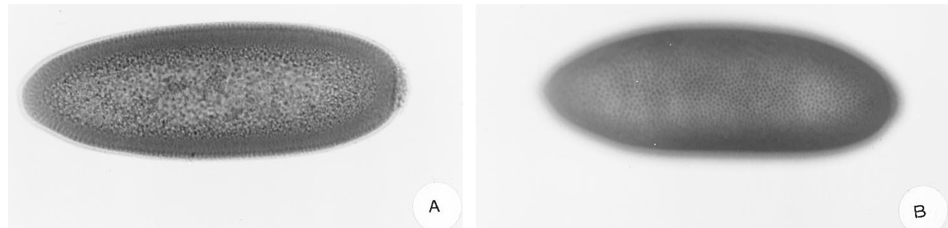


FIG. 5. Monoclonal antibodies to Sc can detect differential levels of the protein. (A) Carefully staged whole-mount embryos (syncytial blastoderm) were stained with monoclonal antibodies to Sc. Shown are two embryos, one strongly stained and one more weakly stained (presumably female and male embryos, respectively). (B) Embryos 0 to 3 h old were stained with Sc monoclonal antibodies, and syncytial blastoderm embryos were grouped on the basis of intensity of staining (weak versus strong). Extracts were prepared from both classes of embryos, and proteins were separated by SDS-PAGE and were blotted onto nitrocellulose. Blots were analyzed for the *Sxl* protein with Sxl monoclonal antibodies. Lane 1, extract from embryos stained weakly with Sc antibody; lane 2, extract from embryos stained strongly with Sc antibody; lane 3, extract from adult males; lane 4, extract from adult female. The same blot was reprobed with antibody specific for lamin (loading control). To assess the sensitivity of our assay we did a reconstruction experiment in which extracts from lightly staining, ostensibly male embryos were doped with increasing amounts of extracts from darkly staining, ostensibly female embryos. In this experiment we were readily able to detect *Sxl* protein in male extracts doped with protein from the equivalent of two female embryos. (We did not try doping with extract equivalent to one female embryo.) Thus, of the 20 lightly stained embryos examined in lane 1, fewer than 2 of these express *Sxl* protein. Of course, this doping experiment does not control for mistakes in the opposite direction, namely, darkly stained embryos which do not express the *Sxl* protein.

it should be in the on state in the more darkly staining female embryos.

To determine if this is the case, staged embryos (0 to 3 h) were stained with the Sc monoclonal antibody. We then grouped and separated the syncytial blastoderm-stage embryos (nuclear cycle 12 to 13) on the basis of their Sc antibody staining intensity (light versus dark). Extracts were prepared from 20 embryos in each group and analyzed by Western blotting using Sxl antibody (Fig. 5B). To control for protein extraction or loading artifacts, we also probed the Western blots with antibody directed against lamin (Fig. 5B). As predicted, we could not detect the *Sxl* protein in the group of



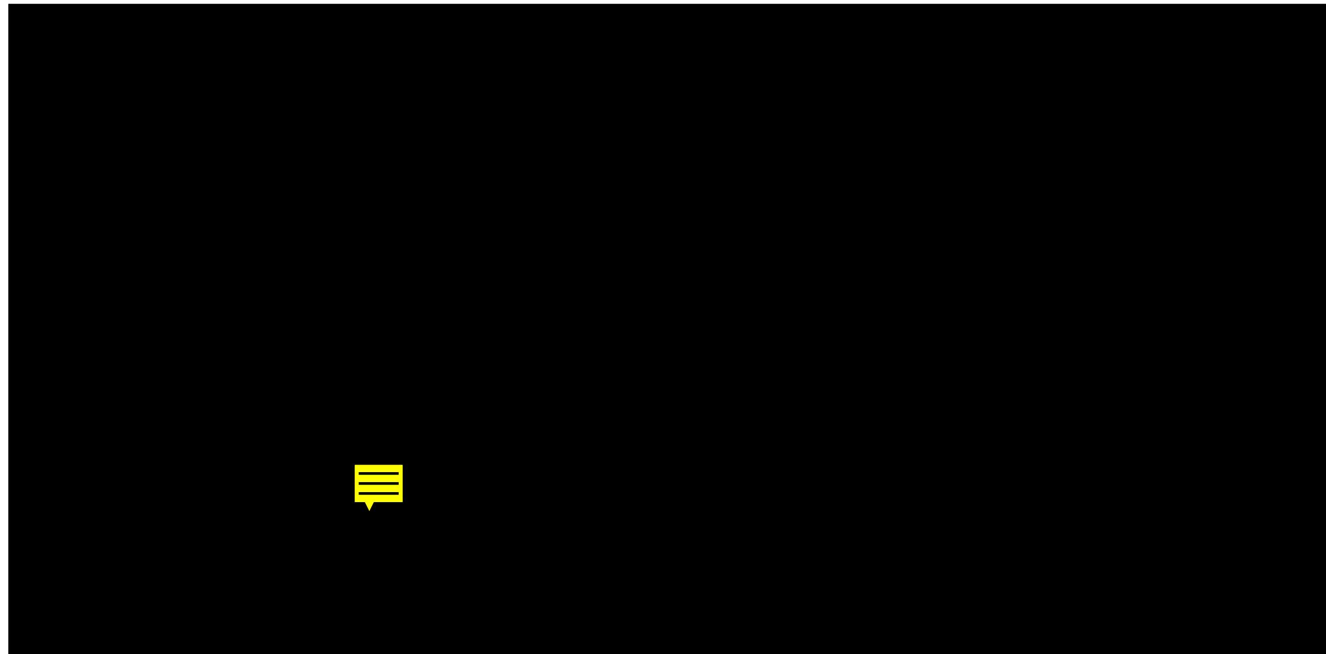


FIG. 6. Sc is a nuclear as well as cytoplasmic protein. *white*[1] embryos were fixed and stained for Sc with monoclonal antibody 5A10. (A and B) Top and surface views of a immunohistochemically stained wild-type syncytial blastoderm embryo, respectively; (C through N) results of confocal analysis. (C, F, I, and L) Embryos were stained with Sc monoclonal antibodies. Sc staining is imaged in red. (D, G, J, and M) Nuclei were stained with the YoPro dye and are imaged in green. (E, H, K, and N) When viewed together, areas of overlap appear yellow. (C, D, and E) Sc appears predominantly cytoplasmic at cycle 7. (F, G, and H) Low levels of protein can first be detected in nuclei after nuclear migration around cycle 10. (I, J, and K) Older syncytial blastoderm embryos show higher levels of Sc in the nuclei but also retain considerable amounts of cytoplasmic protein. (L, M, and N) Magnified (original magnification, ×60) view of syncytial blastoderm embryos.

embryos that stained only lightly for the Sc antigen. In contrast, the *Sxl* protein was present in the group of embryos that stained more darkly for the Sc antigen. This result confirms the inferred sexual identity of the embryos in the lightly and more darkly staining groups and indicates that the level of Sc is correlated with the activity state of *Sxl.*

Subcellular localization of Sc. Sc is a member of the bHLH family of transcription factors and consequently would be expected to predominantly nuclear. However, this did not seem to be the case from our immunohistochemical staining. As can be seen in Fig. 4B, much of the Sc in this nuclear cycle 9 embryo seems to be in the cytoplasm, not the nucleus (compare staining in the wild-type embryo in panel B with the *sc* deletion embryo in panel A) and the strong nuclear dots observed with other nuclear antigens at this stage (e.g., histones [data not shown]) is not evident. In older, pre-cellular blastoderm embryos, nuclear protein becomes evident. This is most clearly seen in the surface view of a immunohistochemically stained syncytial blastoderm embryo shown in Fig. 6A. However, even in this embryo substantial amounts of the protein also appear to be present in the cortical cytoplasm (Fig. 6B).

To more precisely define both the time course of *sc* expression and the distribution of Sc during the early stages of embryogenesis, we used confocal microscopy. Since early embryos sometimes exhibit significant levels of nonspecific staining, we compared the staining pattern in wild-type embryos with that of *Df(1)sc19* embryos of equivalent developmental age. The earliest stage at which Sc antigen could first reliably be detected in wild-type embryos is around nuclear cycle 6 to 7 (Fig. 6C), though staining above background is often observed in even earlier embryos. These observations are consistent with the results of Parkhurst et al. (47), who reported that *sc* transcripts are expressed as early as nuclear cycle 3. The staining at nuclear cycle 6 to 7 is considerably lighter than that observed for older embryos. Moreover, as illustrated by the panels showing Sc (Fig. 6C, F, I, and L), nucleic acids (Fig. 6D, G, J, and M), and the overlapping image (Fig. 6E, H, K, and N), the Sc in these early embryos appears to be predominantly cytoplasmic, with little or no protein in nuclei.

The level of antibody staining increases substantially after the somatic nuclei migrate to the surface of the embryo. Moreover, by nuclear cycle 10 (Fig. 6F to H), Sc can be detected not only in the cytoplasm but also in the nuclei (as judged by the yellower nuclei in panel H than in panel E). The level of nuclear Sc appears to increase during cycles 11 to 13 (Fig. 6I to K), which corresponds to the time in development when the

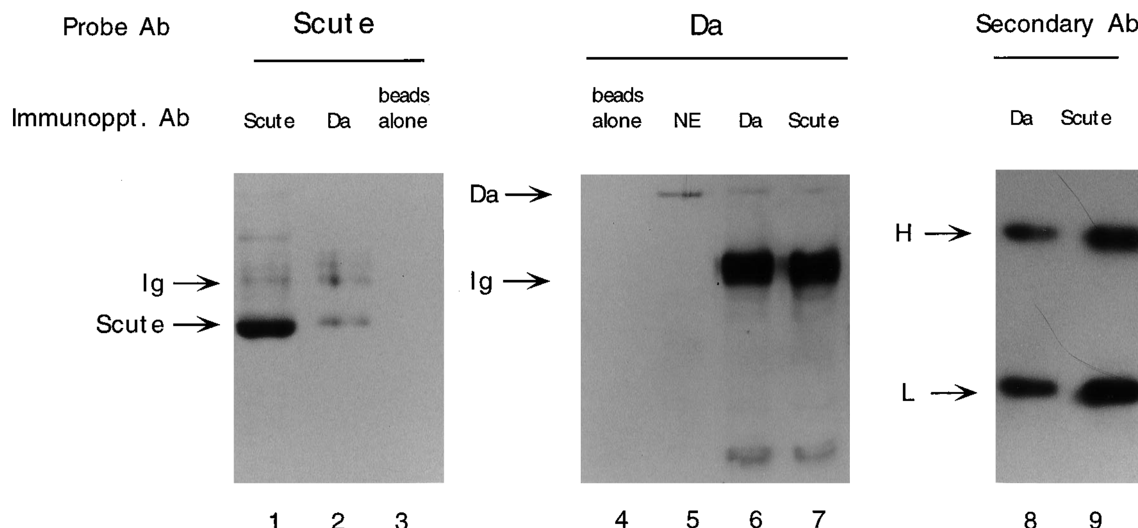


FIG. 7. Coimmunoprecipitation of Da and Sc. Wild-type embryonic extracts (0 to 3 h) were incubated in the presence of either Da- or Sc-specific monoclonal antibodies. The immunoprecipitates (Immunoppt.) were removed by using Sepharose beads conjugated with protein A. The beads were collected by centrifugation and then washed thoroughly as described in Materials and Methods and cited references. Proteins extracted from the beads (e.g., Da, Sc, and the immunoglobulin heavy and light chains) were separated on an SDS–10% polyacrylamide gel, blotted onto a nitrocellulose membrane, and probed with either Da or Sc monoclonal antibodies (Ab). Sc (lane 1) and Da (lane 2) immunoprecipitates were probed with antibodies against Sc. Da (lane 6) and Sc (lane 7) immunoprecipitates were probed with antibodies against the *da* protein. Lanes 3 and 4, immunoprecipitation negative controls using just beads and probed with Sc and Da monoclonal antibodies, respectively; lane 5 (positive control), nuclear extract probed with Da antibody; lanes 8 and 9, immunoprecipitates probed with secondary antibody alone. In the absence of primary Da or Sc antibodies, only the light- and heavy-chain immunoglobulins (Ig) are detected with the secondary antibody.

Sxl-P$_e$ promoter is active. However, even during this critical period, a substantial proportion of the protein appears to remain in the cytoplasm. This partitioning of Sc into both the nuclear and cytoplasmic compartments during the syncytial blastoderm stage is illustrated in the higher magnification of a blastoderm embryo shown in Fig. 6L to N. While high levels of protein are found in the somatic nuclei and cytoplasm, the level of Sc in the nuclei or cytoplasm of the germ line pole cells is consistently much lower (Fig. 6B and I to K). In this regard, it is interesting to note that *Sxl-P*$_e$ is activated in somatic nuclei but not in the pole cells. Presumably this may be due, at least in part, to soma-germ line differences in the level of Sc. During cellularization, there is a further redistribution of Sc into the nuclei, and in subsequent stages of embryogenesis it is preferentially localized in nuclei, not in the cytoplasm (data not shown).

Protein-protein complexes are formed between Sc and Daughterless. The binding of bHLH proteins like Sc to DNA is believed to require the formation of protein-protein complexes, usually in the form of heterodimers (or hetero-oligomers) between two different bHLH proteins. In mammals, such heterodimers often consist of a tissue- or cell-type-specific bHLH protein complexed with a more ubiquitous protein. A very good candidate for the partner of Sc in sex determination (and also neurogenesis) is the ubiquitous bHLH protein encoded by the *da* gene. *da* protein derived from the mother is required in the early zygote for the activation of *Sxl-P*$_e$ (30, 37), and it has been suggested that heteromeric complexes between the numerator element Sc and Da would interact with *Sxl-P*$_e$ to activate expression in female embryos (45). Consistent with this suggestion, in vitro experiments have demonstrated that heteromeric complexes of Da and the various bHLH proteins encoded by the AS-C can be assembled on DNA oligomers containing bHLH E-box consensus binding sites (9, 55).

Balancing the positive effects of Sc-Da complexes would be complexes formed with negative regulatory proteins encoded by denominator elements on the autosomes. Evidence for such negative regulatory interactions comes from previous studies by Parkhurst et al. (45), who showed that ectopic expression of the bHLH protein Hairy from a *hunchback* (*hb*) promoter blocked the activation of *Sxl*, leading to female-specific lethality. Moreover, duplication of *sc*, but not *da*, suppresses the female lethality of the *hb:h* transgene. These findings led to a model in which the bHLH protein Hairy binds to Sc, competing with the formation of Sc-Da complexes and hence preventing *Sxl-P*$_e$ activation (45). Since the endogenous *hairy* gene is not normally expressed until after *Sxl-P*$_e$ is activated (and turned off), it was postulated that some other bHLH protein encoded by an autosomal denominator gene would compete with Da for complex formation with Sc. One candidate for such a denominator element is the autosomal bHLH gene *deadpan* (58). Mutations in *dpn* lead to the inappropriate activation of *Sxl-P*$_e$ in males (30) and cause male lethality.

This model for the regulation of *Sxl-P*$_e$ by the X/A signaling system predicts the formation of two types of Sc complexes in vivo—positive regulatory complexes between Sc and Da and negative regulatory complexes between Sc and Dpn. To test the first part of this model, we determined whether heteromeric complexes between Sc and the *da* protein can be detected in early embryos. For this purpose extracts from 0- to 3-h embryos were immunoprecipitated with antibodies directed against either Da or Sc and protein A-Sepharose beads. The embryonic proteins immunoprecipitated by antibody attached to the Sepharose beads were then analyzed by Western blotting.

In the Western blot shown in the first panel of Fig. 7, immunoprecipitated proteins isolated with Sc or Da antibody (or beads alone) were probed with Sc antibody. As expected, antibodies directed against Sc immunoprecipitate Sc (lane 1), while Sc is not detected when only the Sepharose beads are used for precipitation (lane 3) or when the Western blots are probed with only the secondary antibody (lanes 8 and 9). Of more interest, Sc can also be detected when the embryo extract is immunoprecipitated with Da antibody (lane 2). This finding

suggests that Sc and Da form a heteromeric complex in vivo. If this suggestion is correct, Sc antibodies would be expected to coimmunoprecipitate the *da* protein. To determine if this is the case, we probed the Western blot of the immunoprecipitated proteins with Da antibody, and this is shown in the second panel. Consistent with a direct physical interaction between Da and Sc, Da can be immunoprecipitated with antibodies directed against Da or Sc (lanes 6 and 7, respectively). To test the second part of this model, we attempted to detect complexes between Sc and Dpn by immunoprecipitation. In contrast to Sc-Da, we were unable to detect Sc-Dpn complexes. With the very important caveat that these are negative findings, it appears that Sc and Dpn do not form complexes in vivo.

DISCUSSION

The selection of the male or female developmental pathway depends upon a signaling system that controls the transcriptional activity of the *Sxl-*P_e promoter. The activity of this signaling system is thought to pivot upon the twofold difference between 1X and 2X embryos in the gene dose of X-linked numerators that encode a number of different transcription factors such as the bHLH protein Sc; that is, one copy of the numerators is insufficient to activate *Sxl-*P_e, while the promoter is activated when two copies of the numerators are present. It is believed that this twofold difference in X-chromosome gene dose is amplified by the formation of concentration-dependent protein-protein interactions between these positive transcription factors and other components of the signaling system such as the maternal gene *da* (20, 45–47). Countering the effects of the transcription factors encoded by genes on the X chromosome are factors which function as denominators and act to keep *Sxl-*P_e off. Some of these are thought to form inhibitory protein-protein complexes with numerator proteins, while others may interact directly with *Sxl-*P_e (20, 30, 45, 58). In the studies reported here we have sought to test certain aspects of this model for sex determination by undertaking a detailed in vivo analysis of the protein encoded by the numerator gene *sc*. Our results with *sc* are consistent with many but not all of the major predictions of this model and also suggest that some of the mechanisms involved are more complicated than initially imagined.

The level of Sc is dependent upon X-chromosome gene dose. One key feature of the model is that in the early zygote, at the time when the X/A ratio is being read, the level of expression of X-linked numerator proteins is directly dependent upon gene dose. Several lines of evidence suggest that this is the case for *sc*. We find that wild-type embryos at the syncytial blastoderm stage—the stage at which *Sxl-*P_e is activated—can be divided into two groups on the basis of the intensity of Sc antibody staining. One group of embryos stains lightly, while the other stains more darkly. As would be expected if the level of staining depends upon the number of X chromosomes, a roughly equal number of embryos partition into each group. That this difference in staining intensity reflects the sc gene dose per se and is not the consequence of some other sex-specific difference that occurs independently of the number of *sc* genes is supported by analysis of the Sc staining pattern observed in a genetic background in which embryos have zero, two, or four copies of *sc*. In this case, the *sc*-positive embryos fall into two groups at the frequency expected from the genetic background, not the sex. The fact that the level of Sc appears to depend strictly on gene dose argues that the dosage compensation system must not be functioning (or must be active in both sexes) during the syncytial blastoderm stage. Of course, this is not the case later in development. By the time the neurogenic pattern of *sc* expression develops in a wild-type genetic background, all embryos (1X and 2X) show essentially the same level of staining. Finally, we show that the level of Sc in wild-type pre-cellular blastoderm embryos is directly correlated with their sexual identity. This is demonstrated by the finding that the *Sxl* gene is not turned on in embryos that show light staining with Sc antibody but is on in the more darkly stained embryos.

While these findings support one key prediction of the model, our studies reveal other factors that must ultimately be of some importance in establishing the effective dose of Sc. One of these is the subcellular distribution of the protein. Even though Sc can be detected in the nuclear division cycles prior to the formation of the syncytial blastoderm, very little of this protein is nuclear. Even after nuclear migration (nuclear cycles 9 and 10), much of the protein remains in the cytoplasm, and it is only around nuclear cycle 11 or 12 that a significant amount begins to enter the nucleus. This coincides closely to the time when *Sxl-*P_e is initially activated (29, 38). This raises the possibility that nuclear import of Sc is an important factor in controlling when the promoter can first be turned on. Whether nuclear import has an impact not only on timing but also on sex specificity is less clear. There does not appear to be any sex specificity in nuclear import, and all of the similarly staged embryos (whether in the darkly or lightly staining classes) show an equivalent distribution of cytoplasmic and nuclear protein. On the other hand, it is possible that the delayed nuclear import plays an indirect role in ensuring the appropriate sex specificity of the promoter. Thus, if nuclear import began earlier, the effective concentration of Sc in 1X nuclei might ultimately exceed the threshold required for activating the promoter. In this respect, it is interesting to note that much of the Sc is still in the cytoplasm even during the peak period of *Sxl-*P_e activity. Were all of this cytoplasmic protein localized in the nucleus, the level of nuclear Sc in 1X animals might exceed that required to begin activating *Sxl-*P_e in these embryos (29, 30).

Curiously, incomplete nuclear import of Sc evident at the syncytial blastoderm stage is not observed at later stages of development in which the protein appears to be predominantly nuclear (56). This raises the possibility that the import machinery for Sc is limiting in early embryos. Alternatively, it is possible that different nuclear localization signals (and perhaps somewhat different machinery) are employed early and late. In either case, one might anticipate that at least some loss-of-function mutations in the *sc* gene that are specific for sex determination (but do not affect neurogenesis) lie in the signal sequence used for early nuclear import.

Regulation mediated by protein dimer formation. A second feature of the model is that concentration-dependent protein-protein interactions may help to amplify the twofold difference between males and females in the dose of X-linked numerator genes. Among the known components of the X/A signaling system, the bHLH proteins encoded by *sc*, *da*, *dpn*, and *emc* are very good candidates for proteins whose activity may be modulated by the formation of dimeric or multimeric complexes. Indeed, previous in vitro studies have shown that Da can form heteromeric complexes with Sc and other bHLH proteins from the AS-C on DNA oligomers containing consensus binding sequences (9, 55). Moreover, in vitro-synthesized *da* protein was found to form heteromers with the AS-C proteins Lethal-of-scute, Achaete, and Sc in the absence of DNA; however, the stability of the Da-Sc complex appeared to be much less than that of complexes formed between Da and either Lethal-of-scute or Achaete (9). Since bHLH proteins can be somewhat promiscuous in their interactions, an obvious question is

whether the complexes observed in vitro between Da and Sc can also be detected in vivo. This is especially important to establish given the marginal stability of the in vitro complexes. To assay for complexes between Da and Sc in vivo, we have used reciprocal coimmunoprecipitations of proteins extracted from early embryos. We have shown that Sc antibodies coimmunoprecipitate Da from early embryo extracts, while Da antibodies coimmunoprecipitate Sc. These results provide the first direct evidence for the existence of Da-Sc complexes in vivo.

A number of studies indicate that Sc is limiting, while the *da* protein is normally present in excess. Hence, the formation of complexes between Sc and Da would be expected to depend most critically upon the relative concentration of Sc. In this case the differences in the level of Sc observed in 1X and 2X animals could have a significant effect on the number of such complexes that are formed in each sex. Perhaps also important in this equation is the fact that a substantial fraction of the Sc is partitioned into the cytoplasm rather than the nucleus. By contrast, Da is reported to be predominantly nuclear in the early embryo, with little protein in the cytoplasm (23). This difference in subcellular distribution would reduce the amount of Sc available to form functional complexes inside the nucleus. It could help amplify the effects of the twofold difference in the level of Sc between males and females, perhaps via denominators whose effects could be enhanced at lower effective numerator concentrations.

In many of the current models for measuring the X/A ratio (20, 29, 45) it is supposed that another mechanism for signal amplification involves negative interactions between numerator and denominator proteins. In the case of Sc, the formation of active complexes with Da is thought to be in direct competition with the formation of inactive complexes between Sc and bHLH proteins which function to convey the autosomal signal. One of the candidates for such inhibitory bHLH proteins is the autosomal gene *dpn*. In our experiments we were unable to detect a complex between Sc and Dpn. Although it must be emphasized that this is a negative result, it raises the possibility that Dpn functions by some other mechanism, i.e., interacting directly with targets in the *Sxl*-P_e promoter and repressing transcription. Such a mechanism would be consistent with results obtained in functional studies with the promoter (30). Another possible inhibitory protein which was not tested here is the maternally expressed bHLH gene *emc*. This protein has been shown to interfere in vitro with the DNA binding of Da-Sc complexes (55), and it could prevent these interactions by forming inactive protein-protein complexes with either Da or Sc. Further studies will be required to address this possibility.

ACKNOWLEDGMENTS

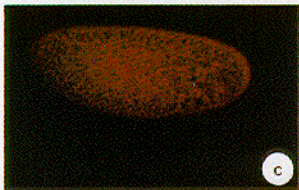
This work was supported by an NIH grant to P.S. J.S. was supported by a postdoctoral NIH fellowship.

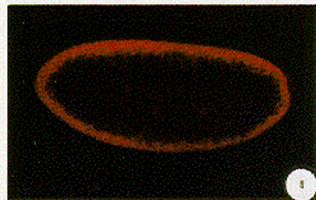
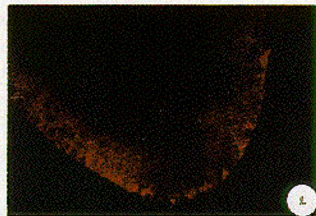
We thank Claire Cronmiller for supplying antibodies against Daughterless, Susan Younger-Shephard and Harold Vassein for antibodies against Deadpan, and Jim Erickson for *Df(1)sc19* flies. We gratefully acknowledge the excellent assistance of Marty Marlow in the generation of monoclonal antibodies to Sc and Joe Goodhouse and Jamila Horabin for their help with the confocal microscopy. Gordon Gray kindly provided fly food. We thank our colleagues from the laboratories of Paul Schedl and Ruth Steward for their critical suggestions and overall encouragement.

REFERENCES

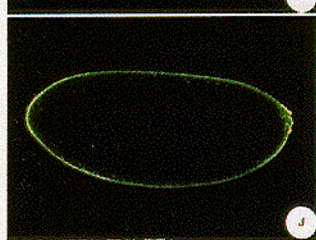
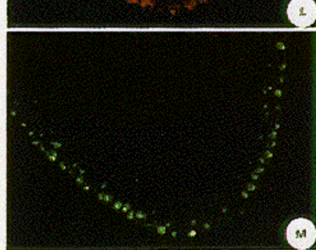
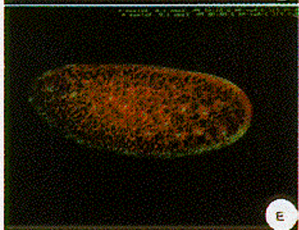
1. **Baker, B. S.** 1989. Sex in flies: the splice of life. Nature (London) **340:**521–524.
2. **Baker, B. S., and M. F. Wolfner.** 1988. A molecular analysis of *doublesex*, a bifunctional gene that controls both male and female sexual differentiation in *Drosophila melanogaster*. Genes Dev. **2:**477–489.
3. **Bell, L. R., J. I. Horabin, P. Schedl, and T. W. Cline.** 1991. Positive autoregulation of *Sex-lethal* by alternative splicing maintains the female determined state in Drosophila. Cell **65:**229–239.
4. **Bell, L. R., E. M. Maine, P. Schedl, and T. W. Cline.** 1988. *Sex-lethal*, a Drosophila sex determination switch gene, exhibits sex-specific RNA splicing and sequence similarity to RNA binding proteins. Cell **55:**1037–1046.
5. **Bier, E., H. Vaessin, S. Younger-Shephard, L. Y. Jan, and Y. N. Jan.** 1992. deadpan, an essential pan-neural gene in Drosophila, encodes a helix-loop-helix protein similar to the hairy gene product. Genes Dev. **6:**2137–2151.
6. **Blackwell, T. K., and H. Weintraub.** 1990. Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. Science **250:**1104–1110.
7. **Bopp, D., L. R. Bell, T. W. Cline, and P. Schedl.** 1991. Developmental distribution of female-specific *sex-lethal* proteins in *Drosophila melanogaster*. Genes Dev. **5:**403–415.
8. **Burtis, K. C., and B. S. Baker.** 1989. *Drosophila doublesex* gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. Cell **56:**997–1010.
9. **Cabrera, C. V., and M. C. Alonso.** 1991. Transcriptional activation by heterodimers of the *achaete-scute* and *daughterless* gene products of *Drosophila*. EMBO J. **10:**2965–2973.
10. **Caudy, M., H. Vaessin, M. Brand, R. Tuma, L. Y. Jan, and Y. N. Jan.** 1988. *daughterless*, a Drosophila gene essential for both neurogenesis and sex determination, has sequence similarities to *myc* and the *achaete-scute* complex. Cell **55:**1061–1067.
11. **Cline, T. W.** 1976. A sex-specific, temperature-sensitive maternal effect of the daughterless mutation of *Drosophila melanogaster*. Genetics **84:**723–742.
12. **Cline, T. W.** 1978. Two closely linked mutations in *Drosophila melanogaster* that are lethal to opposite sexes and interact with daughterless. Genetics **90:**683–698.
13. **Cline, T. W.** 1980. Maternal and zygotic sex-specific gene interactions in *Drosophila melanogaster*. Genetics **96:**903–926.
14. **Cline, T. W.** 1983. The interaction between *daughterless* and *Sex-lethal* in triploids: a lethal sex-transforming maternal effect linking sex determination and dosage compensation in *Drosophila melanogaster*. Dev. Biol. **95:**260–274.
15. **Cline, T. W.** 1984. Autoregulatory functioning of a Drosophila gene product that establishes and maintains the sexually determined state. Genetics **107:**231–277.
16. **Cline, T. W.** 1985. Primary events in the determination of sex in Drosophila melanogaster, p. 301–327. Liss, New York.
17. **Cline, T. W.** 1986. A female-specific lethal lesion in an X-linked positive regulator of the Drosophila sex determination gene, *Sex-lethal*. Genetics **113:**641–663.
18. **Cline, T. W.** 1988. Evidence that *sisterless-a* and *sisterless-b* are two of several discrete "numerator elements" of the X/A sex determination signal in Drosophila that switch *Sxl* between two alternative stable expression states. Genetics **119:**829–862.
19. **Cline, T. W.** 1989. The affairs of *daughterless* and the promiscuity of developmental regulators. Cell **59:**231–234.
20. **Cline, T. W.** 1993. The Drosophila sex determination signal: how do flies count to two? Trends Genet. **9:**385–390.
21. **Cronmiller, C., and T. W. Cline.** 1986. The relationship of relative gene dose to the complex phenotype of the *daughterless* locus in *Drosophila*. Dev. Genet. **7:**205–221.
22. **Cronmiller, C., and T. W. Cline.** 1987. The Drosophila sex determination gene *daughterless* has different functions in the germ line versus the soma. Cell **48:**479–487.
23. **Cronmiller, C., and C. A. Cummings.** 1993. The *daughterless* gene product in *Drosophila* is a nuclear protein that is broadly expressed throughout the organism during development. Mech. Dev. **42:**159–169.
24. **Cronmiller, C., P. Schedl, and T. W. Cline.** 1988. Molecular characterization of *daughterless*, a *Drosophila* sex determination gene with multiple roles in development. Genes Dev. **2:**1666–1676.
25. **Daga, A., J. E. Tighe, and F. Calabi.** 1992. Leukaemia/*Drosophila* homology. Nature (London) **356:**484.
26. **Driever, W., and C. Nusslein-Volhard.** 1988. A gradient of *bicoid* protein in *Drosophila* embryos. Cell **54:**83–93.
27. **Duffy, J. B., and J. P. Gergen.** 1991. The *Drosophila* segmentation gene *runt* acts as a position-specific numerator element necessary for the uniform expression of the sex-determination gene *Sex-lethal*. Genes Dev. **5:**2176–2187.
28. **Erickson, J. W., and T. W. Cline.** 1991. Molecular nature of the *Drosophila* sex determination signal and its link to neurogenesis. Science **251:**1071–1074.
29. **Erickson, J. W., and T. W. Cline.** 1993. A bZIP protein, *sisterless-a*, collaborates with bHLH transcription factors early in *Drosophila* development to determine sex. Genes Dev. **7:**1688–1702.
30. **Estes, P., L. N. Keyes, and P. Schedl.** 1995. Multiple response elements in the *Sex-lethal* early promoter ensure its female-specific expression pattern. Mol. Cell. Biol. **15:**904–917.
31. **Garrell, J., and J. Modolell.** 1990. The Drosophila *extramacrochaete* locus, an

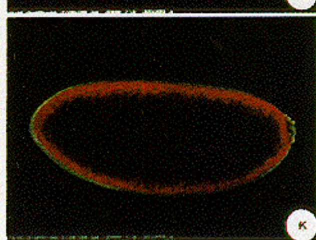
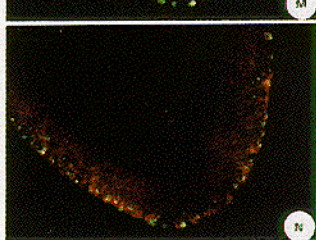
antagonist of proneural genes that, like these genes, encodes a helix-loop-helix protein. Cell **61:**39–48.
32. **Gorman, M., M. I. Kuroda, and B. S. Baker.** 1993. Regulation of the sex-specific binding of the *maleless* dosage compensation protein to the male X chromosome in *Drosophila*. Cell **72:**39–49.
33. **Govind, S., A. Whelan, and R. Steward.** 1992. In vivo self-association of the *Drosophila* rel protein dorsal. Proc. Natl. Acad. Sci. USA **89:**7861–7865.
34. **Hawkes, R., E. Niday, and J. Gordon.** 1982. A dot-immunobinding assay for monoclonal and other antibodies. Anal. Biochem. **119:**142–147.
35. **Hodgkin, J.** 1990. Sex determination compared in *Drosophila* and *Caenorhabditis*. Nature (London) **344:**721–728.
36. **Horabin, J. I., and P. Schedl.** 1993. Regulated splicing of the *Drosophila Sex-lethal* male exon involves a blockage mechanism. Mol. Cell. Biol. **13:** 1408–1414.
37. **Horabin, J. I., and P. Schedl.** 1993. *Sex-lethal* autoregulation requires multiple *cis*-acting elements upstream and downstream of the male exon and appears to depend largely on controlling the use of the male exon 5′ splice site. Mol. Cell. Biol. **13:**7734–7746.
38. **Keyes, L. N., T. W. Cline, and P. Schedl.** 1992. The primary sex-determination signal of *Drosophila* acts at the level of transcription. Cell **68:**933–943.
39. **Koerna, T. J., J. E. Hill, A. M. Myers, and A. Tzagoloff.** 1991. High-expression vectors with multiple cloning sites for construction of *trpE* fusion genes: pATH vectors. Methods Enzymol. **194:**477–490.
40. **Lindsley, D. L., and G. G. Zimm.** 1992. The genome of *Drosophila melanogaster*. Academic Press, Inc., New York.
41. **Lucchesi, J. C., and T. Skripsky.** 1981. The link between dosage compensation and sex differentiation in *Drosophila melanogaster*. Chromosoma **82:** 217–227.
42. **Murre, C., P. S. McCaw, and D. Baltimore.** 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, *daughterless*, *MyoD*, and *myc* proteins. Cell **56:**777–783.
43. **Murre, C., P. S. McCaw, H. Vaessin, M. Caudy, L. Y. Jan, Y. N. Jan, C. V. Cabrera, J. N. Buskin, S. D. Hauschka, A. B. Lassar, H. Weintraub, and D. Baltimore.** 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell **58:**537–544.
44. **Nagoshi, R. N., M. McKeown, K. C. Burtis, J. M. Belote, and B. S. Baker.** 1988. The control of alternative splicing at genes regulating sexual differentiation in *D. melanogaster*. Cell **53:**229–236.
45. **Parkhurst, S. M., D. Bopp, and D. Ish-Horowicz.** 1990. X:A ratio, the primary sex-determining signal in Drosophila, is transduced by helix-loop-helix proteins. Cell **63:**1179–1191.
46. **Parkhurst, S. M., and D. Ish-Horowicz.** 1992. Common denominators for sex. Curr. Biol. **2:**629–631.
47. **Parkhurst, S. M., H. D. Lipshitz, and D. Ish-Horowicz.** 1993. *achaete-scute* feminizing activities and *Drosophila* sex determination. Development **117:** 737–749.
48. **Parkhurst, S. M., and P. M. Meneely.** 1994. Sex determination and dosage compensation: lessons from flies and worms. Science **264:**924–932.
49. **Salz, H. K., T. W. Cline, and P. Schedl.** 1987. Functional changes associated with structural alterations induced by mobilization of a *P* element inserted in the *Sex-lethal* gene of Drosophila. Genetics **117:**221–231.
50. **Salz, H. K., E. M. Maine, L. N. Keyes, M. E. Samuels, T. W. Cline, and P. Schedl.** 1989. The *Drosophila* female-specific sex-determination gene, *Sex-lethal*, has stage-, tissue-, and sex-specific RNAs suggesting multiple modes of regulation. Genes Dev. **3:**708–719.
51. **Smith, D. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene **67:**31–40.
52. **Steinmann-Zwicky, M., H. Amrein, and R. Nöthinger.** 1990. Genetic control of sex determination in *Drosophila*. Adv. Genet. **27:**189–237.
53. **Torres, M., and L. Sanchez.** 1989. The *scute* (T4) gene acts as a numerator element of the X:A signal that determines the state of activity of *Sex-lethal* in *Drosophila*. EMBO J. **8:**3079–3086.
54. **Torres, M., and L. Sanchez.** 1991. The *sisterless-b* function of the *Drosophila* gene *scute* is restricted to the stage when the X:A ratio determines the activity of *Sex-lethal*. Development **113:**715–722.
55. **Van Doren, M., H. M. Ellis, and J. W. Posakony.** 1991. The *Drosophila extramacrochaete* protein antagonizes sequence-specific DNA binding by *daughterless*, *achaete-scute* protein complexes. Development **113:**245–255.
56. **Vassein, H., M. Brand, L. Y. Jan, and Y. N. Jan.** 1994. *daughterless* is essential for neuronal precursor differentiation but not for initiation of neuronal precursor formation in *Drosophila* embryo. Development **120:**935–945.
57. **Villares, R., and C. V. Cabrera.** 1987. The *achaete-scute* gene complex of D. melanogaster: conserved domains in a subset of genes required for neurogenesis and their homology to *myc*. Cell **50:**415–424.
58. **Younger-Shephard, S., H. Vassein, E. Bier, L. Y. Jan, and Y. N. Jan.** 1992. *deadpan*, an essential pan-neural gene encoding an HLH protein, acts a dominator in *Drosophila* sex determination. Cell **70:**911–922.


C

F

I

L

D

G

J

M

E

H

K

N