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Selection and Maintenance of Sexual Identity in the Drosophila Germline

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ABSTRACT

Unlike sex determination in the soma, which is an autonomous process, sex determination in the germline of Drosophila has both inductive and autonomous components. In this paper, we examined how sexual identity is selected and maintained in the Drosophila germline. We show that female-specific expression of genes in the germline is dependent on a somatic signaling pathway. This signaling pathway requires the sex-non-specific transformer 2 gene but, surprisingly, does not appear to require the sex-specific genes, transformer and doublesex. Moreover, in contrast to the soma where pathway initiation and maintenance are independent processes, the somatic signaling pathway appears to function continuously from embryogenesis to the larval stages to select and sustain female germline identity. We also show that the primary target for the somatic signaling pathway in germ cells can not be the Sex-lethal gene.

SEX determination in somatic cells of Drosophila melanogaster is dependent upon an autonomous system that functions transiently in the early embryo (SANCHEZ and NOTHIGER 1983; CLINE 1984). This system measures the relative number of X chromosomes to autosomes (the X/A ratio) in each nucleus and sets the sexual pathway by controlling the transcriptional activity of a special embryonic promoter, Sxl-Pe, of the master regulatory gene, Sex-lethal (Sxl) (KEYES et al. 1992). Sxl-Pe is turned on by the signaling system in female (2X/2A) embryos, while it remains off in male (1X/2A) embryos. The proteins from the Sxl-Pe mRNAs set in motion an autoregulatory feedback loop that serves to maintain the female-determined state during the remainder of development. In this feedback loop Sxl proteins promote their own expression by directing the productive female-specific splicing of transcripts expressed from the late or maintenance Sxl promoter, Sxl-Pm. In males, the maintenance mechanism also operates at the level of RNA splicing. In the absence of the embryonic proteins, transcripts from Sxl-Pm are spliced to include a male-specific exon that contains in frame translation stop signals that prematurely truncate the open reading frame. The continued splicing of Sxl-Pm transcripts in the nonproductive default pattern during the remainder of the life cycle ensures that the male-determined state is remembered.

Sxl directs subsequent somatic sexual development by controlling several subordinate pathways. These include the dosage compensation system and the somatic sexual differentiation pathway. In the former case, the dosage compensation system is turned off by Sxl in females, while it is on by default in males (LUCCHESI and MANNING 1987; GORMAN et al. 1993). In the latter case, Sxl promotes female differentiation by directing the female-specific splicing of transcripts from transformer (tra) (MCKEOWN et al. 1987). This produces tra protein, which, together with the constitutively expressed cofactor, transformer 2 (tra2) protein, activates the splicing of doublesex (dsx) transcripts into the female mode (NA-GOSHI et al. 1988). The female dsx protein produced from these transcripts then executes female differentiation. In males, the Sxl-tra-dsx splicing cascade is in the default mode and the dsx protein produced by the default mRNAs directs male differentiation.

While the key steps in somatic sexual development are now well understood, the mechanisms of pathway initiation, memory and differentiation in the sexual development of the germline remain largely obscure. What is clear is that germline sexual development is likely to be quite different from the soma. First, many of the known components of the X/A signaling system do not seem to play any role in the germline (SCHÜPBACH 1985; GRANADINO et al. 1993; STEINMANN-ZWICKY 1993). Second, the target of the X/A counting system, Sxl-Pe, is not active in the pole cells of female embryos when it is on in somatic nuclei nor does the promoter appear to function in germ cells at later stages of development (KEYES et al. 1992). Third, Sxl has been shown to be required for normal oogenesis (SCHÜPBACH 1985) and it has generally been assumed that Sxl functions as a master regulatory switch in germline sex-determination, much like it does in the soma (NOTHIGER et al. 1989; STEINMANN-ZWICKY et al. 1989; GRANADINO et al. 1989; SANCHEZ et al. 1987; CLINE 1984).
1993; Steinmann-Zwicky 1993). However, from our previous studies (Bopp et al. 1995), it is conceivable that Sxl is required for some aspect of germline development other than establishing and maintaining sexual identity. Fourth, genes downstream from Sxl in the sexual differentiation pathway—tra, tra2 and dsk—are not required in female germ cells but rather must function in the surrounding somatic tissue (Marsh and Wieschaus 1978; Schüpbach 1982). Finally, pole cell transplantation experiments indicate that the choice of female identity in the germline is not strictly autonomous as it is in the soma. Steinmann-Zwicky et al. (1989) showed that XX pole cells develop along the male mode when transplanted into a male somatic background. By contrast, XY or XO pole cells appear to have autonomous information for male development and develop in the male mode irrespective of the somatic environment. If one assumes that the default state of germline development is male (cf. Granadino et al. 1993) this would imply that the female pathway requires both an inductive signal from the soma and an autonomous signal dependent upon the X/A ratio.

The studies reported here have attempted to gain further insights into the mechanisms that might be involved in initiating and maintaining sexual identity in the germline. For this purpose we have used two molecular markers to determine which sexual pathway the germline is following. One of these is the sex-specific germline-specific gene, orb (Lantz et al. 1992, 1994). We describe the expression pattern of these two genes in genetic backgrounds that alter the development of the female germline. Our results indicate that Sxl is unlikely to be the master switch in germline sex determination. Instead, our studies suggest a different mechanism for initiating and maintaining germline sexual identity and implicate players in this process that have yet to be identified.

**MATERIALS AND METHODS**

**Fly strains:** Flies were maintained on standard yeast/cornmeal medium and kept at 25° unless otherwise indicated. Mutants are described by Lindsey and Zimm (1992). The dsk allele is dsk', the dsk' allele is the dsk' allele. The tra' and tra' stocks were obtained from J. Belote, the tra2's allele (tra') from W. Mattox and the tra2's and dsk' (dsk') allele were from B. Oliver. Stocks not in our collection were from the Bloomington Stock center.

**RT-PCR Analysis of gonadal RNA:** RNA was prepared from dissected ovaries or testes as previously described (Bopp et al. 1993). The sample was treated with acid phenol to reduce the amount of contaminating genomic DNA. Analysis of Sxl RNA was as previously described (Bopp et al. 1993); orb RNA was analyzed similarly. A primer that hybridizes to common orb sequences was used to reverse transcribe (Frohman et al. 1988) the RNA. 5% of the cDNA mixture was then amplified with either a male-specific primer and the common primer in Figure 1A or with a female-specific primer and the common primer. Since both male and female PCRs use the same reverse transcribed material, the amount of male vs. female product reflects the proportions of each type of orb RNA within a sample. PCR conditions were: one cycle of 95° for 3 min, 62° for 2 min, 72° for 40 min followed by 30 repeats of the cycle 95° for 15 sec, 62° for 2 min, 72° for 1.5 min. Primer sequences were as follows: reverse transcription primer 5' CTCCATGTCGAGTGCGATGCCC 3', common primer 5' CGAGTTCGAGCGGAGGCAAAGC 3' (5' GTATGGCCTGCTATGG 3' for the products in Figure 1B), male primer 5' CATGTTGGGAGTCGAA- GACCC 3', female primer 3' GAGAGGCGAGATGGTGATGACC 3'. Detection of products was done by Southern analysis with an orb cDNA.

**Temperature shift experiments:** Eight-hour collections (with the exception of the 18° 12-hr time point, which was a 4-hr collection) of tra2's/tra2 embryos (w; bw tra2's/YO × pr cn bw tra2/YO; B') were maintained at the collection temperature of either 18° or 29°. At various time points in development they were then shifted to 29° or 18°, respectively, and left at the new temperature until they reached adulthood. The gonads of the XX pseudomales (non-white, non-Bar eyed, non-Curly) were then dissected and the expression state of orb assayed as described above.

**Immunocytochemistry:** Samples were dissected, fixed, stained and imaged as previously described (Bopp et al. 1993). The older embryo was vigorously shaken during the devitilization step to crack the cuticle and stored in methanol at 4° for several days before staining with antibodies. Antibody incubations and washes for these embryos were extended to at least double the normal time. Fluorescent probes were from Jackson ImmunoResearch Laboratories, Inc.

**Quantitation of PCR products:** Blots were imaged on a Molecular Dynamics Phosphorimager. Counts of the male and female bands were measured less background values to give an estimate of the ratio of products.

**RESULTS**

**Sexual identity of germ cells in mutants that give rise to tumorous ovaries:** Pole cell transplantation experiments in the early eighties (Schüpbach 1982, 1985) showed that female germ cells defective in Sxl function do not differentiate properly but instead form tumorous cysts consisting of many small undifferentiated one- and two-cell cysts. This observation gave rise to the idea that female sterile mutations exhibiting a similar tumorous ovary phenotype define a group of loci involved in germline sex determination (Oliver et al. 1988, 1990; Pauli and Mahowald 1990; Pauli et al. 1993; Wei et al. 1994). Support for this hypothesis came from finding that a constitutive allele of Sxl can either completely or partially alleviate the tumorous phenotype of some of these mutants (Steinmann-Zwicky 1988; Pauli et al. 1993; Bae et al. 1994).

To explore the possible role of these loci in germline sex determination, we analyzed their effects on the splicing of Sxl mRNA and the expression and subcellular distribution of Sxl protein (Bopp et al. 1993; Lantz et al. 1994; see also Oliver et al. 1993). The tumorous ovary mutants could be divided into two classes. In the first, class A, are mutations that, at most, only marginally
perturb the female-specific splicing of Sxl RNA. This group includes the tumorous alleles of fused (fas1), bag-of-marbles (ban1), osv-1 (osv-1/+; data not shown), as well as two female sterile alleles of Sxl (Sxlf4 and Sxlf5). Some of the mutants in this group show abnormalities in the subcellular distribution of Sxl protein, typified by a persistence of cytoplasmic Sxl protein as the undifferentiated germline cysts age (see BOPP et al. 1993). The cytoplasmic rather than nuclear localization of Sxl protein is a characteristic feature of class A mutants with abnormalities in the subcellular distribution of Sxl protein. In these cells, Sxl protein is detected in the unspliced form of Sxl transcripts, which is not expressed in wild-type ovaries. The rt-PCR products from the mutants reveal that the expectations of the first prediction are met: orb transcripts in the class A mutants are expressed exclusively in the female not the male mode. This finding is consistent with the conclusion that the class A tumorous ovary mutations are defective in some aspect of germ-line development other than sex determination. Moreover, it should be noted that this is true even for the Sxl mutations, Sxlf4 (see Figure 1B) and Sxlf5. Thus, the abnormal cystoblast divisions observed in the ovary mutants (BOPP et al. 1993) can not be attributed to a failure to properly establish female identity in the germline.

While the predicted results were obtained for the class A tumorous ovary mutants, this was not true for the class B mutants. In spite of the fact that snf1621 and osv-1 have no detectable germline Sxl protein, the orb transcripts in these mutant ovaries are also exclusively female (Figure 1B and C). This finding indicates that the Sxl gene does not control all aspects of germline sexual identity and can not function in the germline as a cell autonomous master switch. BAE et al. (1994) have drawn similar conclusions from their studies on the sex-specific expression of a variety of other markers.

Effect of the somatic sex determination genes on germline sexual identity: If Sxl is not the master switch in germline sex determination, then the genes and regulatory strategies used in setting and 'remembering' germline sexual identity could be quite different from those in the soma. Indeed, the pole cell transplantation experiments described above have suggested that an important component of germline sex determination is somatic signaling. The obvious candidate for genes involved in generating a somatic signal would be members of the somatic sexual differentiation pathway, tra, tra2, and dsx. These genes are known to be required in the soma for normal oogenesis. Moreover, it has been demonstrated that tra and dsx have an effect on the sexually dimorphic size of the developing gonad as early as the first instar (STEINMANN-ZWICKY 1994a,b). To ascertain if the sexual differentiation pathway is involved in somatic signaling, we asked whether mutations in these three genes affect the sex-specific expression of Sxl and orb in the germline.

dsx: dsx is at the bottom of the sexual differentiation pathway and is expressed in a male- or female-specific form. Null mutations in dsx disrupt the sexual differentiation of both sexes and mutant animals develop as intersexes. In addition, there are dominant mutations of dsx that constitutively express the male form of the dsx protein independently of chromosomal sex. When such a dsxp allele is heterozygous with a wild-type allele in an XX animal, both the male and female forms of the dsx protein are expressed and this results in intersexual development. When dsxp is heterozygous with a defi-
Figure 1.—Sex specificity of orb RNA in tumorous ovary mutants that affect expression of Sxl in the germline. (A) Sex-specific transcripts of orb. Rectangles depict exons and the lines connecting the rectangles introns. The positions of primers used in the RT-PCR analyses are shown by arrows below the exons. Fp, Mp show the positions of the female- and male-specific primers, respectively. Arrows that are unlabeled are the primers to common sequences. Horizontal bar represents 1 kb. (B) RT-PCR of orb RNA in wild-type (wt) ovaries and testes and in the tumorous ovaries of Sxlmutant (SxlP) and stum homozygous females. The short arrow in A shows the position of the common primer used. This results in a 331-bp-male-specific product and a 483-bp-female-specific product. Left 4 lanes show the PCR products when the male-specific primer (Mp) is used, and the right four lanes, when a female-specific primer (Fp) is used. The position of the expected sex-specific product is marked by the appropriate symbol. In testes, the expected product is detected when the male primer is used; no product is detected when a female primer is used. The reverse is true for wild-type ovaries. All ovaries show no male-sized orb RNA. Also in all ovary samples is a band above the position of the male product that has the expected size to be derived from genomic DNA [note the small size of the intron between the male primer (Mp) and the first common exon]. (C) RT-PCR of orb RNA in snf homozygous females. Only the female form of orb RNA is detected. The long unlabeled arrow in A shows the position of the common primer. This results in a 1027-bp-male-specific product and a 1179-bp-female-specific product. The band well above the product expected from male RNA is the genomic band (note the larger second male intron). The position of relevant lambda size markers is shown between B and C.

iciency for dsx, only the male form of the protein is expressed and XX animals develop as males.

If the inducing signal from the soma passes through dsx, then at least one of the genotypes—dsx+/Df, dsx−/dsx-, dsx0/+—should affect the signal being sent to XX germ cells. If the proposed female inducing signal (GRANADINO et al. 1993) is dependent upon the female dsx protein (STEINMANN-ZWICKY 1994a,b), this signal would be disrupted in at least two of the three genotypes (dsx−/dsx- and dsx0+/Df). We should observe a corresponding alteration in the sex-specific expression of genes in the germline; Sxl and orb should be in the male not the female mode. In the case of dsx0/+, whether an effective female signal would be transmitted to the germline would depend on whether the male form of the dsx protein blocks production of the female signal and/or induces a male signal. (Note that even by other scenarios, if the signal that feminizes or masculinizes the germline is dsx-dependent, at least one of these backgrounds should disrupt the signal in XX animals and affect the sex-specific expression of Sxl and orb.)

Shown in Figure 2, A–F, is the pattern of Sxl protein expression in gonads from these dsx mutant females, while Figure 2, G and H, shows the RT-PCR analysis of Sxl and orb transcripts. Morphological analysis of gonads from the different dsx mutant combinations reveals ab-
normalities in the development of both the germline and the soma. The least severe defects are observed in dsx\(^+/^+\) where oogenic differentiation of both germline and somatic cells appears to occur. As illustrated in Figure 2A, egg-chamber-like structures containing polyplid nurse cells, an “oocyte” and surrounding follicle

![Figure 2](image-url)
cells are formed in this mutant background. Sxl protein is clearly expressed in the germ cells. In the chamber shown in Figure 2A Sxl protein is present at high levels not only in the nurse cells but also in the "oocyte." By contrast, in wild-type egg chambers of equivalent developmental stage, high levels of Sxl protein are found in the nurse cells while the oocyte has only very low levels of protein, most of which is in the oocyte nucleus (Bopp et al. 1993). In dsx-/dsx- females the germ cells still appear to develop along an oogenic pathway. On the other hand, differentiation of the somatic follicle cells is abnormal; irregularly shaped egg chamber-like structures containing polyploid "nurse cells" are formed, but these "chambers" are not properly surrounded by somatic cells (see Figure 2, E and F). As in dsx+/+, Sxl protein can be detected in the germline cells of these gonads. The most severe disruptions in gonadal development are observed in dsx+/Df females. In this genetic background, neither the somatic nor the germ cells appear to follow the oogenic differentiation pathway and the gonad resembles the testis of males. In spite of this failure in oogenic differentiation, Sxl protein is evident in the dsx mutant gonads. That the germ cells in these different dsx mutant combinations are following a female pathway is supported by the RT-PCR analysis of orb transcripts (Figure 2H). As might be expected from the oogenic differentiation of the germ cells in dsx+/+ and dsx-/dsx- gonads, orb RNA is exclusively female in both these backgrounds. orb is also exclusively female in the dsx+/Df gonads where, by morphological criteria, the germ cells do not appear to properly execute an oogenic differentiation pathway. These results indicate that XX germ cells have been directed to express both Sxl and orb in the female mode even though the dsx mutations cause the surrounding soma to develop inappropriately along an intersexual or male differentiation pathway. Thus, it would appear that Sxl is not a component of the somatic system that signals "female identity" to the germline (as defined here by the sex-specific expression of these two marker genes).

That the signaling of germline sexual identity can occur independently of dsx is further supported by the analysis of orb transcripts in XY animals that are dsx−. As shown in Figure 2H, orb transcripts in dsx− males are expressed exclusively in the male not the female mode. tra2: The tra2 gene is expressed constitutively in both sexes and encodes multiple proteins all of which contain an RNA Recognition Motif (RRM) domain. In females the tra2 gene is required in the soma, but not the germline, while in males it is not required in the soma, but has an essential function in the late stages of spermatogenesis. The best understood function of tra2 in the female soma is as a co-factor in the tra-dependent female-specific splicing of dsx. In this regulated splice, tra2 protein provides the sequence specificity, recognizing a repeated sequence motif in the dsx female-specific exon. tra2 also functions as a tra co-factor in turning
off a dsx-independent differentiation pathway in females that is responsible for male-specific behavior and the formation of the muscle-of-Lawrence (Taylor 1992). The likely target gene for tra and trn2 in this pathway is fruitless (Gailey et al. 1991). Since fruitless does not appear to be required in females, we reasoned that there could be a second dsx-independent pathway in the soma that is responsible for signaling the feminization of Sxl and orbin in the germline. Like the behavioral pathway (Gailey et al. 1991; Taylor et al. 1994), this second dsx-independent pathway might require tra2 as a co-factor to regulate the processing of RNA produced by an unknown downstream target gene ("Y" in Figure 8). To test this possibility we analyzed orbin and Sxl expression in various tra2 mutant backgrounds.

In the first experiment we examined orbin transcripts in XX animals heterozygous for a tra2 temperature-sensitive allele, tra2s2, and a null allele, tra2. At 18°C, tra2s2/tra2 XX animals differentiate essentially as females but with small or rudimentary ovaries. Consistent with the somatic phenotype, all orbin RNA is in the female mode at this temperature (Figure 3C). When the temperature is elevated to 25°C and 29°C, there is a reduction in tra2 function and XX animals develop as pseudomales. This masculinization is accompanied by a change in the pattern of orbin expression in the germline. At 25°C most of the orbin RNA is expressed in the male mode and only a small amount of female RNA is observed. The switch from female to male is even more complete at 29°C, where essentially all orbin RNA is expressed in the male mode (Figure 3C).

While the loss of tra2 function at the elevated temperature switches orbin from the female to the male mode, similar effects were not observed for Sxl. Regardless of the somatic phenotype or the temperature at which the flies were raised, Sxl protein could be detected in the germ cells of tra2s2/tra2 XX individuals. The Sxl antibody staining pattern and morphology of a gonad from a tra2 pseudomale raised at 25°C is shown in Figure 3A. As might be expected from the male pattern of expression of orbin, neither the soma nor the germ cells show evidence of oogenic differentiation. Instead the gonad resembles a small incompletely developed testis and is populated with many small undifferentiated germ cells. The distribution of Sxl protein in the tra2s2 germ cells is similar to that observed in the class A tumorous ovaries mutants. Relatively high levels of predominantly cytoplasmic Sxl protein are present in the germ cells at the apical end of the tra2s2 gonad, while there is a gradual reduction in protein in the older germ cells in more distal regions of the gonad. PCR analysis confirms that Sxl transcripts are spliced predominantly in the female mode with a small amount of male RNA (Figure 3B).

Two hypothesis could explain why the tra2s2 mutation affects orbin but not Sxl. In the first, the sexual state of orbin and Sxl are controlled by entirely independent mechanisms. In the second, residual tra2 activity of the temperature sensitive allele might be sufficient to initiate the Sxl autoregulatory loop in the germline at some point in development. Once the feedback loop is initiated, autoregulation would ensure that Sxl would be expressed predominantly in the female mode even if the tra2-dependent feminizing signal was too weak to efficiently activate the orbin female promoter.

To distinguish between these two hypotheses we examined Sxl expression in XX animals carrying the tra2 null mutation over a deficiency for the locus. These experiments indicate that the second hypothesis is likely to be correct—the sexual state of both orbin and Sxl in the germline is dependent upon tra2 activity. As illustrated in Figure 3A, this mutant combination affects Sxl expression in the germline, and in most cases we detect little or no Sxl protein in germ cells. Infrequently, in the same individual, the germ cells in one gonad have no detectable protein while the germ cells

![Figure 3](image-url)
in the other gonad have very low, but above background levels of Sxl protein. At present it is not clear why we occasionally observe gonads expressing these very low levels of Sxl protein. It is possible that this reflects some residual tra2 activity of maternal origin since we have noticed that if the tra2 deficiency allele is inherited from the mother, the low levels of Sxl protein are detected much less frequently than if the deficiency allele is received from the father.

**tra:** The tra gene is only active in females and it encodes a 22-D protein with multiple motifs. In the splicing regulation of dxx, it appears to provide an Arg/Ser “activation” domain for the tra2 protein bound to the female-specific dxx exon (HEDLEY and MANIATIS 1991; HOSHIJIMA et al. 1991; RYNER and BAKER 1991). It is likely to function in an analogous manner, using tra2 protein as a sequence-specific co-factor, in the inactivation of the dxx-independent male behavioral pathway. Since our analysis of the sexual mode of Sxl and orb expression implicates tra2 in the somatic signaling of germline sex, we expected that, like the dxx pathway and the fruitless behavioral pathway, tra would regulate the somatic signaling pathway by providing an “activation” domain to tra2 protein bound to an RNA encoded by a germline signaling gene. If this were the case, null mutations of tra should also switch the expression of both orb and Sxl from the female to male mode in the gonads of XX animals.

To test this hypothesis we first examined the expression of orb and Sxl in the gonads of XX animals homozygous for tra`. tra` is a null allele where the tra coding sequences are deleted (J. BELOTE, personal communication). To our surprise, we found that both orb and Sxl are expressed in the female mode in the germ cells of this tra mutant. This is illustrated for orb by the RT-PCR shown in Figure 4D. With the female-specific primer, we detect a fragment of the size expected for the amplification product of female orb RNA. While some bands are also observed in the male lane, none are of the correct size expected for an RT-PCR product amplified from male mRNA. Essentially equivalent results were obtained with Sxl antibody; most of the germ cells in tra` mutant gonads express cytoplasmic Sxl protein (not shown, see photo of the tra`/Df gonad in Figure 4A).

Since these findings were contrary to our expectations, we examined orb and Sxl in two other genetic backgrounds, tra`/Df and tra`/Df, that should also be null for tra activity. The deficiency chromosome removes tra as well as several surrounding genes, while tra` has a nonsense mutation at amino acid 13 (J. BELOTE, personal communication). The results for Sxl in these two mutant combinations were similar to that for the tra` homozygote. As shown for tra`/Df in Figure 4A, the gonad from XX animals deficient in tra function contains many small undifferentiated germ cells. Like some of the class A tumorous ovary mutants, these undifferentiated cells have predominantly cytoplasmic Sxl protein. When the gonads of these pseudomales were analyzed for the Sxl splicing pattern, most of the RNA appeared to be spliced in the female mode. However, significant amounts of male spliced Sxl RNA could also be detected (Figure 4C). The gradual reduction in Sxl antibody staining intensity in more distal regions of the gonad suggests that this is likely to result from a failure in autoregulation as these undifferentiated cells age (see Figure 4A).

While the female-specific activation of Sxl does not appear to be affected by any of the tra mutant combinations, this is not the case for orb. As shown in Figure 4D, RNA expressed from the male promoter can be detected in gonads from tra`/Df and tra`/Df XX animals. For tra`/Df about 10–15% of orb RNA is expressed in the male mode. A curious result was seen for the tra`/Df genotype. Depending on the source of the tra` allele (Bloomington Stock Center or J. BELOTE), we observed either about 10% or about 40% male orb RNA. Since the genotype with respect to tra is the same, this difference in relative amounts of male and female orb RNA is presumably due to genetic background.

Despite this variability, these results show that an XX germline can express germline genes in the female mode in the complete absence of tra. This suggests that while tra may contribute to or augment the feminization of the germline it can not be the sole source of the somatic feminizing activity that is tra2-dependent.

**Sxl:** The findings described above indicate that null mutations in tra have only a small effect on the female-specific expression of Sxl and orb in the germline. Since mutations in tra2 can completely block the female-specific expression of these germline markers, this would imply that there must be some other gene that utilizes tra2 as a co-factor to effect the feminizing signal from the soma. This inference is consistent with our analysis of orb expression in the gonads of Sxl/M1,ns7/ Sxl[M1,ns7,M1] chromosomal females. Critical for our analysis, this mutant combination differentially affects Sxl function in the soma. Approximately 50% of the transheterozygous females survive so that this combination of Sxl alleles appears to retain at least some ability to control the dosage compensation pathway. On the other hand, this allele combination is apparently unable to regulate tra splicing (NAGOSHI et al. 1988) and Sxl[M1,ns7]/Sxl[M1,ns7,M1] animals have a very male-like morphology, including their body size. In spite of the fact that this genotype severely disrupts female tra regulation, it has no apparent effect on the feminization of the germline marker gene orb (Figure 5B). In addition, female-specific expression of Sxl is activated in the germline (Figure 5, A and B). These findings would also suggest that tra is not essential to send a feminizing signal to the germline.

**Germline sexual identity is set in the embryo:** The
results described above indicate that a tra2-dependent somatic signaling pathway is required for the female-specific expression of Sxl and orb in the germline. To better understand how the Sxl-orb pathway functions it is important to ascertain when in development the tra2-dependent feminization signal is first communicated to the germline. Unfortunately, we do not have the tools required to answer this question with a great deal of precision. The only available molecular marker for germline sex early in development is the Sxl gene; however, it is unlikely to be the first sex-specific gene expressed in XX germ cells, and its activation need not correspond to the time when the tra2-dependent feminization signal is first communicated. On the other hand, the timing of Sxl activation can be used to place an upper limit on the stage in development when this germline sex determination pathway must first operate.

Since morphological differences between male and female gonads are already evident by the first instar larva (Kerkis 1931), the pathway, and Sxl expression, should be initiated at an earlier stage—presumably at some point during embryogenesis. Hence, we examined Sxl expression in the germ cells of wild-type embryos at different stages of development. To identify the germ cells in the small embryonic gonads, we counter stained with vasa antibodies. As noted above, activation of Sxl in the germline is uncoupled from the soma and in early embryos the progenitor cells of the germline, the pole cells, do not express Sxl protein (Bopp et al. 1991). Moreover, the pole cells lack Sxl protein for as long as they are visible on the exterior of the embryo. Staining of older embryos indicates that Sxl remains off in female germ cells well beyond the time that pole cells are internalized and the gonad coalesces. Sxl antibody staining in XX germ cells can first be reliably detected in 16–20 hr embryo collections. At this time, female embryos (i.e., those that express Sxl protein in the soma) had Sxl protein in their germ cells (as judged by vasa staining; see embryo in Figure 6). By contrast, none of the male embryos (i.e., those lacking Sxl protein in somatic cells) at this developmental stage had Sxl protein-positive germ cells. As was the case in the larval gonad (see Bopp et al. 1993), Sxl protein in the XX embryonic germ cells is predominantly cytoplasmic. These results indicate that germline sexual development is initiated at least as early as 16–20 hrs of embryogenesis.

**Time frame of the signal:** If the regulatory strategies used in the Sxl-orb germline sex determination pathway are analogous to those employed in the soma, then the tra2-dependent signal should be required only transiently at the time sexual identity is initially selected. Thereafter, germline sexual identity should be controlled by a maintenance mechanism, that functions autonomously in the germ cells independently of the

![Figure 4](image-url)
FIGURE 5.—Germline expression of Sxl and orb in Sxl

initiating signal, perhaps much like the Sxl autoregulatory feedback loop.

If this model for initiation and maintenance is correct, then the tra-dependent somatic signal should become dispensable either just before or around the time when we first detect Sxl protein in female germ cells. To test this model we took advantage of the temperature sensitive tra allele. tra embryos were collected and incubated for different times at the permissive temperature of 18° and then shifted to the nonpermissive temperature of 29° until the adult stage. Control animals were raised continuously at either the permissive or nonpermissive temperature. We then examined the pattern of orb expression in the germline of the resulting XX adults. (Only orb was assayed because the autoregulatory activity of Sxl would complicate interpretation of the temperature shift data.) We expected to find that tra embryos would fail to properly signal female identity when shifted to the nonpermissive temperature prior to pathway initiation, i.e., roughly midway through embryogenesis. Indeed, as shown in Figure 7 this expectation is correct; orb transcripts are expressed in the “male mode” when the temperature shift occurs prior to the time that we first detect Sxl protein in the germline. A different result should be obtained when the temperature shift occurs after pathway initiation; the loss of tra activity should have no effect on sexual identity and orb should be expressed in the “female mode.” Surprisingly this is not the case; orb transcripts are expressed in the “male mode” not only in 24-hr embryos but also through the first and into the second instar larval stage. In fact, the XX tra animals remain sensitive to the temperature shift until midway through the third instar larval stage.

These findings are inconsistent with the model of a transient germline sex determination signal. They suggest that the tra-dependent signaling system is required continuously, at least until the mid-third instar larval stage, to faithfully maintain a commitment to the female pathway. If this is correct, then the soma must be capable of signaling and the germline capable of responding to the signal as late as this larval stage. To test this, tra embryos were collected at 29° and then shifted to 18° at different times. As can be seen in Figure 7, the tra-dependent signal can initiate female-specific orb expression in XX germ cells not only in larval stages, but as late as the early pupal stage.

DISCUSSION

Sxl is not the master regulatory gene in the germline: The results presented here, together with the recent studies of Bae et al. (1994) indicate that the Sxl gene can not function as the master regulatory switch in germline sex determination. Two lines of evidence
support this assertion. The first comes from an analysis of germline sexual identity in mutant backgrounds in which Sxl is not properly autoregulated and XX germ cells in the adult female gonad have no detectable Sxl protein. snf' is a mutation in a gene encoding a generic splicing factor associated with U1 snRNP (Flickinger and Salz 1994), while otu' is a mutation in a gene encoding a germline-specific cytoplasmic protein of unknown function (Steinhauer and Kalfayan 1999). Both mutations have sex-specific effects in the germline; when homozygous in females, they cause an early arrest of oogenesis but they have no apparent effect on gametogenesis in males. The early oogenic arrest in snf' is likely to be the direct consequence of a failure in Sxl autoregulation (and the lack of Sxl protein) since gain-of-function mutations in Sxl, such as Sxl', which constitutively express Sxl protein suppress the oogenesis defect. In contrast, otu' appears to have more pleiotropic effects, and the defects in oogenesis in this mutant appear to involve processes beyond simply failing to produce Sxl protein. In spite of the fact that XX germ cells homoyzous for either mutation do not express detectable amounts of Sxl protein, the sexual identity of the mutant germ cells, as judged by the pattern of orb expression, is female not male (see also Bae et al. 1994).

The second line of evidence comes from the reverse condition which occurs in the germline of tra''/tra2 XX animals grown under permissive conditions. Even though Sxl protein is present in these germ cells, orb is expressed in the male not the female mode. Thus, Sxl expression is not in itself sufficient to induce feminization of the germ cells. Consistent with the view that Sxl does not function as the master switch of sexual identity in the germline, the constitutively active Sxl mutations, Sxl' and Sxl', do not feminize XY pole cells but allow normal gametogenesis in a male soma (Steinmann-Zwicky et al. 1989; Steinmann-Zwicky 1993). Similarly, when Sxl protein is ectopically expressed in the male germline from a cDNA construct, it does not impose female development and the transgenic males are fertile (D. Bopp, unpublished data).

If Sxl is not the master switch in germline sex determination, why is it required in XX germ cells for normal oogenesis? It could function in one of several subordinate germline sex differentiation pathways, perhaps eliciting the female-specific expression of a small group of target genes. Consistent with this possibility is the fact that the expression of several sex-specific germline enhancer trap lines and some genes (e.g., Stellate) appears to depend upon Sxl (see Wei et al. 1994). An alternative view is that the functions of Sxl during oogenesis are not at all related to the system controlling germline sexual identity but rather Sxl is required for the proper elaboration of developmental processes that are unique to oogenesis much like other genes, such as bam, fu, orb, egal, Bic-D, etc., that function in the differentiation of the female germline. This view would be consistent with the germline phenotypes observed in genetic backgrounds deficient in Sxl activity (Schüpbach 1985; Bopp et al. 1993).

Model for sex determination in the germline: How is sex chosen and remembered in the germline? Pole cell transplantation experiments have suggested that autonomous and nonautonomous components are important in germline sex determination. We will discuss the nonautonomous component, the somatic communication pathway, first as our findings bear most directly on its role in the sex determination process.

Neither dsx nor tra are essential for the somatic communication pathway that controls Sxl and orb expression in the germline: Both Oliver et al. (1993) and Steinmann-Zwicky (1994a,b) have argued that the somatic signal for female sexual identity in the germline is mediated by the tra → dsx somatic sexual differentiation pathway. In the
former case, this conclusion was based on the detection of male Sxl RNA in adult gonads of tra and dsx mutants, while in that latter, it was based (in part) on the altered size of the gonad in mutant larvae. Our results are most clear cut for dsx. Like OLIVER et al. (1993), we have found low levels of male-spliced Sxl RNA in gonads of various dsx mutant combinations. However, this male RNA does not appear to arise from a failure in initiating female-specific expression of Sxl in the germline. In fact, high levels of Sxl protein are evident, particularly in germ cells at the apical end of the mutant gonads. A more likely explanation is that the male Sxl RNA arises from an occasional failure in Sxl autoregulation as the germ cells proceed down the gonad and attempt to differentiate. Similar failures in Sxl autoregulation are evident in other mutants such as bam or fu, that perturb the early steps in the formation of a 16-cell cyst and disrupt the redistribution of cytoplasmic Sxl protein. These observations indicate that dsx is not required to activate Sxl expression in the female mode in XX germ cells. That dsx is not essential for the feminization of XX germ cells is supported by our analysis of orb expression. We found that orb is expressed in the female mode in XX germ cells not only in the absence of dsx activity, but also when dsx is exclusively male. In addition, since we find that orb is in the male mode in the germ cells of dsx− XY males, it also appears that dsx activity is not required for the masculinization of XY germ cells.

While dsx does not seem to play a role in the Sxl- orb germ line sex determination pathway, it is clearly required in the female soma for normal oogenesis and fertility. The phenotypic effects of various dsx mutant combinations on gonadal development may be instructive in this regard. The least severe disruptions in oogenic differentiation are evident under conditions where both the male and female forms of the dsx protein are expressed (dsx+/dsP). In these gonads we observe nearly normal looking egg chambers consisting of an oocyte at the posterior, nurse cells at the anterior, and a surrounding array of somatic follicle cells. However, some aspects of oocyte-nurse polarity seem to be perturbed since we observe high levels of Sxl protein in the oocyte. This is presumably a consequence of a failure in some aspect of germline-soma communication. In addition, vitellogenesis which normally initiates around stage 7 or 8 does not occur. In the absence of dsx activity (dsx+/ds−), the germ cells also initiate oogenic differentiation and appear to form 16 cell cysts; however, these egg chambers do not develop properly. Though polyploid nurse cells are present, we can not detect an oocyte at the posterior end, and somatic cells do not properly envelop the cyst. This finding would suggest that the repositioning of the oocyte to the posterior of the 16-cell cyst may require a dsx-dependent signal from soma to germline (see also LANTZ et al. 1994). Finally, in a soma which expresses only the male dsx protein (dsx+/Df) there is no apparent oogenic differentiation, and only small undifferentiated germ cells are observed. This finding would indicate that in a soma expressing only the male form of the dsx protein, the germline cystoblasts are unable to properly execute the pathway that ultimately generates a 16-cell cyst (see Figure 8).

The malfunctioning of the somatic cells in the adult dsx+ gonad may be relevant to the findings of STEINMANN-ZWICKY (1994a) that the gonads of dsx− female larvae are more male-like in size and the gonads of dsx− male larvae are more female-like in size. Gonadal size is related to germ cell proliferation and, if both males and females modulate the rate of germ cell proliferation through dsx, then this signaling process would be disrupted in both sexes by the absence of dsx activity.

As might be expected from the fact that dsx is expressed in the male mode in the absence of tra function, the gonad of tra− females resembles that observed in dsx+/Df females. There is no evidence of oogenic differentiation and the male-like somatic gonad is populated by clusters of small undifferentiated germ cells. Like OLIVER et al. (1993) we detect male-spliced Sxl RNA in the tra− mutant gonads; however, the pattern of Sxl protein accumulation in these germ cells again indicates that this is probably due to an occasional failure in autoregulation, rather than a failure in germline sex determination. The presence of predominately female orb RNA in genetic backgrounds null for tra function would also indicate that tra is not essential for communicating “female identity” to the germline. While tra does not appear to be essential for somatic signaling of germline sexual identity, we can not exclude the possibility that tra may enhance this communication or may have a partially redundant function in the communication pathway. This ambiguity about the role of tra comes from the fact that we detect some male orb RNA in two of three tra null mutant backgrounds. The relationship of tra to the somatic communication pathway will be discussed further below.

The somatic communication pathway requires tra2: Like tra and dsx, tra2 is required in the female soma, but not the germline, for normal oogenesis. However, while neither tra or dsx seem to play a critical role in signaling the feminization of Sxl and orb, tra2 is absolutely essential; in the absence of tra2 function, both these marker genes are expressed in the male not the female mode. Since tra2 is expressed constitutively in both sexes, it is unlikely that a somatic feminization signal could originate from tra2. Rather, one must suppose that as in the tra → dsx sexual differentiation pathway, tra2 functions as a co-factor for some other sex-specific gene, “X.” This is shown in the model diagrammed in Figure 8. In this model we have placed Sxl upstream of X, and it
activates the somatic signaling pathway by directing the female-specific expression of X. (Though it seems reasonable to assume that Sxl regulates X, it should be emphasized that we have no direct evidence for such a somatic Sxl function.) The female X protein, together with tra2 protein, would then regulate the activity of a downstream target gene, "Y." We presume that the regulation of Y is post-transcriptional and involves interactions between X and tra2 that are quite similar to those between tra and tra2 proteins in the regulation of dsx. If tra and X proteins interact with the same domain(s) in the tra2 protein, it is possible that these two genes are partially redundant in the somatic communication pathway. A redundancy of this sort could explain the weak and variable effects of tra mutants on the sexual state of the orb gene. It would also be consistent with the observation that ectopic expression of tra protein from a constitutive hsp83:tra (female) cDNA transgene can partially feminize the germline of chromosomal males, activating both Sxl and orb in the somatic mode. This feminization of the male germline appears to be a consequence of somatic tra expression from the transgene and, like the female signal described here, requires the activity of tra2 (J. I. HORABIN, D. BOPP, J. WATERBURY and P. SCHEL, unpublished observations; see also NAGOSHI et al. 1995).

Since the default state of the signaling pathway appears to be male, we presume that when the female form of Y is expressed it functions to send a feminization signal from the soma to the germline (and not to block the expression of a masculinizing signal). If Y is similar to dsx, which is a transcription factor, there may be several steps between expression of the female Y protein and the actual signal to the germline.

What is the target for the somatic signaling system? The results presented here and in BAЕ et al. (1994) indicate that Sxl can not be the key target of the somatic signaling system. Some other gene (or genes), "Z" in Figure 8, is activated when germ cells receive this signal and it directs the female-specific expression of downstream targets such as Sxl and orb. From pole cell transplantation experiments, it is clear that XX germ cells are much more capable of responding to the feminization signal than are XY germ cells (STEINMANN-ZWICKY 1994a). This could be due to the operation of an autonomous X/A counting system in germ cells that is analogous to (but different from) that found in the soma. Alternatively, it may simply reflect the dose effects of one or several X-linked genes that are the target(s) for the somatic signal. In this view, the presence of two copies of the X-linked genes would enhance the response to the somatic feminization signal. In either case, a good candidate for gene Z, the germline target of the somatic feminization signal, is ovo. Expression of ovo in the germline appears to be dependent on the X chromosome dose (OLIVER et al. 1994) and null mutations have been reported to cause the early death of female germ cells (OLIVER et al. 1987, 1994).

Initiation and maintenance: Our results indicate that the regulatory strategies used for initiation and mainte-
nance in the Sxl-orb germline sex determination pathway are quite different from those employed in the soma. In the soma, an autonomous and transient signal, the X/A ratio, is used in the initial choice of female sexual identity early in embryogenesis. This signal sets in motion an autoregulatory feedback loop which then serves to maintain the determined state in each cell for the rest of the life cycle. The choice of sexual identity in the Sxl-orb germline pathway also depends upon a signal. However, the signal is not autonomous but appears to require the soma to inform the germline of its appropriate sexual identity. Additionally, the somatic signal does not function transiently. Instead, it is required over an extended period of time to commit the germ cells to the female state and thus appears to function not only in the setting but also in the memory of sexual identity. Of course, if the activity of the somatic signaling pathway is controlled by Sxl (as diagrammed in Figure 8), it is the Sxl autoregulatory feedback loop in the soma that ultimately functions to initiate and maintain female identity in the germline.

In our temperature-shift experiments, the timing of the signal defined by up-shifts and down-shifts did not coincide. The former indicated that the somatic signal is required through the mid-third instar larval stage, while the latter showed that the signaling system can feminize the germline as late as the pupal stage. Whether this discrepancy is meaningful is not clear. In the up-shift experiment, it is conceivable that the somatic signal may continue to function for some time after the shift to the nonpermissive temperature. For instance, it may take several days for active tra2 protein to decay after the up-shift (cf. Belote et al. 1985). Additionally, the downstream proteins in the pathway (e.g., Y) may not turnover immediately after the loss of tra2 activity. If this is the case, then the upshifts will underestimate the latest time in development when the signaling pathway is functional. This would suggest that the somatic signal may not only function but may also be required into the pupal stage.

From the time the Sxl-orb germline sex determination pathway is first activated midway through embryogenesis until perhaps as late as the pupal stage, the germline appears to be incapable of autonomously remembering its sexual identity. The obvious question then is why does the somatic signal become dispensable? While it is plausible that an autonomous memory system becomes activated in the germline at or just before the pupal stage, an alternative, and equally attractive possibility, is that the end of the signaling period marks the formation of oogenic stem cells which irreversibly commits the fate of germ cells to the “female” pathway—oogenesis. If this is the case, there may be no key sex determination gene (like Sxl in the soma) in germ cells that controls all aspects of female germline development. Instead, the soma may function as the “master regulator” for the germline, continuously transmitting cues which activate different female-specific differentiation pathways.

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