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Sex-lethal, master and slave: a hierarchy of germ-line sex determination in *Drosophila*

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SUMMARY

Female sex determination in the germ line of *Drosophila melanogaster* is regulated by genes functioning in the soma as well as genes that function within the germ line. Genes known or suspected to be involved in germ-line sex determination in *Drosophila melanogaster* have been examined to determine if they are required upstream or downstream of *Sex-lethal*$^+$, a known germ-line sex determination gene. Seven genes required for female-specific splicing of germ-line *Sex-lethal*$^+$ pre-mRNA are identified. These results together with information about the tissues in which these genes function and whether they control sex determination and viability or just sex determination in the germ line have been used to deduce the genetic hierarchy regulating female germ-line sex determination. This hierarchy includes the somatic sex determination genes *transformer*$^+$, *transformer-2*$^+$ and *doublesex*$^+$ (and by inference *Sex-lethal*$^+$), which control a somatic signal required for female germ-line sex determination, and the germ-line ovarian tumor genes *fused*$^+$, *ovarian tumor*$^+$, *ovo*$^+$, *sans fille*$^+$, and *Sex-lethal*$^+$, which are involved in either the reception or interpretation of this somatic sex determination signal. The *fused*$^+$, *ovarian tumor*$^+$, *ovo*$^+$ and *sans fille*$^+$ genes function upstream of *Sex-lethal*$^+$ in the germ line.

Key words, *Drosophila*, ovarian tumors, RNA splicing, germ-line sex determination

INTRODUCTION

A fundamental biological choice in many organisms is the decision to make eggs or sperm. Control of this sexual choice in *Drosophila melanogaster* females is a particularly intriguing process, since it depends on regulatory signals from both the surrounding somatic tissues and the germ-line cells themselves. We are interested in understanding the nature of the steps controlling female germ-line sexual differentiation and how they are integrated into a regulatory hierarchy.

The regulation of sex determination in the germ line of *Drosophila* is substantially different than in the soma (reviewed by Pauli and Mahowald, 1990; Steinmann-Zwicky, 1992). Of the genes that control somatic sex determination only one, *Sex-lethal*$^+$ (*Sxl*$^+$), is required within germ-line cells for sex determination (Marsh and Wieschaus, 1978; Schüpbach, 1982, 1985; Salz et al., 1987; Steinmann-Zwicky et al., 1989). The finding that *Sxl*$^+$, which is the master regulatory gene in somatic sex determination (reviewed by Cline, 1988, 1989; Baker, 1989; Slee and Bownes, 1990), is also required in female germ-line cells has led to the inference that *Sxl*$^+$ also functions as a master regulatory gene in the germ-line sex determination hierarchy. Thus, the accepted model for *Sxl* function places this gene at the head of three subordinate pathways: (1) somatic sex determination; (2) somatic dosage compensation and (3) germ-line sex determination. However, there are some reasons to question the latter assumption. First, *Sxl*$^-$ mutations affect both sex-specific viability (because *Sxl* controls dosage compensation) and female sexual differentiation in the soma (reviewed by Lucchesi and Manning, 1987; Cline, 1988), but only female sexual differentiation in the germ line (Schüpbach, 1985; Salz et al., 1987; Oliver et al., 1990; Steinmann-Zwicky et al., 1989). Thus, germ-line *Sxl*$^+$ functions are, at best, a subset of its somatic functions. Second, the genes that *Sxl*$^+$ controls directly (*transformer*$^+$; *tra*$^+$) and indirectly (*doublesex*$^+$; *dsx*$^+$) to determine somatic sex are not required in germ-line cells (Marsh and Wieschaus, 1978; Schüpbach, 1982). Thus, *Sxl*$^+$ must function to control the activities of different downstream sex determination genes in the germ line and the soma. Third, at least some obligatory regulators of *Sxl*$^+$ functioning in the soma are unlikely to be required in the germ line (c.f. Cline, 1983, 1988; Cronmiller and Cline, 1987). These observations suggest that *Sxl*$^+$ may have quite different roles in somatic and germ-line cells.

Despite the above caveats to using *Sxl*$^+$ function in the soma as a model for *Sxl*$^+$ function in the germ line, the phenotype observed when female germ-line cells lack *Sxl*$^+$ function has been crucial for understanding germ-line sex determination (reviewed by Pauli and Mahowald, 1990;

Steinmann-Zwicky, 1992). The ovaries of wild-type females contain 16-cell cysts of germ-line cells each of which arises from a stem cell by four nuclear divisions with incomplete cytokinesis (King, 1970). As each cyst matures, one cyst cell becomes an oocyte while the remaining fifteen become polyploid nurse cells. In contrast, the ovaries of 2X females with Sxl^- germ lines are filled with large numbers of fairly uniformly sized small germ-line cells (Schüpbach, 1985). These Sxl^- germ-line cells are not morphologically female and appear either undifferentiated or similar to primary spermatocytes (Schüpbach, 1985; Oliver et al., 1988, 1990; Steinmann-Zwicky et al., 1989). Similarly, it has been suggested that the gonadoblastomas frequently found in phenotypic human females are the result of male-like germline develoment in a female soma (Scully, 1970; Page, 1987). Thus, the formation of ovarian tumors may also be associated with defective germ-line sex determination in humans.

The phenotype produced by the absence of Sxl^+ expression in germ-line cells is similar to the phenotype observed in *Drosophila* mutants at a number of so-called 'ovarian tumor' genes (reviewed by King, 1970; King and Storto, 1988). These ovarian tumor genes have been widely thought of as *Drosophila* oncogenes or cell-cycle regulators (King, 1970; King and Storto, 1988; Steinhauer et al., 1989; McKearin and Spradling, 1990). However, the similar phenotypes of *Sxl* and ovarian tumor gene mutations have led to recent studies that have supported the idea that many ovarian tumor genes function in female germ-line sex determination (Oliver et al., 1987, 1988, 1990; Steinmann-Zwicky, 1988). Most strikingly, mutations in *ovo*, *ovarian tumor* (*otu*), or *sans fille* (*snf)* are suppressed by the constitutive expression of Sxl^+ function, implying that these ovarian tumor genes function prior to Sxl^+ in germ-line sex determination (Steinmann-Zwicky, 1988; Oliver et al., 1990; Salz, 1992; Pauli et al., 1993; this study). Here we provide molecular evidence that at least some ovarian tumors are the result of switched germ cell sexual identity.

Another important component of the female germ-line sex determination mechanism has been revealed by the discovery that a signal from the soma is necessary for female germ-line sex determination (Steinmann-Zwicky et al., 1989; also see Dobzhansky, 1931; Schüpbach, 1982; Cline, 1984): wild-type 2X germ-line cells in a 1X male soma have a spermatocyte morphology. A somatic signal may be only required in females for germ-line sex determination, as 1X germ-line cells show a spermatocyte morphology in a 2X or 1X female soma [i.e. wild-type 2X flies or 1X flies constitutively expressing tra^+ function (Steinmann-Zwicky et al., 1989)]. This cell-cell communication between a 2X soma and 2X germ line is probably dependent on the somatic sex determination genes. Thus 2X germ-line cells in a 2X soma transformed to male by tra^-, $tra\text{-}2^-$ or *dsx* gain-of-function mutations develop some male characters (Brown and King, 1961; Nöthiger et al., 1989; Yanicostas and Lepesant, 1990). The roles of the tra^+, $tra\text{-}2^+$ and dsx^+ genes in germ-line sexual differentiation must reside in the soma, as 2X germline cells mutant for any of these genes develop as fully functional eggs in a wild-type 2X female soma (Marsh and Wieschaus, 1978; Schüpbach, 1982). The somatic signal is likely to be upstream of germ-line Sxl^+ function because 2X germ-line cells of 2X tra^- individuals develop more female characteristics when female Sxl^+ activity is expressed constitutively (Nöthiger et al., 1989).

Although it is likely that Sxl^+ functions in the germ line and soma in different genetic contexts, one would expect similarities in regulation in these two tissue types at the molecular level. In the soma sex-specific splicing is the basis of the sex-specific function of Sxl^+ (Bell et al., 1988, 1991; Bopp et al., 1991; Keyes et al., 1992). The female-specific Sxl^+ mRNA encodes a full-length Sxl^+ protein that functions to autoregulate the splicing of its own pre-mRNA, as well as the pre-mRNA of the tra^+ gene (Sosnowski et al., 1989; Inoue et al., 1990; Bell et al., 1991). In males, an alternative pattern of Sxl^+ pre-mRNA splicing results in the inclusion of an exon bearing premature in-frame stop codons precluding synthesis of a functional polypeptide (Bell et al., 1988; Bopp et al., 1991). Less is known about the expression of Sxl^+ in germ-line cells, but the presence of sex-specific germ-line-dependent Sxl^+ transcripts suggests that alternative splicing may also be an important regulatory mechanism in the germ line (Salz et al., 1989; Samuels et al., 1991).

In this report, we first establish that the region of the Sxl^+ pre-mRNA bearing the key regulatory stop codons is spliced in the same sex-specific manner in the germ line as in the soma. We then use this molecular distinction between the male and female germ line to order the functions of the somatic sex determination genes and ovarian tumor genes relative to *Sxl*. The results indicate that both the somatic sex determination genes and most ovarian tumor genes are required for the female-specific germ-line splicing of Sxl^+ pre-mRNA. These findings together with information about (1) which of these genes also control vital germ-line functions and (2) the cell types in which these genes function have enabled us to infer how the functions of these genes are integrated into a hierarchy controlling female germ-line sex determination.

MATERIALS AND METHODS

Flies

The following alleles are believed to retain some gene activity based on genetic criteria: otu^1, otu^7, otu^9, otu^{10}, ovo^{rM1}, ovo^{rM2}, fu^1 and snf^{1621}. The following alleles have no gene activity based on genetic criteria and might therefore be null: lzl^G (an *ovo* allele with an additional gain-of-function eye phenotype) and ovo^{D1rS1}. The following alleles are molecularly or cytogenetically defined null mutations: $Df(1)Sxl^{7BO}$, tra^1, $Df(3)st^4$ (a tra^- chromosome) $tra\text{-}2^1$, $tra\text{-}2^B$, *Df(2)TRIX* (a $tra\text{-}2^-$ chromosome) and $Df(3)dsx^{r+M43}$. The $Sxl^{M\#1}$ mutation has female gene activity even when the normally obligatory level of $daughterless^+$ positive regulatory activity is greatly reduced. A special combination of *dsx* alleles is required to generate 2X males. The dominant dsx^{swe} allele results in a male-specific dsx^+ RNA. In *trans* to a deletion, the resulting 2X flies express only male-specific dsx^+ gene product and develop as somatic males.

In many crosses with somatic sex determination mutations, the chromosomal sex of a fly may not correspond to its phenotypic sex. Sex-linked markers were used to determine the chromosomal sex. 1X and 2X males were distinguished by the X-linked marker *y* (for tra^- and $tra\text{-}2^-$), size and gonad morphology. The Y-linked marker B^s was used for $tra\text{-}2^-$ and dsx^{swe}. Finally, any X0 males in the RT-PCR experiments would be detected by the presence of male-specific Sxl^+ RNA in the carcass lanes.

See Lindsley and Zimm (1992) and the text for descriptions of mutant chromosomes and for additional references.

PCR

Poly(A)$^+$ RNA from approximately 50 flies, gonadectomized flies, or gonads was reverse transcribed with Moloney murine leukemia virus reverse transcriptase and oligo(dT) (Sambrook et al., 1989). These cDNAs were amplified by the polymerase chain reaction utilizing Taq DNA polymerase, in the presence of [^{32}P]dATP (Saiki et al., 1988). DNA amplification products were separated by electrophoresis through 5% denaturing polyacrylamide gel electrophoresis and detected by autoradiography. The sequences of the primers we have used are:

#847 = 5′ ACAACGACAGCAGCAGGCCA 3′;
#1614 = 5′ CACAGCCCAGAAAGAAGCAG 3′;
#958 = 5′ TCGTTGGCGAGGAGACCATG 3′;
#1613 = 5′ GGGCTTTGGAGGTGTCCTCG 3′;
#848 = 5′ GTAACCACGACGCGACGATG 3′;
#2078 = 5′ AGCGAGTTGTTGCTGGCCAT 3′.

See Bell et al. (1988) for the alignment of these primers on the *Sxl* cDNA sequences. The identity of PCR amplification products was confirmed by chain termination sequencing (Sanger et al., 1977) with Sequenase brand T7 DNA polymerase (USB) and the above primers. The manufacturers' instructions were followed with the following exception. Template and primer DNA were resuspended in sequencing buffer, boiled for 3 minutes and flash annealed in a dry ice/ethanol bath (Kusukawa et al., 1990).

RNA and protein localization

In situ hybridization to gonads was based on the protocol of Tautz and Pfeifle (1989). Experimental gonads and control gonads can be distinguished based on morphology, therefore one set of experimental gonads and either a positive or negative control were processed in the same tube. Gonads were fixed in 4% formaldehyde and were digested for at least 10 minutes in 50 μg/ml Proteinase K at room temperature. Gonads were also prepared by freeze substitution (Lasko and Ashburner, 1990). Digoxigenin probes were synthesized according to the Genius digoxigenin labeling kit instructions (Boehringer Mannheim) or by polymerase chain reaction with the following NTP concentrations (1 mM each dATP, dCTP, dGTP, 0.65 mM dTTP, and 0.35 mM digoxigenin-UTP). Probes were made from *Sxl* clones or by RT-PCR products amplified by the polymerase chain reaction.

For antibody localization, dissected gonads were prepared by freeze substitution and incubated with a 1:10 dilution of anti-*Sxl* (mAb 104; Bopp et al., 1991) for 3 hours at room temperature or overnight at 4°C. Alkaline phosphatase-conjugated anti-mouse antibodies were used as secondary antibodies.

RESULTS

Sxl$^+$ pre-mRNA splicing in wild-type germ-line cells

Given that the functions of *Sxl*, the somatic sex determination genes, as well as a number of the ovarian tumor genes have been implicated in germ-line sex determination we wanted to determine (1) if we could confirm molecularly the proposals that ovarian tumor genes functioned in germ-line sex determination; (2) whether the functions of these genes are integrated into a single regulatory hierarchy; and (3) the structure of that hierarchy. One simple way of determining whether mutants in the ovarian tumor genes caused sexual transformations is to see if male-specific molecules are found in mutant females. Likewise, a simple way to determine the order of function of two genes is to inquire whether one gene regulates the expression of the other. To this end, our initial experiments address whether the sex-specific functioning of *Sxl* in the germ line is regulated by alternative pre-mRNA processing as it is in the soma. A finding that *Sxl*$^+$ produces sex-specific transcripts within the germ line enables us to use these sex-specific transcripts as a phenotype to determine the likely sexual identity of germ cells and to order the functioning of other genes involved in germ-line sex determination relative to *Sxl*$^+$.

We first examined whether the sex specificity of *Sxl*$^+$ function in the germ line was associated with sex-specific patterns of pre-mRNA splicing identical to those used to control *Sxl*$^+$ activity in the soma (Fig. 1). We used reverse transcription followed by the polymerase chain reaction (RT-PCR) to ask whether *Sxl*$^+$ mRNAs in male and female gonads (which contain both germ-line and somatic cells) differed with respect to the presence of the male-specific *Sxl*$^+$ exon whose inclusion in mRNA leads to premature termination of *Sxl*$^+$ protein in somatic cells. RT-PCRs using one primer homologous to the male-specific exon and a second primer homologous to a more 5′ common exon (Fig. 1A), led to the amplification of two male-specific, Sxl$^+$-dependent, products (Fig. 1B; lanes 1 and 2). The presence of two male-specific products is also observed in male somatic cells where two alternative acceptor sites at the beginning of the male-specific exon are used (Samuels et al., 1991; Fig. 1C). Sequencing confirmed that these two amplification products are due to the presence of male-specific *Sxl*$^+$ templates arising from the use of the same alternative splice sites used in somatic cells (Fig. 1C). *Sxl*$^+$ primers homologous to the two common exons that flank the male-specific exon used in RT-PCRs (Fig. 1A) result in two female-specific products (see later figures). Sequencing confirmed that these two female-specific products are derived from *Sxl*$^+$ templates differing because of alternative splicing (Bell et al., 1988; Samuels et al., 1991; not shown). Indistinguishable sex-specific amplification products are observed in experiments using dissected gonads, gonadectomized flies, or intact flies (Fig. 1), suggesting that the same splice sites are used in somatic and germ-line cells in the region of the transcription unit that we examined. The *Sxl*$^+$ mRNAs produced in the germ line and the soma do differ (Samuels et al., 1991), but the germ-line-specific regions are located elsewhere on the primary transcript. While these differences may be important for the different roles of *Sxl*$^+$ in the germ line and the soma, the inclusion of male-specific exon with its premature termination codons is likely to be the overriding determinant of the sex-specific functioning of *Sxl* in the germ line.

To determine if the sex-specific gonadal *Sxl*$^+$ RT-PCR products are generated from mRNAs in the soma, or germ line, or both, we did in situ hybridization to gonads using a male-exon-specific probe. Signal is detected in the germ-line cells of wild-type males (Fig. 1D) indicating that male-specific *Sxl*$^+$ mRNAs are present in germ-line cells. In the ovaries of wild-type 2X females (Fig. 4F) or the testis of 1X males completely deleted for *Sxl* (Fig. 1E), hybridization signal is essentially absent [There is limited signal seen in *Sxl*$^-$ males that must be due to cross hybridization

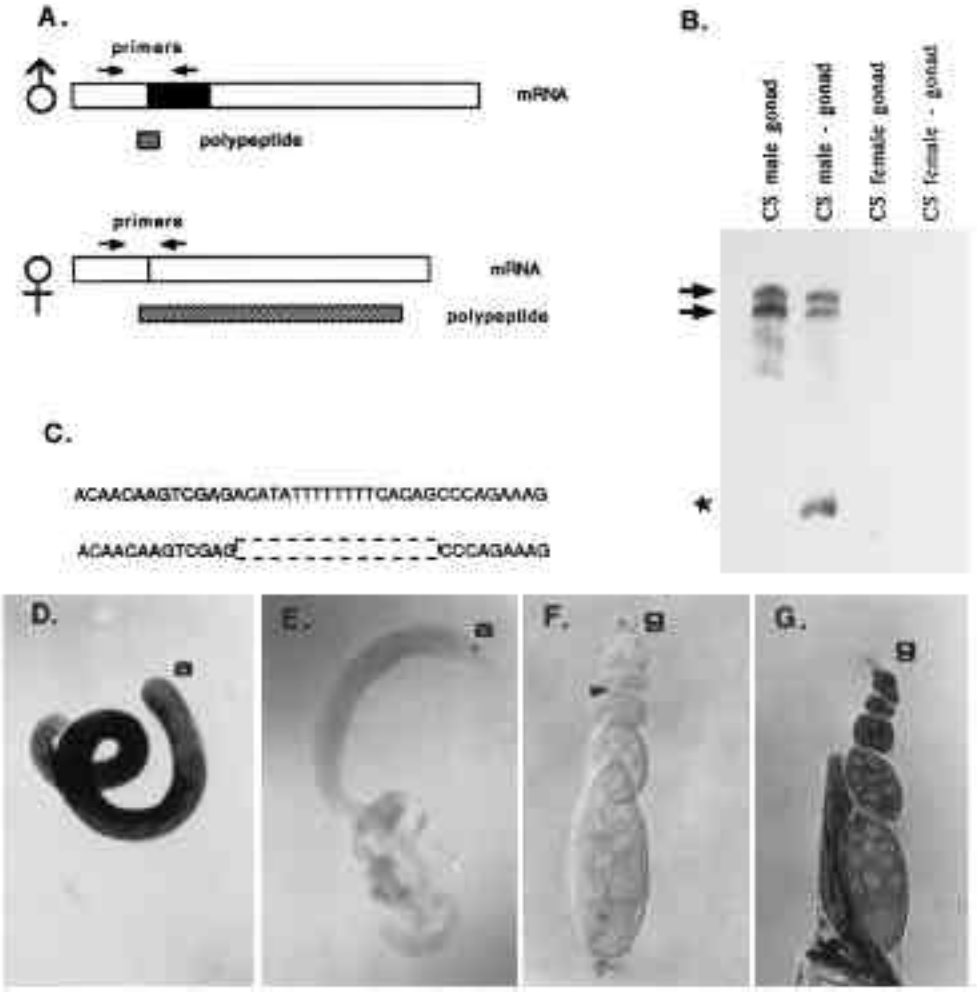


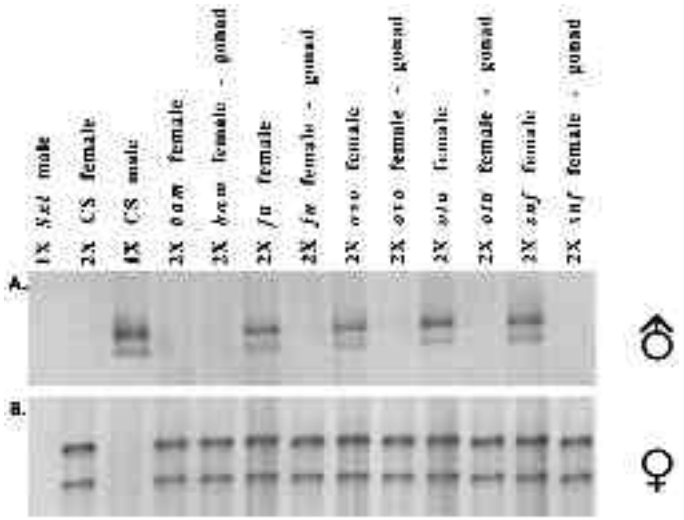
Fig. 1. Sex-specific *Sxl*$^+$ pre-mRNA splicing in gonads. (A) The male-specific *Sxl*$^+$ mRNAs differ from the female-specific form by virtue of the male-specific exon (in black). Predicted polypeptides are shown below the mRNAs. The arrows indicate the relative positions of primers used in RT-PCRs. (B) The result of RT-PCRs using the primer pair (#847 and #1613) amplifying only the male-specific *Sxl*$^+$ cDNA (the upper doublet indicated by arrows). Canton S (CS) is a wild-type strain. The band marked with a star is an artifact (sequencing revealed no homology to *Sxl*$^+$). (C) Part of the sequence of the male-specific amplification products are shown. Note the presence of the AG and poly-pyrimidine tract in the longer product. (D) The gonads of wild-type males (CS) show hybridization signal in the bulk of the testis when probed with the male-specific exon. (E) Gonads of *y cm Df(1)Sxl*^{fp7BO} */Y* males hybridized with the male-specific exon. Sometimes a few cells near the apex of the testis (a) show background staining. (F) Hybridization signal from the *Sxl*$^+$ male exon is not observed in wild-type (CS) female gonads. (G) The germ cells of wild-type females (CS), at all stages including in the germarium (g), do hybridize with non-sex-specific *Sxl*$^+$ probes. Flies were grown at 25°C.

(Fig. 1E)]. When a common region of the *Sxl*$^+$ mRNA is used as a probe, it hybridizes to germ-line cells of wild-type females (Fig. 1G) and males (not shown), suggesting that *Sxl*$^+$ mRNAs found in female germ-line cells are spliced female specifically. These in situ data, together with the results of the RT-PCRs, indicate that the examined region of the *Sxl*$^+$ pre-mRNA is sex specifically spliced in germ-line cells.

The finding that *Sxl* produces male-specific and female-specific transcripts within the germ line provides us with a tool to characterize molecularly possible germ-line sexual transformations. More importantly, the functioning of other genes involved in germ-line sex determination can be ordered, relative to *Sxl*, by determining if they regulate the pattern of *Sxl* pre-mRNA splicing.

Ovarian tumor genes required for germ-line *Sxl* pre-mRNA splicing

We used RT-PCRs and in situ hybridization to examine whether mutations in ovarian tumor genes affect *Sxl*$^+$ expression at the level of pre-mRNA splicing. When the *Sxl* male-exon-specific primer pair is used, RT-PCR products can be generated from 2X *fu*$^-$, *ovo*$^-$, *otu*$^-$, or *snf*$^-$ females (Fig. 2A). These amplification products depend on the presence of mutant gonads, indicating that *Sxl*$^+$ pre-mRNA splicing is not disrupted in somatic tissues outside of the gonad. For example, male-specific amplification is detected when mRNAs from whole 2X *snf*$^-$ females are used as the RT-PCR substrate (Fig. 2A, lane 12), but not when mRNAs from 2X *snf*$^-$ gonadectomized flies are used (Fig. 2A, lane 13). The male-specific *Sxl*$^+$ RT-PCR products from 2X *snf*$^-$



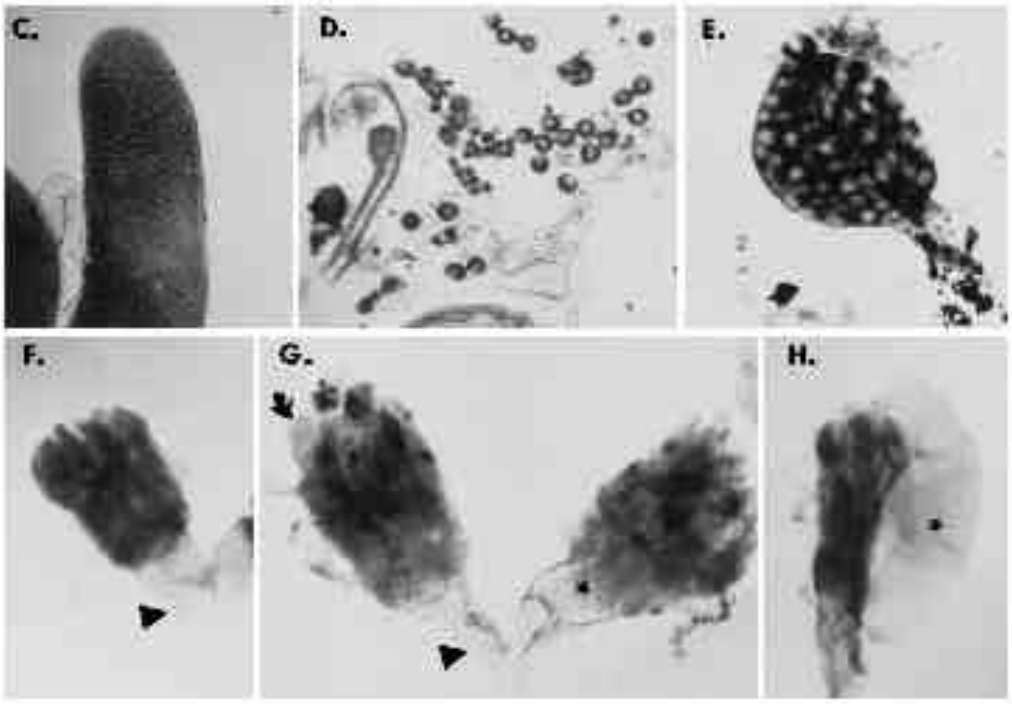


Fig. 2. Sex-specific *Sxl*$^+$ pre-mRNA splicing in ovarian tumor-bearing flies as assayed by RT-PCR and in situ hybridization. (A) RT-PCRs using male-specific primer pairs (#847 and #1613). (B) RT-PCRs using primers (#847 and #2079) recognizing the female-specific *Sxl*$^+$ cDNA. Similar results were obtained using primers #847 and #848 (not shown) Several male-specific *Sxl*$^+$-dependent amplification products were also seen using #847 and #2079, but amplification was weak, these probably represent additional *Sxl*$^+$ splice forms (not shown). The chromosomal sex, genotypes and tissues used in the RT-PCR reaction are shown. C-H were hybridized with the male-specific *Sxl*$^+$ exon probe. (C) The apex of a wild-type (CS) testis whole-mount preparation. Staining is sometimes less intense at the most apical portion. (D) A freeze-substituted wild-type testis (CS) preparation showing cytoplasmic localization of hybridization signal i.e. signal is not due to unprocessed nuclear RNA. (E) A freeze-substituted 2X *otu*$^-$ ovarian tumor chamber also showing cytoplasmic localization of hybridization signal. (F-G) Whole-mount preparations of 2X *snf*$^-$ ovaries. (F) Hybridization signal is restricted to the tumorous ovary where it appears uniform and is not seen in the oviduct (arrowhead). (G) Hybridization signal is found throughout the ovaries but is mottled. Staining is absent from the oviduct (arrowhead), a maturing egg (star), and some tumorous chambers (arrow). (H) Hybridization signal is found in all the tumorous ovarioles to the left, but is absent from the ovariole containing an egg (star). We did not observe male-exon hybridization in the ovaries of *bam*^{1}*mwh red e*, *bam*^{105}*red e*, *y cv v fu*^{1}, *y cv ovo*^{rM1}*v f*, or *y cv ovo*^{rM2} *v f* females (not shown). Remaining genotypes are (1) *Sxl = y cm Df(1)Sxl*^{fp7BO}*/Y;* (2) *ovo = lzl*^{G}*/ovo*^{D1rS1}*v*^{24}*;pr mle*^{1}*/mle*^{roma}; (3) *otu = ct otu*^{1} *v* and (4) *snf = snf*^{1621} *v*^{24}. The wild-type strain is Canton S (CS). The *bam*$^-$, *ovo*$^-$, *otu*$^-$ and *snf*$^-$ females contained ovarian tumors. We did not observe clear examples of ovarian tumors in the *fu*$^-$ females during this experiment. Flies were grown at 25°C except the *ovo*$^-$ flies that were grown at 18°C and *snf*$^-$ flies that were grown at 29ºC.

females have been sequenced and are identical to the male-specific RT-PCR products derived from wild-type 1X males; products detected in all other mutants show similar electrophoretic mobility, suggesting that *Sxl*$^+$ pre-mRNA splicing occurs in the precise male pattern in the gonads of *fu*$^-$, *ovo*$^-$, *otu*$^-$, or *snf*$^-$ 2X females. These results strongly support the notion that the *ovo*$^+$, *otu*$^+$ and *snf*$^+$ genes function in germ-line sex determination and that they function upstream of *Sxl*$^+$ in the germ line (Oliver et al., 1988, 1990; Steinmann-Zwicky, 1988; Wei et al., 1991). In addition, these data provide the first evidence that the *fu*$^+$ gene has a germ-line sex determination function.

The *bam*$^+$ gene is a member of the ovarian tumor class of genes, but differs from *fu*$^+$, *otu*$^+$, *ovo*$^+$ and *snf*$^+$ in that *bam*$^+$ activity is required for germ-line development in males and females (McKearin and Spradling, 1990). RT-PCRs from *bam*$^+$ mutant females result in no male-exon-specific *Sxl*$^+$ products (Fig. 2A, lane 4). Thus *bam*$^+$ activity does not appear to be required for female-specific *Sxl*$^+$ pre-mRNA splicing, implying that a mutant pattern of *Sxl*$^+$ pre-mRNA splicing is not an obligatory consequence of ovarian tumor formation. This result might be expected for a 'true' ovarian tumor gene functioning independently of sex determination or a sex determination gene functioning downstream of *Sxl*.

As the ovarian tumor genes that function upstream of *Sxl*$^+$ are all germ-line dependent, and all but *fu*$^-$ show germ-line restricted phenotypes (Lindsley and Zimm, 1992), we expected that the misregulation of *Sxl*$^+$ pre-mRNA splicing in the gonads of 2X *ovo*$^-$, *snf*$^-$, *otu*$^-$ and *fu*$^-$ females occurs in the germ cells themselves. However, as cell-cell commu-

nication is an important component of germ-line sex determination, this idea was tested directly. To determine if male-specific Sxl^+ RT-PCR products from ovarian tumor females (Fig. 2A) are of germ-line origin, we did in situ hybridization to gonads using the Sxl^+ male-specific probe. RNA containing the Sxl^+ male exon is detectable by in situ hybridization in the cytoplasm of germ-line cells of 2X females mutant for otu^- (Fig. 2E), snf^- (Fig. 2F-H) or ovo^-, (not shown). There is no evidence that the Sxl^+ male exon is expressed in the somatic tissues of the ovaries in these mutations (Fig. 2F,G). We did not detect male-exon sequences by in situ hybridization to fu^- females. We also failed to detect the male exon in 2X females mutant for weak *ovo* alleles. The failure to detect the male-specific *Sxl* splice form may be due to the low frequency of ovarian tumor cells observed in these flies. Taken together these in situ data, and the RT-PCRs, suggest that otu^+, ovo^+ and snf^+ activities are required for female-specific Sxl^+ pre-mRNA splicing within 2X germ-line cells. The products of these genes need not act directly, and might act to either promote female-specific splicing or block male-specific splicing.

The ovo^- ovarian tumor phenotype is partially suppressed by $Sxl^{M\#1}$

The constitutive *Sxl* allele $Sxl^{M\#1}$ is of particular value in genetic studies because the suppression of a loss-of-function mutant in another gene by $Sxl^{M\#1}$ is strong evidence that the wild-type copy of the suppressed allele acts upstream of Sxl^+ (Cline, 1984). The converse is not true; failure of $Sxl^{M\#1}$ to rescue a mutant phenotype is uninformative in terms of ordering gene function. Because $Sxl^{M\#1}$ fails to rescue the death of germ cells caused by ovo^- mutations in females, it had been suggested that ovo^+ acts downstream or independent of Sxl^+ in the female germ line (Steinmann-Zwicky, 1988). This conclusion is contradicted by our finding that ovo^+ function is required for female-specific Sxl^+ pre-mRNA splicing in the germ line, which establishes that ovo^+ functions upstream of Sxl^+ in the germ line. One way that the molecular and genetic results can be reconciled is if ovo^+ functions prior to Sxl^+ and controls at least two processes, one an *Sxl*-independent vital function (the absence of which results in germ cell death) and the other an *Sxl*-dependent sex determination function (Cline, 1988; Oliver et al., 1990).

To test genetically the hypothesis that there is a branch in the germ-line sex determination pathway between ovo^+ and Sxl^+, we have employed a genotype in which ovo^- germ cell viability is enhanced, but the ovo^- germ-line sex determination phenotype is still present. In these flies, we asked whether $Sxl^{M\#1}$ would suppress the ovo^- germ-line sex determination phenotype. The requisite *ovo* phenotype is provided by the finding that the survival of 2X ovo^- germ-line cells is enhanced in a $maleless^-$ (mle^-) background (Oliver, 1989; these data will be presented in full elsewhere). The survival of germ-line cells in 2X ovo^- mle^- females is variable (0-82% of ovaries have germ cells). Surviving cells usually form small ovarian tumors (Fig. 3A; 75-100% of ovaries with germ cells have exclusively tumors or a mix between tumors and nurse cell cysts), and show male-specific Sxl^+ pre-mRNA splicing by RT-PCR. In the presence of the $Sxl^{M\#1}$ allele, the germ-line cells of 2X ovo^- mle^- females are much more likely to contain female-like egg chambers with 16 cells (Fig. 3B; 42-100% of ovaries with germ cells have nurse cells or a mix between nurse cells and tumors), indicating that $Sxl^{M\#1}$ suppresses the germ cell sex determination phenotype of ovo^- mutations. The rescue of ovo^- ovarian tumor cells sexual morphology by $Sxl^{M\#1}$ is partial. The best rescue of the 2X ovo^- phenotype results in chambers having a polyploid nurse-cell morphology, indicating that there has been a failure to specify or differentiate one of the cells as an oocyte (Fig. 3B); additionally some tumorous chambers remain (not shown). The partial rescue could be due to remaining viability problems, involvement of ovo^+ in a third pathway, or insufficient levels of 'constitutive' Sxl^+ product from $Sxl^{M\#1}$ (Fig. 3C; see Cline, 1984; Cronmiller and Cline, 1987; Steinmann-Zwicky, 1988 for discussion of $Sxl^{M\#1}$ function when Sxl^+ positive regulators are limited). Regardless of the reason for partial rescue, the interaction between $Sxl^{M\#1}$ and ovo^- is in agreement with our molecular data suggesting that ovo^+ functions upstream of Sxl^+.

Somatic sex determination genes are required for *Sxl* pre-mRNA splicing in the germ line

Loss-of-function mutations in the somatic sex determination

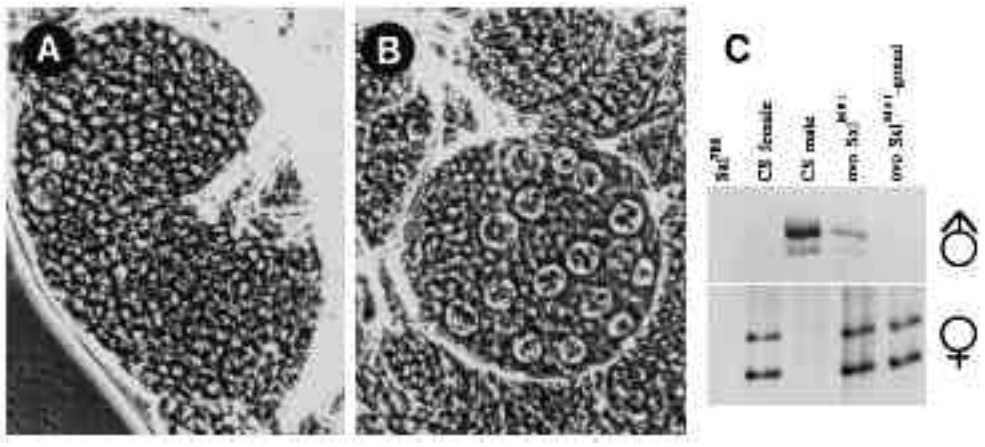


Fig. 3. Suppression of ovo^- germ-line sexual morphology by $Sxl^{M\#1}$. (A) Micrograph of an ovarian tumor from a ovo^-; mle^- female. Note the presence of many small cells. (B) Micrograph of a female-like egg chamber from a ovo^- $Sxl^{M\#1}$; mle^- female. There are 16 cells in this chamber that have a nurse cell morphology. No identifiable oocyte is present. (C) RT-PCRs (the same format as Fig. 1) reveals male-specific amplification products in the presence of $Sxl^{M\#1}$. Full ovo^- $Sxl^{M\#1}$ genotypes are lzl^G/ovo^{D1rS1}; $pr\ mle^1/mle^{roma}$ and lzl^G $Sxl^{M\#1}$ sn/ovo^{D1rS1}; $pr\ mle^1/mle^{roma}$. Flies were grown at 18°C.

genes, *tra*⁻ and *tra-2*⁻ and gain-of-function *dsx* alleles, transform females somatically into males (Baker and Ridge, 1980; Nöthiger et al., 1989) and also cause partial sex transformations of the germ line (Schüpbach, 1982; Nöthiger et al., 1989). These partial sex transformations of the germ line must be a consequence of the roles of these genes in somatic cells, since *tra*⁺, *tra-2*⁺ or *dsx*⁺ do not function in germ-line cells. We have used RT-PCR and in situ hybridization experiments to ask whether *tra*⁺, *tra-2*⁺ and *dsx*⁺ are required for correct female-specific splicing of germ-line *Sxl*⁺ pre-mRNA.

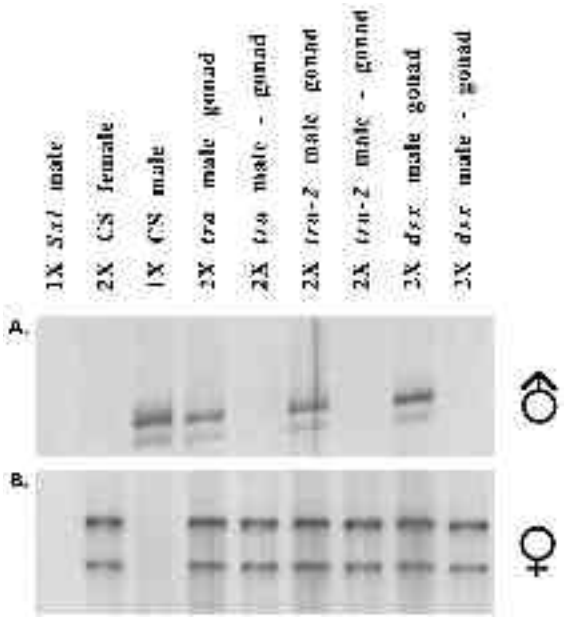
The male-specific *Sxl*⁺ amplification products are observed following RT-PCRs with gonadal RNA from 2X flies that are transformed to male by *tra*⁻, *tra-2*⁻ or *dsx* gain-of-function mutations (Fig. 4A, lanes 4, 6 and 8) suggesting that somatic *tra*⁺, *tra-2*⁺, and *dsx*⁺activities are required for female-specific *Sxl*⁺ pre-mRNA splicing in gonadal tissue. However, female-specific amplification products are also observed following RT-PCRs on 2X male gonadal RNA. Male-specific amplification products are absent following RT-PCRs on gonadectomized 2X males (Fig. 4A, lanes 5, 7 and 9) indicating that somatic sex determination genes are required only for female-specific *Sxl*⁺ pre-mRNA splicing in the gonad.

To determine if the male-specific *Sxl*⁺ pre-mRNA splicing seen in 2X male gonads occurs in the germ-line cells, we used in situ hybridization. When *Sxl*⁺ male-specific exon sequences are used as probe, hybridization signal is detected in the testis of 2X male flies (Fig. 4C-E), but never in the testicular sheath or other tissues of obvious mesodermal origin. Hybridization signal is restricted to clusters of presumptive germ-line cells within the testis. Since the germ-line sex determination functions of *tra*⁺, *tra-2*⁺ and *dsx*⁺ are required only in somatic cells (Marsh and Weischaus, 1978; Schüpbach, 1985), our results indicate that *Sxl*⁺ pre-mRNA splicing in germ-line cells requires a signal from the soma that is controlled in part by the somatic sex determination genes.

Germ-line phenotypic variability in somatic sex determination and ovarian tumor mutations

For both the somatic sex determination and the ovarian tumor genes that regulate sex determination, the sexual morphology of germ-line cells is variable in all of the alleles that we have examined. This variability is manifest as some germ-line cells retaining female characteristics (Smith and King, 1966; Gollin and King, 1981; King et al., 1986; Oliver et al., 1988; Schüpbach, 1982; Nöthiger et al., 1989). Since this phenotypic variability is observed even when known molecularly null alleles of the somatic sex determination genes are examined (Schüpbach, 1982; Nöthiger et al., 1989), we believe it is revealing something important about the regulation of germ-line sex determination.

The phenotypic variability seen morphologically in these mutations is also evident at the molecular level. Our molecular data on *Sxl*⁺ expression in the germ line of these 2X males also suggest that they are of mixed sexual identity: some cells express the *Sxl* male mRNA and some cells express the *Sxl*⁺ female-specific protein. For both the



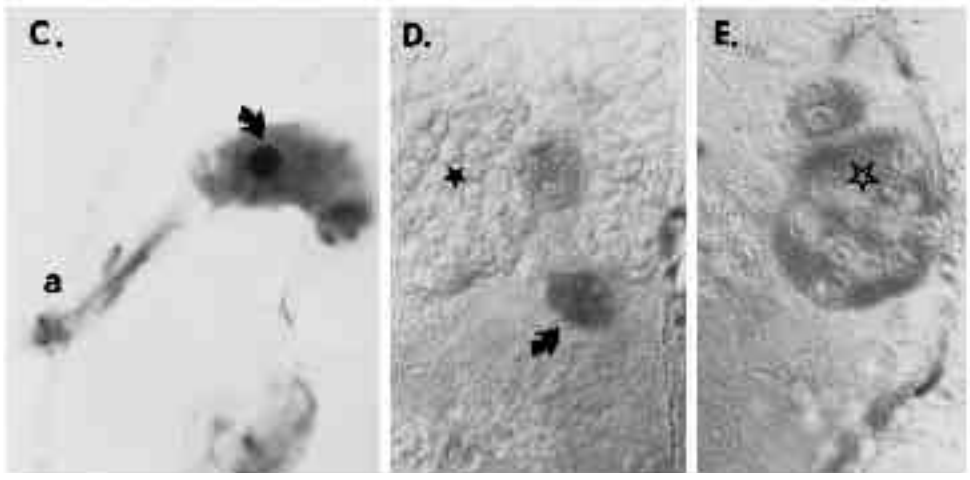


Fig. 4. 2X male flies express *Sxl*⁺ male exon. (A) RT-PCRs using primers that can only amplify male-specific *Sxl* exon containing cDNAs. (B) RT-PCRs using primers that can amplify female-specific *Sxl* cDNAs (see Fig. 1 for details). (C-E) 2X males have germ cells staining for the male-specific exon. (C) The apex (a) is practically devoid of germ-line cells (see Ota et al., 1981; Nöthiger et al., 1989). A cluster of germ cells hybridize with the male-specific exon probe is shown (arrow). (D) A magnified view of 2X germ cells. One cluster of small germ cells (with male morphology?) shows hybridization within the male-exon probe (arrow). A larger cluster of cells with a nurse cell morphology show no hybridization (star) (E) Another view of 2X male germ cells. In this case, there are clusters of germ cells with a nurse cell morphology that also hybridize with the male-exon probe (open star). The full genotypes are: *tra = y/+; tra*^{1} *jv/Df(3)st*^{4} *gl e. tra-2 = y/+; tra-2*^{1}*/Df(2)TRIX. dsx = dsx*^{swe}*/Df(3)dsx*$^{r+M43}$. Flies were grown at 25°C.

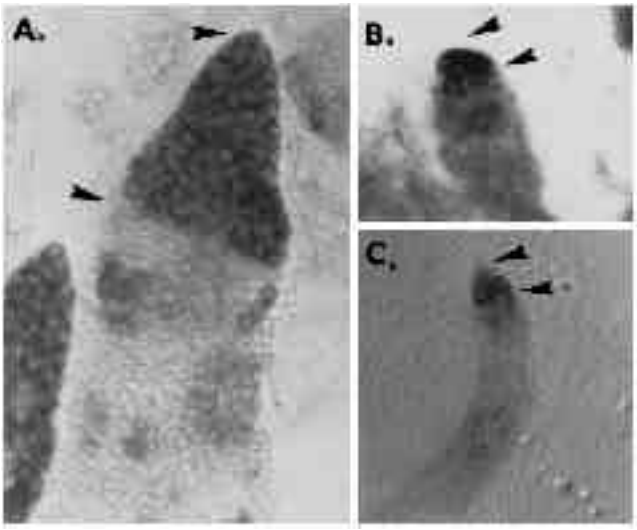


Fig. 5. The *Sxl* antigen is readily detectable in the germ cells of wild-type females and is expressed in greater numbers of *bam*⁻ germ cells. (A) The enlarged germaria of 2X *bam*⁻ females contain large numbers of cells that express the Sxl^+ antigen at high levels. This is consistent with a role in the control of germ cell divisions (McKearin and Spradling 1990). (B) Sxl^+ antigen levels are highest in two to eight cells at the anterior terminus of the wild-type germarium. As there are believed to be about two stem cells per germarium (Weischaus and Szabad 1979), the stained cells are likely either dividing cyst cells or a mix between dividing cyst cells and stem cells. In addition to the prominent staining in the germ cells at the anterior most part of the germarium, there is weak staining in early egg chambers (stage 1 and 2). Later egg chambers (stage 3) show moderate levels of Sxl^+ antigen. Detection of Sxl^+ antigen fades in later egg chambers (not shown). (C) When the Sxl^+ antigen is detected in the ovarian tumors of *snf*⁻ females (which it frequently is), the pattern of staining in the germarium is similar to wild type. Note that Sxl^+ antigen detection is cytoplasmic in all cases, raising a number of interesting questions about the function of Sxl^+ protein in early egg chambers. The limits of strong Sxl^+ antigen detection in the germaria are indicated with arrowheads. Full genotypes are: *bam*⁻ = bam^1 *mwh red e*. *snf*⁻ = snf^{1621} v^{24}. Sxl^+ antigen is never seen in wild-type male gonads (not shown).

ovarian tumor and somatic sex determination mutations, the presence of Sxl^+ male-exon hybridizable RNA sequences in germ-line cells is positively correlated with germ cell sexual phenotype. For example, 2X *snf*⁻ ovaries bear ovarioles with tumors and occasionally one or more ovarioles with wild-type appearing female cysts and eggs. In such cases, Sxl^+ male-exon hybridization is detected in the male-like tumor cells (Fig. 2G,H). However, the correlation between sexual morphology and Sxl^+ expression is not absolute, since some male-like cysts in 2X *snf*⁻ ovaries fail to show hybridization with the Sxl^+ male-specific exon probe (Fig. 2G). Similarly, in somatic sex determination mutants, male-exon hybridization signal can be seen in many clusters of male-like germ cells (Fig. 2D), but some clusters of germ-line cells show none. In addition, in some clusters with more female characteristics (Fig. 2E), male-exon hybridization is also seen. Although the expression of male-exon sequences in female cysts might be irrelevant (it is likely to be the presence of functional female product, not the absence of male-specific product that is critical), the expression of Sxl^+ antigen almost always occurs in some germ-line cells of 2X ovarian tumor females (Fig. 5) or 2X males (Fig. 6), suggesting that the sexual transformations are incomplete.

DISCUSSION

Germ-line sex determination is a fundamental process in essentially all higher eukaryotes, but is not well understood. We have built upon previous genetic studies suggesting that ovarian tumor genes (Oliver et al., 1987, 1988, 1990; Pauli and Mahowald, 1990; Steinmann-Zwicky, 1988; Wei et al., 1991), along with the more well known somatic sex determination genes (Nöthiger et al., 1989), control this process in *Drosophila*. The primary goal of the experiments reported here was to gain insight into how the genetic functions controlling germ-line sex determination are organized into a regulatory hierarchy.

We initially addressed whether the female-specific germline functioning of the prototypical germ-line sex determination gene, Sxl^+, is achieved by the sex-specific germ-line

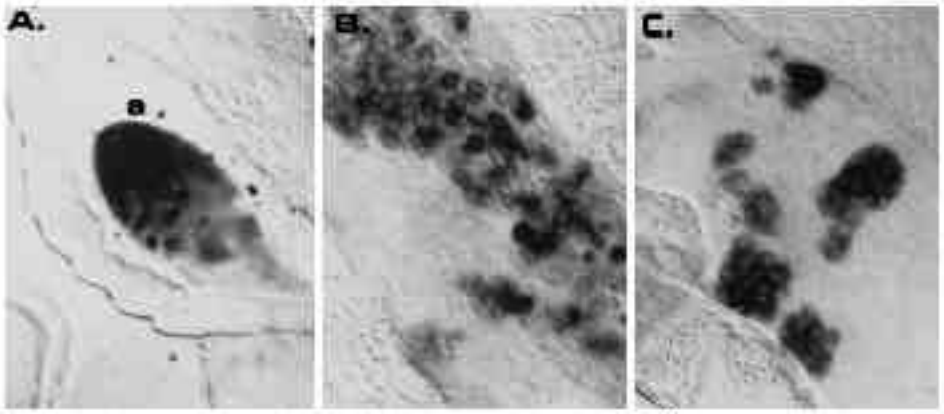


Fig. 6. The Sxl^+ antigen is detected in 2X males. Antigen-positive cells can be present in large numbers at the testicular apex (a) of 2X males. This may be analogous to the situation seen in 2X *bam*⁻ females (c.f. Fig. 5). We suggest that the presence of Sxl^+ protein in excessive numbers of 2X male germ cells is due to a secondary effect of an improper gonadal environment and is not of major significance in terms of hierarchical order [see McKearin and Spradling (1990) for the converse form of this argument]. As in wild-type or mutant 2X females, cytoplasmic antigen is present. The genotypes shown are: (A) *y/+; tra*^{1} *jv/Df(3)st*^{4} *gl e* (B) *y/+; tra-2*^{B}*/Df(2)TRIX* and (C) $dsx^{swe}/Df(3)dsx^{r+M43}$. Flies were grown at 25°C.

splicing of Sxl^+ pre-mRNA. Our results demonstrate that the same sex-specific alternative splices are used to regulate Sxl^+ function in the germ line and soma (Salz et al., 1989 and Samuels et al., 1991). This finding enabled us to use the female versus male patterns of Sxl^+ pre-mRNA splicing in the germ line as phenotypes to provide molecular confirmation of the germ-line sexual transformations in ovarian tumor mutations. Most importantly, the Sxl^+ probes provided a tool for ordering the functions of the wild-type products of other genes implicated in female germ-line sex determination.

A model summarizing our current ideas about how the genetic functions controlling germ-line sex determination are organized into a regulatory hierarchy are presented in Fig. 7. In the following sections we discuss the results that led to each aspect of this model.

Somatic sexual identity and *Sxl* expression in the germ line

Previous genetic and transplantation studies have shown that female germ-line sexual development in *Drosophila melanogaster* requires a female soma (Schüpbach, 1985; Steinmann-Zwicky et al., 1989). The generation of a signal molecule regulated by the somatic sex determination hierarchy is the simplest model to account for the role of the soma in female germ-line sex determination. Consistent with this idea are the findings from genetic studies which indicate that Sxl^-, tra^-, $tra\text{-}2^-$ and *dsx* gain-of-function mutations that cause chromosomally female individuals to differentiate somatically as males, and have no effects on germ-line sex determination within the germ line, cause germ-line sexual transformations [Nöthiger et al., 1989; c.f. Cline (1984) and Oliver et al. (1990) for discussion of somatic-line-dependent germ-line transformations by Sxl^-].

Our results examining the effects of tra^-, $tra\text{-}2^-$ and *dsx* gain-of-function mutations on the expression of Sxl^+ in the germ line are in agreement with these previous genetic findings and provide molecular confirmation of the model that the somatic sex determination hierarchy acts to control germ-line sexual differentiation in females (Fig. 7). For each of these three genes, mutant combinations that transform 2X individuals into males somatically result in the male splicing pattern of germ-line Sxl^+ pre-mRNA. Thus the role of the somatic sex determination hierarchy in germ-line sex determination is mediated through its control of Sxl^+ expression in the germ line. One possibility is that *dsx*, the final gene in the somatic sex determination hierarchy, controls the expression of a diffusible molecule or trans-membrane protein involved in transmitting a signal from the soma to the germ line.

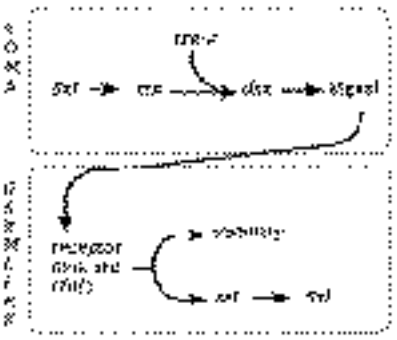


Fig. 7. A working model of germ-line sex determination in *Drosophila*. Some of the genes functioning in *Drosophila* sex determination are shown. The arrows denote the proposed regulatory hierarchy; not direct positive or negative interactions. The identity of the proposed signal and receptor molecules are unknown. Some of the genes are clearly required for other functions: the snf^+ gene product is also required in the soma when Sxl^+ is limited, suggesting that snf^+ might be a redundant regulator of Sxl^+ in the soma (Oliver et al., 1988); The $tra\text{-}2^+$ gene is required in the male germ line, but it is not clear what relationship this role has (if any) to sex determination (Belote and Baker, 1983); the fu^+ is required for multiple processes in development (Busson et al., 1988). The ovo^+ locus is complex and also encodes for functions (*shavenbaby*) unrelated to sex determination (Oliver et al., 1987). We have placed a question mark next to fu^+ because of the limited data supporting a role in germ-line sex determination and because we are unsure if it is important for germ-line viability. A large number of genes acting in the soma have been obmitted. See text for details.

The sex determination hierarchy within the germ line

Turning to the regulation of sex determination within the germ line, our results show that the functions of four of the five ovarian tumor genes that we assayed (otu^+, ovo^+, fu^+ and snf^+) are required for the female-specific splicing of Sxl^+ pre-mRNA. These data strongly suggest that Sxl^+, otu^+, ovo^+, fu^+ and snf^+ function together in a pathway specifying female differentiation of the germ line and that Sxl^+ functions subsequent to the other four ovarian tumor genes in this pathway (Fig. 7). Genetic data on the interactions of these genes are also consistent with this conclusion (Oliver et al., 1988, 1990; Steinmann-Zwicky, 1988; Pauli et al., 1993; this study).

The final ovarian tumor gene that we have analyzed is bam^+. As Sxl^+ pre-mRNA is spliced to the female-specific form in the germ line of 2X bam^- females, our results provide no information as to the regulatory relationship, if any, between these two genes. Additionally, the *female lethal (2) d*$^+$ gene, which we have not examined, is likely to function also upstream of Sxl^+ in this pathway (Granadino et al., 1990, 1992).

A branch in germ-line sex determination pathway

A further ordering of the sex determination genes that function within the germ line can be inferred from the difference between the phenotypes produced by Sxl^- and snf^- versus otu^- and ovo^-. Mutations in *otu* or *ovo* result in the absence of 2X germ-line cells and sexual transformations of 2X germ-line cells (the latter are seen in leaky alleles; King et al., 1986; Oliver et al., 1987, 1990). While Sxl^- or snf^- also result in sexual transformations, they do not have any effect on germ cell viability. The simple explanation for the differences between these two groups of genes is a branched pathway (Fig. 7). Genes that function prior to the branch would be required for both germ cell viability and sex determination, as are ovo^+ and otu^+, whereas sex determination genes functioning after viability and sex determination come under separate control would be required only for sex determination, as are snf^+ and Sxl^+.

This idea rests on the supposition that the extant alleles of *Sxl* and *snf* accurately reflect the full range of wild-type functions of these genes. It is clear from studies on an extensive number of *Sxl*$^-$ alleles that the lack of viability effects in the germ line is characteristic of *Sxl* mutations (Cline, 1983; Schüpbach, 1985; Salz et al., 1987) and hence *Sxl*$^+$ function in the germ line is not essential for germ cell viability. However, the available *snf*$^-$ alleles retain some function (Gollin and King, 1981; Salz, 1992). Thus it is possible that the null *snf*$^-$ phenotype includes 2X germ cell death, in which case *snf*$^+$ would function prior to the branch in the pathway.

The *fu*$^+$ gene is required in multiple pathways, and this has had made it difficult to determine whether *fu*$^+$ is required for the full viability of germ cells (Smith and King, 1966; Busson et al., 1988).

Connecting the somatic signalling and germ cell autonomous sex determination pathways

The final important fact that has contributed to our model of how the functions of the somatic and germ-line components of germ-line sex determination are integrated into a hierarchy is provided by the observation that, like *ovo*$^-$ and *otu*$^-$, the *tra*$^-$, *tra-2*$^-$ and *dsx* gain-of-function mutations result in both reduced numbers of germ-line cells, as well as germ-line sexual transformations. These observations suggest that the somatic sex determination genes impact on germ-line sex determination at the same level as *ovo*$^-$ and *otu*$^-$ (Fig. 7).

While the nature of the cell-cell signaling process that connects the somatic and germ-line components of germ-line sex determination is currently unknown, the molecular analyses of *fu*$^+$ and *ovo*$^+$ are consistent with these genes being part of such a pathway. The *fu*$^+$ gene is a member of the serine/threonine protein kinase gene family (Préat et al., 1990), suggesting that *fu*$^+$ protein might be fairly directly involved in signal transduction. The *ovo*$^+$ gene is a member of the zinc-finger transcription factor family (Mével-Ninio et al., 1991). Thus, the *ovo*$^+$ gene product could be a *fu*$^+$-kinase-dependent transcription factor for downstream genes like *snf*$^+$ or even an early germ-line *Sxl*$^+$ promotor [c.f. the reduction in 'germ-line-dependent' *Sxl*$^+$ transcripts in *ovo*^{D1}/+ females (Salz et al., 1989; Samuels et al., 1991)]. Alternatively, *ovo*$^+$ might be required to transcribe a receptor for the somatic sex determination signal. The sequence of *otu*$^+$ is currently less helpful, since there is no homology to recognized gene families (Steinhauer et al., 1989).

On the role of *Sxl* in the germ line

Most of the previous thinking on the role of *Sxl*$^+$ in the germ line presumed that *Sxl* was the master regulatory gene for germ-line sex determination, since *Sxl*$^+$ is the master regulatory gene for somatic sex determination and dosage compensation. Our data are inconsistent with this interpretation. *Sxl*'s function within the germ line appears to be subordinate to all other currently identified genetic functions regulating germ-line sex. Thus, rather than considering *Sxl*$^+$ as the master regulatory gene in both the soma and germ line, it may be more appropriate to consider *Sxl*'s germ-line versus somatic functions as another case of an important regulatory function which is reused in multiple developmental processes and acts in different regulatory contexts in each process. For example, the *daughterless*$^+$ (Cline, 1989), *scute*$^+$ (Torres and Sánchez, 1989; Erickson and Cline, 1991) and *deadpan*$^+$ (Younger-Shepherd et al., 1992) genes, which are required for early regulation of *Sxl*$^+$ transcription in the soma, are also required for the development of the nervous system. Similarly, the *tra-2*$^+$ gene is required in the male germ line for fertility and for somatic sex determination (Belote and Baker, 1983). Likewise the *cut.*$^+$ (Blochlinger et al., 1990; Lui et al., 1991), *fushi tarazu*$^+$ (Doe et al., 1988; Pick et al., 1990), *Antennapedia*$^+$, *Sex combs reduced*$^+$, *Ultrabithorax*$^+$, and *abdominal-A*$^+$ (Tremml and Bienz, 1989; Reuter and Scott, 1990) genes function in multiple development processes.

The model that we have presented for the female germ-line sex determination hierarchy (Fig. 7) is a framework and is certainly incomplete. Most obviously, additional steps are likely to be added to this hierarchy as new genes are identified and characterized. We turn below to several aspects of the extant data which suggest some of the ways this model may be refined.

Is there a second pathway of germ-line sex determination?

The germ-line phenotypes produced in 2X males by *tra*$^-$, *tra-2*$^-$ and *dsx* gain-of-function mutations are intriguing in that they suggest there may be an alternative, low efficiency pathway for specifying female germ-line sexual differentiation. The germ-lines phenotypes in 2X males produced by these somatic sex determination mutations are quite similar and include few, or no germ-line cells in the testis and, most strikingly, a mixture of sexual morphologies among the cells that are present. Our molecular data on *Sxl*$^+$ expression in the germ line of these 2X males also suggest that they are of mixed sexual identity: some cells express the *Sxl* male mRNA and some cells express the *Sxl*$^+$ female-specific protein. The findings from both molecular and genetic studies that the germ lines of 2X males have a mixed sexual identity is probably revealing an important feature of germ-line sex determination.

A common explanation for the incomplete penetrance of a phenotype, i.e. that the mutant alleles in question are not null, does not seem to be applicable in this situation. For example, the *tra*^{1} mutation is a DNA null (McKeown et al., 1987), yet in the presence of this mutation both male-and female-like 2X germ cell development and male- and female-specific *Sxl*$^+$ splicing occur (Brown and King, 1961; Nöthiger et al., 1989; this study). Similarly, 2X; *dsx*^{swe}/*dsx*$^-$ flies are known to produce only the male-specific *dsx* product (Nagoshi and Baker, 1990), yet both male-like and female-like 2X germ cell development and *Sxl*$^+$ splicing occurs in 2X *dsx*^{swe}/*dsx*$^-$ flies (Nöthiger et al., 1989; this study). Thus, in at least these cases, the variable sexual characters observed in 2X flies mutant for sex determination genes can not be due to partially functional regulatory genes. The fact that female sexual identity appears to be achieved by at least some germ-line cells in the absence of an operative somatic sex determination hierarchy strongly suggests that while a somatic signal generated by this hierarchy is important for the attainment of female sexual identity by all germ-line cells, there is an alternative regu-

latory pathway that is capable of bringing about female differentiation of at least some germ-line cells.

The ovarian tumor genes are also characterized by germ lines with mixed sexual identities. While the alleles of these genes are less well characterized, and thus may not be nulls, the congruent mixed germ-line phenotypes seen in both the germ-line and somatic line-dependent mutations raises the possibility that an alternative pathway may also exist for the functions specified by the germ-line genes.

If there is an alternative regulatory pathway, it will be important to determine whether it also functions in the soma, or is autonomous to the germ-line cells, or has components in both tissues. In this regard, it is interesting to note that, in pole cell transplantation experiments, 2X germ-line cells transplanted into 1X (wild-type) males were reported to always differentiate as male (Steinmann-Zwicky et al., 1989). While the sample size in these experiments was small, these results, when taken at face value and contrasted with the findings that in 2X flies with a male soma mixed sexual differentiation is observed, suggest that there may be a somatic signal independent of *tra*$^+$, *tra-2*$^+$ and *dsx*$^+$ that is capable of bringing about female differentiation of germ-line cells.

Death and dosage compensation?

One facet of our model for germ-line sex determination suggests that genes acting early in the hierarchy (such as *ovo*$^+$, *otu*$^+$ and the somatic sex determination genes) control both germ-line sex determination and a vital function, whereas genes functioning later in the hierarchy (*snf*$^+$ and *Sxl*$^+$) regulate just sex determination (Fig. 7). This raises a question as to the nature of the vital function that is regulated by the early germ-line genes.

The model most compatible with the data is that the vital function controlled by the early acting female germ-line sex determination genes is the repression of dosage compensation in the female germ line (Oliver et al., 1987, 1990; Oliver, 1989). Dosage compensation is a process that equalizes the amount of transcripts produced by X-linked genes in 1X and 2X flies by doubling the transcription rate of the male's X chromosome (reviewed by Lucchesi and Manning, 1987). In somatic cells, dosage compensation is achieved by the male-specific functioning of the *mle*$^+$, *male specific lethal-1*$^+$, *male specific lethal-2*$^+$ and *male specific lethal-3*$^+$ genes. Whether dosage compensation occurs in the male germ line is unclear, since only one (*mle*$^+$) of the dosage compensation genes that have been tested for germ-line function in males, has a vital function in 1X germ-line cells (Bachiller and Sanchez, 1986). Thus, if the *mle*$^+$ male germ-line function is in dosage compensation, the process has different genetic requirements there than it does in somatic cells. Results showing that in 2X germ-line cells *mle*$^-$ mutations partially suppress the lethality of *ovo*$^-$ (Oliver, 1989; data outlined in this study will be presented in detail elsewhere) are intriguing, since they suggest that the inappropriate activation of *mle*$^+$ may be causally related to the cell lethality phenotype of *ovo*$^-$ mutations in 2X germ-line cells. While these data are consistent with the idea that a vital function controlled by the early acting female germ-line sex determination genes is the repression of dosage compensation, there is a clear need for further studies aimed at defining: (1) the function of *mle*$^+$ in the germ line; (2) the cause of the variable response of *ovo*$^-$ mutations to the absence of *mle*$^+$ and (3) whether mutations at other upstream genes are also suppressed by the absence of *mle*$^+$.

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