Sexual Backtalk with Evolutionary Implications: Stimulation of the Drosophila Sex-determination Gene *Sex-lethal* by its Target *transformer*

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Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession no.s EU670259, EU636097, and EU636098.
RUNNING TITLE: Effect of tra on Sxl Autoregulation

KEYWORDS: sex-determination, Drosophila

Sex-lethal positive autoregulation

transformer maternal effect

ovary development, Drosophila

evolution of sex determination

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ABSTRACT

We describe a surprising new regulatory relationship between two key genes of the Drosophila sex-determination gene hierarchy, *Sex-lethal (Sxl)* and *transformer (tra)*. A positive autoregulatory feedback loop for *Sxl* was known to maintain somatic cell female identity by producing SXL-F protein to continually instruct the target gene *transformer (tra)* to make its feminizing product, TRA-F. We discovered the reciprocal regulatory effect by studying genetically sensitized females: TRA-F from either maternal or zygotic *tra* expression stimulates *Sxl* positive autoregulation. We found female-specific *tra* mRNA in eggs as predicted by this *tra* maternal effect, but not predicted by the prevailing view that *tra* has no germline function. TRA-F stimulation of *Sxl* seems to be direct at some point, since *Sxl* harbors highly conserved predicted TRA-F binding sites. Nevertheless, TRA-F stimulation of *Sxl* autoregulation in the gonadal soma also appears to have a cell-nonautonomous aspect, unprecedented for somatic *Sxl* regulation. This *tra-Sxl* retrograde regulatory circuit has evolutionary implications. In some Diptera, *tra* occupies *Sxl*’s position as the gene that epigenetically maintains female identity through direct positive feedback on pre-mRNA splicing. The *tra*-mediated *Sxl* feedback in Drosophila may be a vestige of regulatory redundancy that facilitated the evolutionary transition from *tra* to *Sxl* as master sex switch.
INTRODUCTION

Diplo-X somatic cells of *D. melanogaster* maintain their female sexual identity epigenetically through the operation of a direct positive feedback loop on pre-mRNA splicing for transcripts from the master feminizing switch gene *Sex-lethal (Sxl)* (Bell *et al.* 1991). The female-specific SXL-F protein thereby generated acts on various regulatory gene targets, including the feminizing switch gene *transformer (tra)* to elicit female differentiation. In some fly species that do not use X-chromosome dose to determine sex (Pane *et al.* 2002; Lagos *et al.* 2007), and possibly even in the honeybee (Hasselmann *et al.* 2008), *tra* occupies the position of *Sxl* as the master developmental switch gene that epigenetically maintains the female sexual fate commitment by direct positive autoregulation of its own pre-mRNA splicing. In those species, *Sxl* itself does not appear to be sex-specifically regulated and its function is unknown. Thus an important evolutionary question is how *Sxl* was recruited to the Drosophila sex-determination pathway. Here we describe functional and structural evidence for an unanticipated additional circuit driving *Sxl* positive autoregulation that seems relevant to this evolutionary question.

We discovered this surprising regulatory circuit for *Sxl* while investigating the developmental basis for a unique female-sterile mutant phenotype encountered when *Sxl* autoregulation was discovered: *Sxl* mutant females' ovaries seemed to disappear during metamorphosis (Cline 1984). We show here that these ovaries disintegrate due to their ambiguous sexual phenotype, rather than from an upset in the vital process of X-chromosome dosage compensation, which *Sxl* also controls.
The functional relationship between the two promoters of *Sxl* and the way their products are affected by particular *Sxl* mutations are central to this female-sterile phenotype. For Drosophila, diplo-X somatic cells become female while haplo-X somatic cells become male because the level of regulatory proteins generated from a double dose of X-chromosome signal element genes (XSEs) is sufficient to activate the "sexual pathway establishment" promoter, *Sxl*<sub>Pe</sub>, while the level generated from just a single dose of XSEs is not (reviewed in Cline and Meyer 1996; see also Erickson and Quintero 2007). Although *Sxl* is required throughout female development to control sexual differentiation and dosage compensation, *Sxl*<sub>Pe</sub> is only sensitive to the level of XSE proteins for a 45-minute period that ends soon after fertilization as the embryo transitions from relying predominantly on maternal gene products to relying on zygotic gene products instead. As *Sxl*<sub>Pe</sub> shuts off, the "sexual pathway maintenance" promoter, *Sxl*<sub>Pm</sub>, becomes active. *Sxl*<sub>Pm</sub> remains active thereafter to provide females with the feminizing SXL-F protein they need throughout development for appropriate sexual differentiation and dosage compensation.

*Sxl*<sub>Pm</sub> also becomes active in males, but they produce no feminizing SXL-F protein because transcripts from *Sxl*<sub>Pm</sub>, unlike those from *Sxl*<sub>Pe</sub>, are only productively spliced into mRNAs that encode full-length SXL-F protein if SXL-F protein is already present. SXL-F is an RNA-binding protein that acts directly on its own pre-mRNA to prevent incorporation of a male-specific exon that would otherwise abort translation early and thereby keep *Sxl* functionally off. Thus the transient early burst of transcription from *Sxl*<sub>Pe</sub> in diplo-X embryos provides a pulse of SXL-F protein that triggers engagement of a positive feedback loop for the productive splicing of *Sxl*<sub>Pm</sub> transcripts. This feedback loop then maintains the female ("ON") state for *Sxl*.
epigenetically thereafter. Because haplo-X embryos lack this triggering pulse of SXL-F, they do not engage the Sxl feedback loop. Instead, they include the male-specific exon in their Sxl mRNA by default and Sxl remains off. SXL-F continually promotes the productive pre-mRNA splicing of transcripts from the downstream target gene tra, thereby ensuring a continual supply of that gene's actively feminizing RNA-binding protein, TRA-F. TRA-F in turn imposes female-specific pre-mRNA splicing on its gene targets.

The fly's gonad was known to be peculiar with respect to sex determination (reviewed in Oliver 2002) ever since the discovery that tra could be eliminated from diplo-X germ cells without apparent effect (Marsh and Wieschaus 1978). This observation led to the conclusion that tra functioning was limited to somatic cells. In somatic cells, tra seemed only to be a slave to Sxl, continually depending on female-specific SXL-F protein to elicit production of its own feminizing protein product (Nagoshi et al. 1988; Sosnowski et al. 1989). Here we show that female-specific tra expression is not limited to somatic cells, and that tra expression in the mother’s germ line, or in the very young embryo itself, can stimulate Sxl positive feedback in the female gonadal soma while having no adverse affect on the sexual development of males.

Although Sxl regulation in the Drosophila soma was believed to be strictly cell-autonomous, its regulation in germ cells was known to have a cell-nonautonomous component (Nöthiger et al. 1989). Responding to Sxl in the gonadal soma, somatic tra triggers female-specific splicing of pre-mRNA from Sxl itself in the neighboring diplo-X germ cells (Bopp et al. 1993; Oliver et al. 1993; Steinmann-Zwicky 1994; Waterbury et al. 2000; Janzer and Steinmann-Zwicky 2001; Evans and Cline 2007). As in somatic cells, in germ cells SXL-F is necessary for
female development (Schüpbach 1985), but unlike in somatic cells, it is not sufficient (Hager and Cline 1997).

The female-sterile phenotype that is the focus of the present study was first seen in a situation where it was inferred that the \textit{Sxl} positive feedback loop had become engaged in nearly all somatic cells except those of the gonad (Cline 1984). The disappearance of those \textit{Sxl} mutant ovaries during metamorphosis was surprising in two respects. First, it was a phenotype that had not been previously reported, much less attributed to mutations affecting sex determination or dosage compensation. While it was known that ovaries partially or completely masculinized by mutations in any of the four somatic sex-determination genes downstream of \textit{Sxl} (\textit{tra}, \textit{transformer-2}, \textit{doublesex}, and \textit{intersex}) were abnormal, they remained intact (reviewed by Laugé 1980) -- as did ovaries suffering X-chromosome dosage compensation upsets (Kelly \textit{et al.} 1995). Second, only the gonad appeared to be defective in these adult females, suggesting that there might be something different about the gonadal soma that made it a particularly difficult somatic tissue type in which to trigger full engagement of the \textit{Sxl} positive feedback loop.

This unusual female-sterile phenotype is found in situations where the normal XSE-based mechanism for initiating female-specific expression of \textit{Sxl} has been impaired, but \textit{Sxl}$^+$ alleles are activated instead by the products of the double-mutant \textit{Sxl} allele, \textit{Sxl}$^{P,MI}$ (Fig. 1A) (Cline 1984; Cline 1986; Granadino \textit{et al.} 1991). The \textit{Sxl}$^{M1}$ lesion in \textit{Sxl}$^{P,MI}$ forces female splicing of some \textit{Sxl}$^p_m$ transcript (Cline 1984; Bernstein and Cline 1994). The mutant SXL-F7 protein thereby produced can activate an \textit{Sxl}$^+$ allele in \textit{trans}, but far less effectively than wildtype protein. Furthermore, since SXL-F7 cannot direct female-specific expression of \textit{tra} (Cline 1984;
Sosnowski et al. 1989), any feminization of somatic tissues that occurs when Sxl\(^{f7,M1}\) is in the presence of a second Sxl allele must result from the productive splicing of Sxl transcripts from that allele. Although Sxl\(^{f7,M1}\) lacks somatic feminizing activity, its germline feminizing functions are intact, as first shown by the observation that homozygous Sxl\(^{f7,M1}\) germline clones produce functional eggs (Cline 1983a) -- another indication of profound differences between germline and somatic sex determination.

Analysis of this female-sterile phenotype was facilitated by our identification of a purely zygotic mutant genotype in which ovarian disintegration occurred with high penetrance, yet female viability remained high. One key feature of this genotype is an increased dose of sans-fille, a gene that enhances the ability of Sxl\(^{f7,M1}\) to activate Sxl alleles in trans (Cline et al. 1999). The other key feature is an "initiation-defective" mutant allele Sxl\(^{f9}\) whose lesion disrupts the ability of Sxl to respond to X chromosome dose in the soma without disrupting any other Sxl function (Maine et al. 1985). The Sxl\(^{f9}\) lesion (Fig. 1A) is a nonsense mutation in the exon that is unique to mRNAs derived from Sxl\(_{Pe}\) in response to higher X-chromosome dose (see Materials and Methods). Because mRNAs derived from Sxl\(^{f9}_p\) are therefore wildtype, if the products of an allele like Sxl\(^{f7,M1}\) acting in trans can induce full engagement of the Sxl\(^{f9}\) feedback loop, the subsequent functioning of Sxl\(^{f9}\) will be indistinguishable from that of Sxl\(^{+}\) in that cell (see Fig. 1). But if instead the level of SXL-F protein from Sxl\(^{f9}\) never reaches that self-sustaining threshold -- as seems to happen particularly frequently in somatic cells of the mutant gonad -- constitutive SXL-F7 from Sxl\(^{f7,M1}\) will nevertheless allow Sxl\(^{f9}\) to maintain a sub-threshold level of wildtype SXL-F in that cell throughout development. Such cells are potentially intersexual, and they are sensitized to even small enhancements of Sxl positive autoregulation that would raise them above
threshold. The subtle enhancement by TRA-F was revealed in this sensitized genotype and led us to highly conserved predicted binding sites for TRA-F in Sxl that had escaped notice for decades.
MATERIALS AND METHODS

**Drosophila culture and genetics:** Flies were raised at 25° in uncrowded conditions on a standard cornmeal, yeast, sucrose, and molasses medium. Markers, balancers, and transgenes are described at http://flybase.bio.indiana.edu except as follows: *Df(tud)* and *tud*°45-6 were a gift from R. Lehmann; *P{U2af50-traF w+mW.hs}2B* allows one to maintain *tra* homozygous stocks (Evans and Cline 2007). The *Binsinscy, y w snx2B let P{w+mC,hs-hid}* balancer carries a recessive lethal and the dominant, temperature-sensitive lethal *hs-hid* transgene described at http://flybase.bio.indiana.edu. The balancer's dominant lethality is tight and remarkably rapid after a 1 h 37° treatment of embryos or first instar larvae.

**The Sxl\(^{P9}\) molecular lesion:** Genetic fine-structure mapping (Bernstein and Cline 1994) incorrectly located *Sxl\(^{P9}\). The strategy was based on intragenic complementation between *Sxl\(^{P9}\) and *Sxl\(^{M1,f3}\), but we subsequently discovered that such females have an unusual propensity for nonhomologous recombination. Consequently, what had seemed to be wildtype intragenic recombinants were later found to be cytologically subtle tandem duplications with both parental alleles in cis. DNA sequencing ultimately showed *Sxl\(^{P9}\) to be an A>T substitution in exon E1 at nucleotide 5,362 (numbered from the *Sxl\(^{Pm}\) start site) (Fig. 1A).

**rtPCR:** Tissues were homogenized in Trizol (Invitrogen), and RNA was isolated according to the manufacturer’s protocol. 4ng of total RNA were reverse-transcribed using random primers. The following primer pairs were used for PCR amplification of cDNAs:

\(tra\) exon 1 5’-CCGATGAAAATGGATGCCG-3’
*tra* exon 2 5’-TGCTCTCTCTGATGGACGACTGTG-3’

*dxs*<sup>+</sup> exon 2 5’-TGGTAGGTCATCGGGAACATCG-3’

*dxs*<sup>+</sup> male-specific exon 5’-GCCATCGGGGTGTAATGTTGTAG-3’

*dxs*<sup>-</sup> exon 3 5’-CGCAGACGCAAACATTGAAG-3’

*dxs*<sup>-</sup> female-specific exon 5’-TCGGGGCAAAGTAGTATTCGTTAC-3’

**Immunohistochemistry:** Embryos were fixed, stained, and photographed as described by Bernstein *et al.* 1995. Ovaries were dissected from females 1-3 days after eclosion. Fixation and washes were carried out as described previously (Hager and Cline 1997). Antibodies were used at the following concentrations: mouse anti-SXL (Bernstein *et al.* 1995) at 1:2000; polyclonal rabbit anti-GFP (Molecular probes) at 1:1000; mouse anti-Eya 10H6 (Bonini *et al.* 1993) at 1:25; mouse anti-En 4D9 (Patel *et al.* 1989) at 1:2; goat anti-mouse-Alexa546 (Molecular Probes) at 1:500 for SXL, and 1:400 for all other primaries; goat anti-rabbit-Alexa488 (Molecular probes) at 1:400. Images were taken on a Leica A OBS confocal microscope and analyzed with ImageJ software.

**Light microscopy:** Live gonads were dissected in Ephrussi and Beadle Ringer’s solution (128mM NaCL, 4.7mM KCl), and viewed by phase contrast or Nomarski optics.

**Generation of labeled FLP-out clones in ovaries:** Female larvae of the genotype described were heat-shocked for five minutes in a 37° water bath 3-27 hours after egg laying. Adults were dissected 3 days after eclosion, and live ovaries were scored for GFP expression.
using a Zeiss Axioskop. An ovariole was scored as containing a somatic clone if any or all follicle cells expressed GFP.

**Cloning of Sepsid tra:** Advantage was taken of synteny between tra and *l(3)73Ah* (Pane et al. 2002; Lagos et al. 2007). We PCR–amplified *l(3)73Ah* using the primers (forward) 5’-CAAGAGTTGCTGGTGAAGCAC-3’ and (reverse) 5’-GTACTTTTCGATGCCGTTGAGG-3’. Having sequenced *S. cynipsea l(3)73Ah*, we used that information to design probes (5’-ACACGTTGACCTGCTCGTCTCGAGTC-3’ and 5’-GAATCCCGACTTCCATCGACTCGAC-3’) with which we screened a *S. synipsea* fosmid library (generously provided by M. B. Eisen) by the protocol of Han et al. (2000). We then directly sequenced the fosmid containing *l(3)73Ah* to obtain *S. cynipsea tra* genomic sequence. Partial *S. neoscynipsea tra* sequence was obtained by sequencing the fragment PCR-amplified by the following primers. Forward: 5’-AAATGCCTGTACTCACC CGAGAG -3’ Reverse: 5’-TGGCATGAGTAACGTCAGCACG -3’.
RESULTS

**High viability Sxl mutant females whose ovaries disappear:**  $Sxlf^{7, M1} / Sxlf^{9}$ mutant females carrying a transgenic copy of *sans fille* + ($P\{snf^+\}$) to boost viability were the focus of this study. Although their viability and the penetrance of their ovarian defects were affected somewhat by genetic background, temperature, and the parent of origin of the extra $snf^+$ allele, their viability was always at least 40% and most females lacked both ovaries.

The molecular basis for the phenotype of these females can be inferred from what is known about *Sxl* positive autoregulation, these two mutant *Sxl* alleles, and the effects of $snf^+$ dose. Above a particular threshold level of SXL-F protein activity, a cell will ramp up to full engagement of the *Sxl* positive feedback loop—the normal female state in which all $Sxl_{Pm}$ transcripts are processed into mRNAs that lack the translation-terminating, male-specific exon. These mRNAs encode full-length SXL-F proteins that direct female somatic development and impose a rate of X-chromosome dosage compensation appropriate for diplo-X somatic cells. Below that triggering threshold, cells will instead damp down to the pre-mRNA splicing state characteristic of males, in which all $Sxl_{Pm}$ transcripts are processed by default into mRNAs that only encode truncated SXL proteins. These truncated proteins lack all somatic feminizing activity and allow a level of dosage compensation that is appropriate only for haplo-X cells.

The fact that there are generally only two stable *Sxl* expression states -- fully ON and fully OFF -- is dramatically illustrated in Fig. 1C by $Sxlf^9$ mutant female embryos. Their salt-and-pepper pattern of SXL-F expression reflects genetically identical somatic cells having settled
stochastically into one or the other of the two stable Sxl pre-mRNA splicing modes as a consequence of the pulse of Sxl\textsubscript{Pe}-derived activity they generated earlier in response to X-chromosome dose having been abnormally close to the threshold at which feedback loop engagement is triggered. Sxl\textsuperscript{9} is a recessive, female-specific lethal allele impaired in its ability to respond to the primary sex-determination signal in the soma, but otherwise wildtype with respect to all Sxl functions (Maine \textit{et al.} 1985). As diagrammed in Fig. 1A, Sxl\textsuperscript{9} carries a nonsense point mutation in exon E1 (see Materials and Methods). Since exon E1 is only included in Sxl\textsubscript{Pe} mRNAs (Keyes \textit{et al.} 1992), this leaky nonsense mutation reduces the initial pulse of SXL-F that normally triggers engagement of the Sxl\textsubscript{Pm} transcript splicing feedback loop in diplo-X embryos. On the other hand, any Sxl\textsubscript{Pm} transcripts that are spliced in the female mode are fully wildtype. Hence, any Sxl\textsuperscript{9} diplo-X somatic cell in which the level of SXL-F protein reaches the threshold for engaging the Sxl\textsubscript{Pm} splicing feedback loop will ramp up to full female splicing. From that time forward, the Sxl mutant cell will be indistinguishable from a wild-type diplo-X cell with respect to Sxl functions. Conversely, any Sxl\textsuperscript{9} cell that fails to reach that threshold will damp down to the male splicing pattern and become indistinguishable from a Sxl diplo-X cell instead. The mutant embryo in Fig. 1C is at stage 12, by which time cells have fully ramped up or damped down with respect to immunostaining of protein generated from Sxl\textsubscript{Pm}-derived mRNAs. Contrast this mosaic pattern to the uniformly dark immunostaining in Fig. 1B of a Sxl\textsuperscript{9}/Sxl\textsuperscript{+} sister at the same stage. The pattern of immunostaining of Sxl\textsuperscript{9} female embryos initially is uniform but much lighter than wildtype (not shown), reflecting the reduced level of SXL-F protein generated from the mutant Sxl\textsubscript{Pe} mRNA.
Because \( Sxl_{Pe} \) and the XSEs that activate it seem not to operate in the germ line (Keyes et al. 1992; Steinmann-Zwicky 1993), an allele like \( Sxl^0 \) that is only defective with respect to \( Sxl_{Pe} \)-mediated functions should be wildtype in germ cells. Although a definitive test of this expectation by pole-cell transplantation has not been made, three observations (data not shown) argue that \( Sxl^0 \) is functionally indistinguishable from \( Sxl^+ \) in germ cells: first, \( Sxl^0 \) and \( Sxl^+ \) germline clones induced in young larvae behave the same; second, \( Sxl^0 \) complements all \( Sxl \) mutants that are defective only in germline functions; and third, the rare \( Sxl^0 \) escaper females one finds in 18\(^o\) cultures are fertile.

As Fig. 1A diagrams, the other key allele in this study, \( Sxl^{f7,M1} \), has two significant lesions (Bernstein et al. 1995). \( M1 \) is a \textit{roo} transposon in the sex-specifically spliced region of \( Sxl \). It disrupts splicing control by allowing a significant level of female \( Sxl_{Pm} \) transcript splicing even in the absence of SXL-F activity. Through positive feedback, \( Sxl^{M1} \) ultimately ramps up to full female activity in most, though not all, haplo-X cells (Cline 1979), killing the chromosomal males by upsetting dosage compensation (Cline 1983b). Nevertheless, \( Sxl^{f7,M1,Y} \) males are fully viable and fertile because the \( f7 \) missense mutation affects all SXL-F protein isoforms, eliminating their ability to regulate \textit{transformer} (\( tra \)), and reducing their autoregulatory and dosage compensation activities (Cline 1984; Bernstein and Cline 1994). Homozygous \( Sxl^{f7,M1} \) females are only poorly viable and their soma is completely masculinized, while \( Sxl^{f7,M1}/Sxl^f \) females are inviable. The \( f7 \) lesion, like \( f9 \), seems not to affect germline functions, since \( Sxl^{f7,M1} \) complements all \( Sxl \) mutants that are defective only in germline functions (data not shown) and \( Sxl^{f7,M1} \) germline clones support oogenesis (Cline 1983a).
The utility of \textit{Sxlf}^{7,M1} in studies of \textit{Sxl} positive autoregulation stems from the fact that it has low but significant constitutive autoregulatory activity but no ability to feminize on its own. Thus any feminization observed in \textit{Sxlf}^{7,M1} heteroallelic animals must be due to the expression of the other \textit{Sxl} allele. In situations where that other allele (e.g. \textit{Sxl}^{p9}) would not be able to express its feminizing potential by itself, \textit{Sxlf}^{7,M1} can elicit in \textit{trans} at least some of that cryptic feminizing activity. Its effectiveness in this regard is greatly enhanced by increased maternal or zygotic \textit{snf}^{+} dose (Cline \textit{et al.} 1999). As Table 1 shows, one extra copy of \textit{snf} introduced from the father increased viability of \textit{Sxlf}^{7,M1}/\textit{Sxl}^{p9} females more than 50 fold (compare cross 1 to cross 3), and did so without greatly reducing the penetrance of the ovarian defect: 95% of the adult females in cross 3 lacked both ovaries, and the remaining 5% had only one. These ovary defects are illustrated in panels B and C of Fig. 2.

It follows from the information presented above that the abnormal gonadal phenotype of \textit{Sxlf}^{7,M1}/\textit{Sxl}^{p9} females is caused by a failure of \textit{Sxlf}^{7,M1} to induce \textit{Sxl}^{p9} to fully engage its feedback loop in the gonadal soma. The apparent tissue specificity of this effect in the surviving adults suggested that it is more difficult to induce \textit{Sxl} autoregulation in the gonadal soma than in any other somatic cell type.

\textbf{A somatic sexual identity crisis causes \textit{Sxlf}^{7,M1}/\textit{Sxl}^{p9} ovaries to disintegrate during metamorphosis:} To better understand the developmental fate of \textit{Sxlf}^{7,M1}/\textit{Sxl}^{p9};+\textit{P}\{\textit{snf}^{+}\} ovaries during metamorphosis, we tagged their germ cells using a \textit{nos:GAL4} germcell-specific driver and a \textit{UAS-GFP} target. The gross morphology of the mutant ovaries (Fig. 3D) is remarkably normal prior to metamorphosis (compare to the wildtype in Fig. 3C). In contrast, the
phenotype of homozygous $Sxlf^{7,MI}$ larval ovaries (Fig. 3B) much more closely resembles that of their brothers' testes at the same stage (Fig. 3A). The fact that $Sxlf^{7,MI}/Sxlf^{7,MI}$ larval ovaries are clearly more masculinized than $Sxlf^{7,MI}/Sxlf^{9}+/P\{snf^{+}\}$ larval ovaries shows that $Sxlf^{7,MI}$ must be inducing some female splicing of $Sxl_{pm}$ transcripts from the $Sxlf^{9}$ allele. That induced female splicing must be below the threshold for triggering $Sxlf^{9}$'s self-sustaining splicing feedback loop, since such ovaries soon disintegrate.

Molecular analysis of $Sxlf^{7,MI}/Sxlf^{9}+/P\{snf^{+}\}$ larval ovaries confirmed that $Sxl$ functioning was not as fully female as morphology had implied. We used *doublesex (dsx)* mRNA as an indicator of $Sxl$ female functioning. *dsx* is a target of *tra* (Baker and Ridge 1980; Nagoshi *et al.* 1988). In the absence of TRA-F protein, *dsx* generates male-specific *dsx^{M}* mRNA, while in the presence of TRA-F, *dsx* splicing follows the alternative, female-specific splicing pattern, generating *dsx^{F}* mRNA.

Lanes 1 and 2 of Fig. 4A show that $Sxl^{+}$ and $Sxlf^{7,MI}$ male larvae carrying $P\{snf^{+}\}$ produce only *dsx^{M}* mRNA, while $Sxl^{+}/Sxlf^{9}+/P\{snf^{+}\}$ control female larvae (lane 4) produce *dsx^{F}* mRNA, with only a trace of *dsx^{M}* . In contrast, $Sxlf^{7,MI}/Sxlf^{9}+/P\{snf^{+}\}$ female larvae (lane 5) produce a significant amount of *dsx^{M}* mRNA in addition to *dsx^{F}* mRNA, clearly signaling molecular intersexuality. To test our hypothesis that the larval ovary was a primary source of this male mRNA, we compared ovaries separated from the fat body tissue in which they were embedded (lane 6) to that fat body tissue (lane 7) from the same $Sxl$ mutant animals. Both tissues produced *dsx^{F}* mRNA, but only the isolated larval gonads produced a strong *dsx^{M}* band as well.
Fig. 3 shows that the ovarian morphology of these mutant gonads breaks down during the first half of the pupal period. By pupal stage 5 (Bainbridge and Bownes 1981), wildtype ovaries are organized into a series of parallel ovarioles comprised primarily of germaria (Fig. 3E). Although the Sxl mutant ovaries still had a full complement of germ cells at this stage, very few were organized into recognizable germaria (Fig. 3F). The ruptured appearance of the mutant ovary reflects disorganization, not an artifact of dissection. By the time of eclosion (3H), disorganization is extreme and most germ cells have been lost (compare to the wild type in 3G). Scored on the basis of morphology alone, 96% of the adult females in this cross appeared to lack gonads; however, a germ cell-specific marker revealed that 92% of those without recognizable ovaries nevertheless had some undifferentiated germ cells on each oviduct.

To further explore the phenotype of these disorganized adult ovaries, we immunostained them with an antibody against the EYES-ABSENT (EYA) protein, a non-sex-specific marker for gonadal soma, while also marking nuclei with DAPI and germ cells with GFP. As shown in Fig. 5A for wildtype ovaries, prior to stage 9 EYA marks the highly organized single-cell layer of somatic cells that surrounds each egg chamber (Boyle and Dinardo 1995; Bai and Montell 2002). Fig. 5B shows a Sxl mutant adult female reproductive tract that differentiated a few fairly normal egg chambers on one side, but only highly disorganized gonadal tissue on the other. In that disorganized region (magnified further in 5B'), somatic as well as germ cells were clearly labeled, but both occurred in amorphous multilayered clumps. These clusters show that mutant somatic and germ cells can proliferate significantly during metamorphosis even if they fail to form a recognizable ovary.
To assay whether these grossly abnormal somatic cells had retained some female character, we immunostained for the product of the *engrailed* (*en*) gene. EN does not label adult testes, but in the adult ovary it marks terminal filament and cap cells (Forbes *et al.* 1996), somatic cells normally located at the apical tip of the germarium (Fig. 5C). EN immunostaining revealed recognizable terminal filaments that were not otherwise associated with organized ovarioles (Fig. 5D). Thus even in the most grossly disorganized regions of these mutant adult ovaries, at least some somatic cells retain some female character.

Male differentiation was also apparent in these disorganized regions. The testes sheath is an epithelial covering (Fig. 5E) whose bright yellow pigmentation serves as a marker of maleness for the underlying gonadal mesoderm (Fung and Gowen 1957). 18% of the *Sxl* mutant ovaries (n=40) contained sporadic patches of cells producing this distinctive pigment (Fig. 5F). Hence by the adult stage, some of the *Sxlf7,M1/Sxlf9;+/* explants must have become at least partially masculinized.

Because there was no precedent for intersexuality alone causing such gross disorganization of a diplo-X gonad, we wondered whether the disorganization might instead be caused by an upset in dosage compensation. If it were, uniformly masculinizing or feminizing the *Sxl* mutant gonads by manipulating *tra* or *dsx* should not improve the situation, since neither *tra* nor *dsx* affects dosage compensation. In contrast, if confusion over sexual identity was the sole problem, such manipulations should allow *Sxl* mutant females to differentiate organized gonads.
The results for uniform masculinization are shown in Fig. 2 and Table 2. The gonads of \( Sxl^{7,M1}/Sxl^{9};+/P\{snf^+\} \) females were invariably rescued when they were masculinized by loss of \( tra^+ \). All 62 \( tra^- \) gonads (Table 2, second row) were recognizable as pseudotestes (Fig. 2F), with a phenotype indistinguishable from that of their \( Sxl^+ tra^- \) control sisters (Fig. 2E). In contrast only three of 26 \( tra^-/+ \) gonads of \( Sxl \) mutant females (Table 2, first row) were recognizable.

Uniform feminization by a TRA-F transgene also rescued, as shown in Fig. 2D, and Table 1, crosses 2 and 4. Rescue by the \( u2af50-tra^F \) transgene was complete even in cross 2 where the penetrance of the ovarian defect was expected to be highest because no extra copy of \( snf^+ \) was included to boost autoregulation. The surprising maternal effect of TRA-F is discussed below.

Since \( dsx \) controls fewer aspects of sexually dimorphic differentiation than its regulator \( tra \) (McRobert and Tompkins 1985; Taylor 1992; Taylor et al. 1994), we wondered whether masculinization of ovaries by DSX-M protein would rescue less effectively than masculinization by loss of TRA-F. To answer this question we used \( dsx^D \), which can only be spliced in the male pattern (Nagoshi & Baker 1990). Hence, \( dsx^D/Df(dsx) \) females only generate DSX-M protein. Masculinization by DSX-M did rescue, generating pseudotestes in both \( Sxl^+ \) control and \( Sxl \) mutant females that were indistinguishable from those generated by \( tra^- \) (compare Fig.s. 2G and 2H to 2E and 2F). Although not all masculinized females had both gonads (Table 2, Cross 2), the difference between \( Sxl \) mutant females and their \( Sxl^+/Sxl^9;+/P\{snf^+\} \) control sisters in this respect was not significant, thus we could conclude that masculinization by DSX-M rescues as effectively as masculinization by \( tra^- \).
The experiments with *tra* and *dsx* show that the problem with *Sxl* mutant gonads is one of sexual identity, not dosage compensation. Moreover, since *tra* and *dsx* only directly affect the sexual identity of somatic cells, rescue by genetic manipulation of these genes argued that a defect in *Sxl* autoregulation in somatic cells alone caused disintegration. As a direct test of the strictly somatic basis for this gonadal defect, we asked whether gonadal disintegration would be ameliorated either by genetically eliminating germ cells (Boswell & Mahowald 1985), or by artificially expressing *SXL-F* specifically in germ cells to stimulate their engagement of the *Sxl^9* autoregulatory loop. Although gonads without germ cells, whether sexually transformed or not, are even more rudimentary than sexually transformed gonads with abnormally developing germ cells, they are nevertheless recognizable as organized gonads. We found that *Sxl^7,M1/Sxl^9*; +/P(*snf^+*) females lacking germ cells invariably lacked one or both ovaries, while their *Sxl^+* sisters, also without germ cells, always had both (Table 3, cross 1). Moreover, a *Sxl* cDNA expression construct transcribed specifically in the germ line (Hager and Cline 1997) also failed to ameliorate the *Sxl^7,M1/Sxl^9* gonadal defect (Table 3, cross 2).

**Clonal analysis of unilateral *Sxl* mutant ovaries shows that *Sxl^9* feedback loop engagement in the gonadal soma is likely to be cell-nonautonomous:** When the probability of a *Sxl^7,M1/Sxl^9* ; +/P(*snf^+*) adult female having recognizable ovaries is low, most recognizable ovaries are unilateral: an ovary averaging half normal size will be present on one side of the female, with none on the other side (Table 1). Such asymmetry must reflect the occurrence of some essential event in the developing gonad whose probability is so low that it seldom happens even once in an individual, much less twice. The low-probability event responsible for ovarian differentiation in this case must be activation of the self-maintaining splicing feedback loop for
Sxl⁹ in precursors of the gonadal soma. Based on previous results in situations where feedback loop engagement for a given precursor cell was stochastic (Cline 1984; Cline 1985), we expected that engagement in one cell would not influence the probability of engagement in neighboring cells.

If feedback loop engagement were cell-autonomous in these unilateral Sxl mutant ovaries, the simplest explanation for their gross asymmetry would be that engagement happened early in development in a single gonadal soma precursor cell, which then grew much more rapidly and extensively than it otherwise would have, compensating for its neighbors that had not engaged and therefore could not contribute to the differentiated ovary. If that precursor cell were genetically tagged sufficiently early in development, all the somatic cells in the ovary that developed would be its progeny and be tagged. On the other hand, if Sxl⁹ feedback loop engagement in this situation were the consequence of a locally cell-nonautonomous process in which a group of neighboring precursor cells participated, the low-probability event might instead be that group collectively reaching a consensus to engage the Sxl⁹ feedback loop. The consensus to engage might or might not be triggered by a single cell initially engaging the feedback loop, but one way or another, cell-cell interactions among neighbors would be involved so that the cells that had engaged would not necessarily be clonally related. Whether this cell-nonautonomous model would require compensatory growth of the cells that had engaged would depend on the timing of the engagement event, the fraction of the precursor cells that were involved, and the final size of the differentiated ovary.
The key distinction between these two alternatives is the growth dynamics predicted for the somatic cells in the unilateral ovaries. We measured those dynamics by genetically tagging precursor cells at random in embryos, then scoring the frequency and size of the marked clones in adults (Fig. 6). The simple cell-autonomous engagement model requires that the clones in Sxl mutant female ovaries be much less frequent per differentiated ovary than for the cell-nonautonomous model, since in the former case a smaller fraction of presumptive precursor cells available to be tagged would have engaged their Sxl feedback loop so they could contribute to the differentiated ovary. Moreover, since the mutant ovaries were over half the size of the controls, by the simple cell-autonomous model the clones produced by the tagged precursor cell should be much larger in the mutant than in control ovaries, since the progeny of that tagged precursor would have to compensate for their untagged neighbors whose failure to engage had kept them from contributing. In contrast, the cell-nonautonomous model makes no such demands regarding clone frequency and clone size.

We tagged embryonic precursors of the gonadal soma by administering heat shocks three to 27 hours after egg laying to females carrying a FLP-out GFP cassette and an hsp-FLPase transgene (Pignoni and Zipursky 1997). Analysis of genetic mosaics has argued that for wild-type females, a single initial embryonic primordium with eight to ten cells contributes somatic cells to both ovaries (Szabad and Nöthiger 1992). In our experiment, these founder cells would have had some chance to divide before being tagged. The high fraction of unilateral ovaries argues that the stochastic event determining whether a mutant ovary can differentiate normally must occur after the primordia for the two ovaries have separated. The frequency of ovarioles whose follicle cells were GFP-positive was compared for 30 three-day-old ovaries from Sxl^{f7,M1}/
Sxlf^9/+; +/P{snf^*} adult females and 30 from their Sxlf^9/+; +/P{snf^*} sisters. The genetic background for this experiment proved to be optimal for distinguishing between the two models, since the probability of Sxlf^9 feedback loop engagement in this case was particularly low: only 3% of the 1,120 mutant females dissected had ovaries, 93% of which were unilateral. Based on ovariole number, the mutant ovaries were about half the size of control ovaries (mutant median 9.5, mean 9.9; and range 2-16 vs. 17, 16.8, and 12-22 for controls).

The simple cell-autonomy model's predictions of lower clone frequency and larger clone size were not met: 28 of the 30 mutant ovaries had clones vs. 22 of 30 for the controls. The high frequency of ovaries with clones in both cases indicates that many mutant and wild-type ovaries had more than one clone, but because we could not distinguish between clones within a single ovary, clone size in Fig. 6 is simply the total number of ovarioles with tagged follicle cells. The fact that no ovary had all its somatic cells tagged showed that we were far from saturation with respect to clone induction. Both the total number of tagged ovarioles (140 mutant vs. 136 control) and the distribution of those tagged ovarioles among the two sets of 30 ovaries scored were indistinguishable (Wilcoxon rank-sum test p=0.81).

A more complicated version of the cell-autonomous model can be imagined that might fit these data, although the number of additional assumptions required for such a fit makes it vulnerable to Occam's razor. Perhaps when metamorphosis begins, any significant salt-and-pepper intermingling of unengaged ovarian somatic precursor cells with their engaged counterparts would cause disintegration. By this hypothesis, the rare event generating unilateral ovaries would not be cell-autonomous feedback loop engagement in a single somatic precursor
cell early in development. Indeed, stochastic cell-autonomous engagement could occur at a relatively high frequency perhaps even throughout the larval period. Instead the rare event would be having a sufficiently large number of contiguous engaged precursor cells at metamorphosis to avoid disintegration. Additional assumptions are necessary to account for the fact that so few mutant gonads reach this threshold, yet those few that do go on to generate adult ovaries that range in size from only 12% to 94% of the median for control ovaries.

**Early zygotic and even maternal expression of TRA-F rescues Sxl mutant ovaries by stimulating Sxl<sup>+</sup> autoregulation:** In the experiments described in Table 1 showing that constitutive zygotic expression of TRA-F from our <i>u2af-tra<sup>F</sup></i> transgene rescues mutant ovaries, we were surprised to see evidence of partial rescue even when the transgene was present only maternally (compare control cross 3 with the experimental cross 4 line immediately below). Extraneous genetic background differences were unlikely to be responsible, since we had taken pains to minimize them so that cross 3 would be an appropriate control for cross 4. Since the sons in cross 4 who did not inherit the feminizing transgene had normal morphology and were fertile, a maternal effect of this feminizing transgene would have to leave male development unscathed.

The reality of a <i>tra<sup>F</sup></i> maternal effect was established beyond question when we observed even stronger maternal rescue with the independently isolated transgene, <i>hsp83-tra<sup>F</sup></i> (Waterbury <i>et al.</i> 2000). This transgene was explicitly designed to express TRA-F at a high level in germ cells. With <i>hsp83-tra<sup>F</sup></i>, all the daughters who had not inherited the transgene had two normal ovaries (Table 1, cross 5). Again, all the sons without the transgene were normal.
We assayed for \( \text{tra}^F \) mRNA in unfertilized eggs to determine whether rescue of the daughters' ovaries could be a direct effect of \( \text{tra} \) in the mothers' germ cells. Data in Fig. 7A show that eggs generated from wild-type mothers clearly do indeed contain female-specific \( \text{tra} \) mRNA (lane 3). Thus even though females do not need a functional \( \text{tra} \) allele in their germ cells in order to make functional eggs (Marsh and Wieschaus 1978), female germ cells appear to transcribe \( \text{tra} \) and splice its transcripts in the female pattern. Lane 4 in Fig. 7A shows that mothers carrying \( hsp83-\text{tra}^F \) load nearly twice as much \( \text{tra}^F \) mRNA into their eggs as mothers without the transgene (line 3), measured relative to an actin internal control. Endogenous and transgenic maternal \( \text{tra}^F \) RNA is gone at least by the late third instar stage (Fig. 7B, lanes 1-3). Curiously, unlike wildtype female larvae and adults, which have a significant amount of non-sex-specific (non-functional) \( \text{tra} \) mRNA (Fig. 7A, lane 2, and 7B, lane 4), eggs contain only the female-specific species. Note that \( \text{Sxlf}_{17/M1}/\text{Sxlf}^{0};+/\text{P}\{\text{sod}^+\} \) larvae have significantly more of the non-sex-specific splice form than their \( +/\text{Sxlf}^{0};+/\text{P}\{\text{sod}^+\} \) sisters (compare lanes 4 and 5 of Fig. 7B), a result consistent with the indications in Fig. 4 of their cryptic intersexuality.

Why would a \( \text{tra}^F \) maternal effect sufficient to rescue the ovaries of mutant daughters not interfere with the sons' sexual development? Perhaps the maternal product that stimulates \( \text{Sxl} \) autoregulation in this highly sensitized situation acts only early in development and does not persist to later stages where males would be affected. Since antibodies to TRA-F are not available, we could not determine directly whether maternally encoded TRA-F protein fails to perdure. Instead, we used female-specific splicing of \( \text{dsx} \) transcripts as a proxy for TRA-F. Fig.4A, lane 3, shows that at least by the late third instar stage, males whose mothers carried a
tra^F transgene were indistinguishable from males whose mothers did not: neither had any female
dsx splice product. Hence, any TRA-F protein made from maternal mRNA must be gone well
before this point.

We used the GAL4 two-component expression system to determine whether full rescue
of mutant ovaries by tra^F could be achieved without a maternal contribution, and to explore the
question of when zygotic tra^F mRNA could rescue. The only UAS-tra^F GAL4 target transgene
available for this purpose (Ferveur et al. 1995) is a pUAST construct that is not expected to be
expressed well in germ cells (Rørth 1998). Hence, it was not surprising that we failed to mimic
the tra^F transgenes' maternal effect by driving this GAL4 target with a maternal germline source
of GAL4 (nos-GAL4-VP16, data not shown). In the course of attempting to determine whether
this UAS-tra^F target would rescue the tra null phenotype, we were surprised to discover that
ubiquitous high-level expression of UAS-tra^F driven by tub-GAL4 greatly reduces the viability of
both sexes—especially males—and even partially masculinizes females! This potential for
paradoxical behavior limited the utility of UAS-tra^F for some but fortunately not all of our
purposes.

Zygotic expression of tra^F in the mesoderm was sufficient to fully rescue the Sxl mutant
gonadal phenotype. twist-GAL4 and GAL4-24B are mesoderm-specific drivers (Brand and
Perrimon 1993; Andrews et al. 2002). Either transgene driving UAS-tra^F rescued all the ovaries,
while no ovaries differentiated properly among sisters carrying only UAS-tra^F (Table 1, crosses 6
and 7). We tested the fertility of 40 GAL4-24B females whose ovaries had been rescued and
discovered that over a seven-day test period, 42% failed to lay eggs. Ovulation, rather than
oviposition, was impaired in the non-laying females. Moreover, only 61% of the laying females produced viable progeny, suggesting that some females may not have mated. We cannot say whether these behavioral problems reflect something interesting about normal Sxl function, or instead just the potential of UAS-tra$^F$ for paradoxical behavior.

To determine when in development tra$^F$ expression could rescue Sxl mutant ovaries, we used a GAL4 driver whose ubiquitous expression could be induced by heat shock to drive UAS-tra$^F$ any time after the blastoderm stage. To induce zygotic tra$^F$ mRNA even earlier, we used an NGT source of GAL4 (Tracey et al. 2000). Maternal germline expression of this driver activates zygotic UAS targets as early as nuclear cycle 11 (ten Bosch 2006). Data in Table 4 show that only pulses of tra$^F$ generated during the first six hours of embryogenesis will rescue the Sxl mutant ovaries. The fact that pulses of TRA-F in very young embryos prevented their Sxl mutant ovaries from exhibiting differentiation defects during metamorphosis is compelling evidence that rescue is a consequence of TRA-F stimulating Sxlf9 feedback loop engagement -- a self-maintaining event.

If maternally-deposited tra$^F$ rescues mutant ovaries by triggering Sxlf9 autoregulatory feedback loop engagement early in development, we would expect this maternal effect to eliminate the male dsx mRNA that we had seen in immature Sxl mutant ovaries from late third-instar larvae (Fig. 4A, lane 5). Data in Fig. 4B, lane 3 (compare to lane 2) show that indeed it does. Moreover, elimination of dsx$^M$ RNA in these female larvae must be due to an effect of TRA-F much earlier, rather than to perdurance of maternally encoded TRA-F protein, since the
brothers of these female larvae lacked the \(\text{\textit{dsxF}}\) mRNA that perduring TRA-F protein would generate.

If the stimulation of \(\text{\textit{Sxl}}^0\) autoregulation in the gonadal soma by increased TRA-F reflects a normal activity of \(\text{\textit{tra}}\) rather than an artifact of TRA-F overexpression, then decreasing TRA-F should have the opposite effect. Data in Table 5 show that it does. In this experiment, 18% of \(\text{\textit{Sxl}}\) mutant daughters had differentiated ovaries when both they and their mothers carried two copies of \(\text{\textit{tra}}^+\) (cross 1). That number dropped to 9% when mothers were heterozygous for \(\text{\textit{tra}}^+\) but daughters were homozygous (\(p<0.01\)), and to 0% when both mothers and daughters were heterozygous (cross 2; \(p<0.01\)). As in all other experiments designed to reveal maternal effects, the mothers used for this comparison were sisters, and the relevant chromosomes had been allowed to freely recombine to homogenize the genetic background.

\textbf{TRA-F affects \(\text{\textit{Sxl}}\) autoregulation even outside the gonad.} An effect of TRA-F on the viability of \(\text{\textit{Sxl}}^{7, M1}/\text{\textit{Sxl}}^9\) females would indicate effects on \(\text{\textit{Sxl}}^0\) autoregulation outside the gonad. Data in Tables 1 and 5 show that there are such effects but their magnitude must is small relative to those in the gonad. Recall that the viability of \(\text{\textit{Sxl}}^{7, M1}/\text{\textit{Sxl}}^9\) females without an extra copy of \(\text{\textit{snf}}^+\) is very low. Raising the level of \(\text{\textit{tra}}^F\) both maternally and zygotically increased their relative viability five fold (Table 1, crosses 1 and 2). However, even with this additional TRA-F activity, 95% of the females died. Moreover, the rescuing effect of \(\text{\textit{tra}}^F\) disappeared when the viability of \(\text{\textit{Sxl}}^{7, M1}/\text{\textit{Sxl}}^9\) females was raised by any additional copy of \(\text{\textit{snf}}^+\) (compare crosses 3 and 4). Decreasing the level of \(\text{\textit{tra}}^F\) activity from the endogenous alleles led to a statistically significant
opposite effect on viability: it dropped from 82% when mothers and daughters both had two \( tr^{+} \) alleles, to 57% when both had only one (Table 5).

If TRA-F protein can stimulate \( Sx\) autoregulation in nongonadal tissues even weakly, the stimulation should also be apparent in \( Sx^{f7,M1}/Y \) males that carry \( Sx^{+} \) and an extra copy of \( snf^{+} \). Like \( Sx^{f7,M1}/Sx^{f9}; +/P[snf^{+}] \) females, these males are sensitized to effects on \( Sx\) autoregulation, but stimulation for them will be deleterious rather than beneficial. \( Sx^{f7,M1}/Y \) males can tolerate a copy of \( Sx^{+} \) or an extra copy of \( snf^{+} \) individually, but when presented with both, the probability of \( Sx^{f7,M1} \) engaging the \( Sx^{+} \) allele's feedback loop becomes significant and viability drops dramatically (Cline et al. 1999). Data in cross 2 of Table 6 illustrate this point. Extra copies of \( snf^{+} \) and \( Sx^{+} \) that had no significant adverse effect on \( Sx^{f7,M1}/Y \) male viability individually (109% and 96% respectively), together reduced viability to 11%.

Table 6, cross 1, shows that the \( hsp83-tra^{F} \) transgene reduced the viability of these sensitized males from 16% without \( tra^{F} \) to 0.8% with it. There was no maternal effect of \( tra^{F} \) on these males: their viability was no lower when their mothers carried \( hsp83-tra^{F} \) (16%) than when their mothers did not (11%). \( Sx^{f7,M1} \) males not carrying \( Sx^{+} \) were unaffected by \( tra^{F} \), whether or not they carried an extra copy of \( snf^{+} \); however, when \( Sx^{+} \) was present, \( hsp83-tra^{F} \) had a small but significant effect even without the extra copy of \( snf^{+} \).

Conserved sequences near the \( Sx\) male-specific exon point to direct stimulation of \( Sx\) by \textbf{TRA-F}: The consensus binding site for TRA-F protein is \( TC[t/a][t/a]C[a/g]ATCAACA \) (Hoshijima et al. 1991). Anticipating that the effect of \( \text{TRA-F} \) on \( Sx\) autoregulation would be
indirect, we were stunned to discover a highly-conserved tandem pair of TRA-F sites in the sex-specifically regulated region of Sxl (Fig. 8). One site is a perfect match to the consensus and is located just 422 bp upstream of the male-specific exon 3 in D. melanogaster. The other site, only 3 bp upstream of the first, is a 1 bp (first position) degenerate sequence. There are no TRA-F consensus sites, or even 1-bp degenerates, anywhere else in Sxl. Although it had been believed that the eight different 13-mers designated as TRA-F sites were functionally equivalent, we found that the one 13-mer in melanogaster Sxl was matched exactly at a comparable position in all but one of the 12 published Drosophila species genomes (Drosophila 12 Genomes Consortium 2007), and in Sceptodrosophila lebanonensis, a fly just outside the Drosophila genus (Powell 1997), whose Sxl locus we partially sequenced (EU670259). The one exception is D. ananassae, which has only a single 1-bp (first position) degenerate sequence in the region. All species except lebanonensis and virilis also have a 1-bp (first position) degenerate sequence very near the consensus site. This degenerate sequence is missing in lebanonensis, but in virilis it is replaced by another exact copy of the conserved consensus sequence. A perfect match to this TRA-F consensus site occurs only 34 other times in the D. melanogaster genome. Just as striking, all the various 1-bp degenerate sites shown in Fig. 8 together occur only 42 times in melanogaster. The presence of such conserved sites so strategically positioned in the sex-specifically spliced region of Sxl argues that the stimulation of Sxl positive autoregulation by TRA-F is direct at some point and occurs by the inhibition of splicing to the male exon.
DISCUSSION

It had been believed that the regulatory relationship in Drosophila between the sex-determination switch gene Sex-lethal (Sxl) and one of its immediate downstream targets, transformer (tra), was simply one of master to slave. Our effort to understand the developmental basis for an unusual female-sterile phenotype observed in a highly contrived genetic situation revealed that the regulatory relationship between Sxl and tra is not so simple: the slave influences its master, and thereby effects itself. Although we could only see evidence of this indirect positive feedback of tra through Sxl in genetically-sensitized situations, our discovery that TRA-F binding sites in Sxl have been evolutionarily conserved for over 45 million years argues that this unanticipated feedback loop has significance for wildtype Drosophila. Our study generated three additional surprises: (1) intersexuality can cause Drosophila tissues to disintegrate; (2) not all aspects of somatic Sxl regulation may be cell-autonomous; and (3) female-specific tra mRNA is present in unfertilized eggs, with tra exerting a maternal effect on Sxl.

The discovery of a "functionally redundant" positive feedback loop for Sxl that operates through tra -- a regulatory circuit that can equally well be thought of as a non-redundant positive feedback loop for tra that operates though Sxl -- is of evolutionary interest in light of the fact that in Tephritid flies, tra occupies the position that Sxl holds in Drosophila: the master sex switch gene that epigenetically maintains the female developmental commitment through direct positive feedback control of its own pre-mRNA splicing (Pane et al. 2002). Very recent work suggests that the honeybee tra ortholog may function like tra in the Tephritids (Hasselmann et al. 2008).
In flies for which \textit{tra} is the master sex switch, \textit{tra} is expressed in the female germ line and that germline expression has been proposed to be an important element in the sex-determination mechanism by which the \textit{tra} feedback loop is initiated (Lagos \textit{et al.} 2007). We suggest that both the functionally redundant, \textit{tra}-mediated circuit for \textit{Sxl} positive feedback in \textit{D. melanogaster} and female germline expression of \textit{tra} in \textit{melanogaster} are vestiges of the evolutionary transition between \textit{tra} and \textit{Sxl} as the autoregulated master sex switch.

\textbf{Ambiguities in somatic cell sexual identity, not upsets in dosage compensation, cause} \textit{Sxlf}^{7,\text{M1}}/\textit{Sxlf}^{9}; +/\textit{P[snf]}^{+} \textit{ovaries to disintegrate during the pupal stage:} We were led to the discovery of \textit{tra}'s effects on \textit{Sxl} in the course of investigating why some \textit{Sxl} mutant ovaries disintegrate. While precedents exist for genetic imbalances caused by upsets in X-chromosome dosage compensation disrupting somatic cell growth and differentiation (Cline 1976; Tanaka \textit{et al.} 1976; Belote 1983), upsets in the functioning of sexual differentiation switch genes downstream of \textit{Sxl} such as \textit{tra} and \textit{doublesex} (\textit{dsx}) that are not involved in X-chromosome dosage compensation had not been reported to cause such disruptions. Although the gonads of intersexual and transsexual flies generated by mutations in such genes are certainly abnormal, they are easily recognized as gonads. For that reason we were surprised to discover that the disintegration of \textit{Sxlf}^{7,\text{M1}}/\textit{Sxlf}^{9}; +/\textit{P[snf]}^{+} \textit{ovaries during metamorphosis is caused by problems with sexual identity, not dosage compensation.}

Although \textit{Sxlf}^{7,\text{M1}}/\textit{Sxlf}^{9}; +/\textit{P[snf]}^{+} mutant ovaries appear morphologically normal prior to metamorphosis, we found them to be intersexual at the molecular level even at this early, rather quiescent stage. We hypothesize that their disintegration soon after the onset of metamorphosis
is a consequence of a sudden increase in level of $d\times_x^F$ required to promote the rapid female
differentiation and counteract any $d\times_x^M$ present, a level that cannot be reached by these mutant
cells that have not fully engaged the $S\times_l^@$ positive feedback loop. In this connection it may be
relevant that Le Bras and Van Doren (2006) reported that the gonad's requirements for $d\times_x^F$ and
$d\times_x^M$ qualitatively different during embryonic development than later. We can imagine that a
sudden shift in sexual identity during a period of rapid differentiation might be more disruptive
than an intersexual orientation held consistently throughout development, such as that imposed
by a loss of the $d\times x$ or $i\times$ genes. Another potential factor that might contribute to the ovaries'
disintegration is phenotypic sexual mosaicism reflecting cells that had fully engaged the $S\times_l^@$
feedback loop mixed with cells that had not.

**Cell-cell interactions may trigger $S\times_l$ feedback loop engagement in the gonadal soma:** All previous evidence had indicated that the regulation of $S\times_l$ in the soma is strictly cell-
autonomous (discussed in Cline and Meyer 1996). Nevertheless, the lack of compensatory
growth of somatic cells in the unilateral ovaries of $S\times_l^{7,M1}/S\times_l^@; P\{snf^+\}+/+$ adults, and the
wildtype frequency at which those somatic cells could be genetically tagged, showed that the
rare stochastic event that allowed these exceptional ovaries to differentiate properly must have
involved more than a single somatic stemcell precursor. We believe that the simplest
explanation for these results is that normal ovarian differentiation occurs when full $S\times_l^@$ feedback
loop engagement in one somatic gonadal precursor cell stimulates feedback loop engagement in
neighboring somatic cells poised just under the threshold for engagement. As mentioned in
RESULTS, we cannot exclude a rather complex cell-autonomous alternative model for $S\times_l^@$
feedback loop engagement, but we favor the cell-nonautonomous alternative since female
somatic cells in the gonad have already been shown to trigger female expression of *Sxl* in neighboring germ cells that have been "sensitized" by having two X chromosomes rather than one (Steinmann-Zwicky *et al.* 1989). Indeed, Evans and Cline (2007) showed that forced expression of TRA-F even in chromosomally male gonadal soma with no SXL-F is sufficient to stimulate SXL-F expression in neighboring diplo-X germ cells to a level adequate to produce fully functional eggs. Hence, our cell-nonautonomous model has these somatic cells simply doing to each other what they are already known to do to germ cells.

Cell-nonautonomy in the sexual differentiation of Drosophila gonads was first reported by Fung and Gowen (1957), and shown most recently by DeFalco *et al.* (2008). However, because none of these examples seemed to involve effects on the sexually determined state of those cells *in sensu stricto* -- the functional state of *Sxl* -- there was no reason *a priori* to expect autonomous behavior more than nonautonomous. In contrast, cell-nonautonomy for *Sxl* in the gonadal soma would be more surprising, because *Sxl* is clearly capable of maintaining its activity state within somatic cells without outside input, and because *Sxl* controls somatic dosage compensation. This link to dosage compensation makes it essential that *Sxl's* expression state in each somatic cell be appropriate for that cell's own X chromosome dose. For that reason we suspect that the influence of any cell-nonautonomy in this case would be relatively minor -- able to increase the fidelity of the process by which the *Sxl* feedback loop is first engaged in diplo-X somatic cells, but not able to affect haplo-X neighbor cells in a genetic mosaic.

Conserved TRA-F binding sites in *Sxl* argue that the indirect *tra* positive feedback circuit is functionally relevant: The discovery of highly conserved predicted TRA-F binding
sites in \textit{Sxl} indicates that some effect of \textit{tra} on \textit{Sxl} must be direct, and that this direct effect must be relevant to wild-type flies. While it is formally possible that a cell-nonautonomous effect of \textit{TRA-F} on \textit{Sxl} could be mediated by transport of this SR-like splicing factor between cells, such transport would be unprecedented. It seems more likely that the direct effect of \textit{TRA-F} on \textit{Sxl} is mechanistically distinct from cell-nonautonomous effects. A direct effect might occur either in zygotic cells that have sequestered maternally-synthesized \textit{tra}^F product, or in cells that have transcribed \textit{tra} themselves at a point in development when they have enough \textit{SXL-F} to direct some female \textit{tra} transcript splicing. Since maternal \textit{tra}^F does not disrupt male zygotic development, we can deduce that maternal \textit{tra}^F products must not persist. That they need not perdure beyond embryogenesis was indicated by our finding that a pulse of \textit{TRA-F} generated zygotically can rescue \textit{Sx}l7,M1/\textit{Sx}l9; +/P\{snf+\} ovaries well only within six hours after egg laying, and does not rescue at all after embryogenesis. We propose that the direct effect of \textit{TRA-F} on \textit{Sxl} in the gonadal soma influences the initial probability of at least one somatic gonadal precursor cell fully engaging the \textit{Sx}l9 feedback loop, but that once one such cell has fully engaged, it stimulates engagement in its somatic neighbors through the same indirect, cell-nonautonomous mechanism by which these cells stimulate \textit{Sxl} in the germ line.

Since we have not yet eliminated \textit{tra}^+ from both the maternal germline and the zygote, the question remains whether such a double loss of \textit{tra} would affect \textit{Sxl} autoregulation even in females that had not been genetically sensitized by mutations in \textit{Sxl}. Marsh and Wieschaus (1978) went partway towards answering that question by transplanting \textit{tra} female germ cells into \textit{tra}^+ female embryos and mating the resulting chimeric adults to \textit{tra} males. Since the masculinized \textit{tra} daughters produced by these chimeric mothers were viable and not malformed,
one can infer that $Sxl^+$ activation was normal in most tissues. Nevertheless, since the gonads seem not to have been examined, the question remains whether the aspect of development that seems most sensitive to the effect of $tra$ on $Sxl$ was in fact unaffected. Now that we know $tra^F$ transcripts are present in germ cells and can affect $Sxl$, and know as well that $Sxl$ is required for meiotic recombination (Cook, 1993; Cline, unpublished; and see Bopp et al., 1999), we need to examine more closely $tra^-$ germ cells and the eggs that they generate.

The predicted TRA-F binding site sequences in $Sxl$, as well as their positions relative to the $Sxl$ male exon, are remarkably conserved. The location of those sites argues that TRA-F binding facilitates $Sxl$ positive autoregulation by directly inhibiting use of the splice acceptor for the male-specific $Sxl$ exon. Although TRA-F has only been shown to function as a splicing activator in $D. melanogaster$ (Hoshijima et al. 1991; Heinrichs et al. 1998), in Tephritid flies it appears to promote the female-specific pattern of $tra$ pre-mRNA splicing by directly inhibiting use of male-specific splice acceptor sites (Ruiz et al. 2007).

It is intriguing that of the 13 fly species whose $Sxl$ sequence we examined, only $D. ananassae$ did not have at least one perfect match to the 13 bp TRA-F consensus binding sequence near the male-specific exon. $D. ananassae$ is far more closely related to $D. melanogaster$ than many other species in this group; however, as Singh (2000) points out, $ananassae$ is an unusual Drosophila species in many respects, including having a high rate of male genetic recombination. Meiotic recombination is generally a female-specific function in Drosophila, one that requires $Sxl$ (Cook, 1993; Cline, unpublished; and see Bopp et al., 1999). Perhaps the acquisition by
ananassae males of a function normally limited to females is related to the loss of TRA-F binding sites in Sxl.

**Redundant positive autoregulation and the evolution of a new master sex switch:**

Many years have passed since *D. melanogaster* was discovered to maintain its female sexual fate decision epigenetically through the operation of a direct positive feedback loop for *Sxl* pre-mRNA splicing. Surprisingly in all that time, the Tephritid ortholog of *tra* is the only other gene found to maintain a cell fate decision by this mechanism. Tephritidae, the "true" fruit flies, are evolutionary cousins of the Drosophilidae. *Sxl* orthologs have also been identified in the Tephritidae (Saccone *et al.*, 1998) and in insects as distant as honeybees (Dearden *et al.*, 2006) and moths (Niimi *et al.*, 2006); nevertheless, only in the Drosophilidae does *Sxl* appear to be sex-specifically regulated (Bopp *et al*. 1996; Dorsett *et al.* 2003). The view that in the Drosophilidae, *Sxl* took over *tra*’s ancestral developmental role is supported by the recent discovery and experimental manipulation of *feminizer* (*fem*), the honeybee *tra* ortholog (Hasselmann *et al*. 2008). The possibility that *fem* is a positively autoregulating master sex switch is supported by the observation that injection into extremely young female embryos of dsRNA directed against the *fem* regulator *complementary sex determiner* (*csd*) induced those embryos to develop into fertile adult males. Our discovery that TRA-F positively influences *Sxl* autoregulation in *D. melanogaster* shows that even in the Drosophilidae, a positive autoregulatory loop exists for *tra*, albeit one that is weaker and less direct than that in the Tephritidae. We propose that functional redundancy with respect to positive autoregulation was important in the evolutionary transition between *tra* and *Sxl* as the autoregulating master sex switch gene.
For modeling that evolutionary transition, it would be helpful to know with more certainty whether direct positive autoregulation of *tra* is the ancestral condition. Hoping to answer this question, we sequenced a 5.7 kb region from 1.9 kb upstream to 3.0 kb downstream of the putative *tra* ORF for *Sepsis cynipsea* (EU636097)(Fig. S1). The family Sepsidae is closer to the Drosophilidae than to the Tephritidae (Yeates and Wiegmann, 1999). Finding TRA-F binding sites in the appropriate region of *S. cynipsea tra* would indicate direct *tra* positive autoregulation and hence point to *tra* autoregulation as being ancestral. The absence of such sites would leave the question open.

Our results leave the question open. Even allowing up to a 3bp degeneracy in the established 13bp TRA-F consensus binding site, we found only two overlapping candidate sites in *S. cynipsea tra*, neither of which seemed likely to be functional: gaATCAATCAACA and TCAACAATCgeCA (degenerate sites lower case and overlap underlined). TRA-F from *D. melanogaster* had been shown not to utilize the first sequence (Inoue et al. 1992), and the second sequence lacks the TCAAxx 3’ sequence common to every predicted TRA-F binding site for *tra*, *dsx*, and *fru* orthologs all the way to mosquitos (Pane et al. 2002; Lagos et al. 2007; Scali et al. 2005). Moreover, we sequenced a 722-bp fragment of *tra* from the sister species *S. neocynipsea* (EU636098) centered on homology to the *S. cynipsea* degenerate TRA-F sites and found that neither site was conserved, despite 85% overall sequence identity for the two corresponding regions. The only sequence in *S. neocynipsea* resembling the TRA-F consensus was a single, different 2-bp degenerate.
Functional redundancy arising by gene duplication has long been considered a basic raw material of evolution (reviewed in True and Carroll 2002). Indeed duplication of the tra ortholog fem to generate csd in an ancestor of the honeybee appears to have led to the evolution of a new sex-determination signal (Hasselmann et al. 2008). Until very recently with the discovery by Hong et al. (2008) of "shadow enhancers," much less attention seems to have been given to the possible evolutionary role of functional redundancy in regulatory circuits, such as that we have discovered between Sxl and tra.

One can imagine an evolutionary route from tra to Sxl as the autoregulated master sex switch gene based on the sequential development and loss of functional redundancy in positive autoregulation (Figure 9). First, Sxl would become a TRAF splicing target by acquiring TRAF binding sites (Fig 9B). The fact that D. melanogaster Sxl has such sites today makes this possibility more plausible than it would otherwise be. Next, tra would acquire SXL-F binding sites that facilitate female tra pre-mRNA splicing, like those it has today, thereby establishing a functionally redundant circuit for tra positive autoregulation (Fig 9C). With two positive feedback pathways operating for tra, the indirect circuit running through Sxl could become stronger while the direct circuit weakened (Fig 9D) and eventually disappeared (Fig 9E). That disappearance may have been hastened by Sxl’s acquisition of its own SXL-F binding sites that generated a weak but direct autoregulatory circuit (Fig. 9E). At that point, Sxl rather than tra would be the gene with functionally redundant positive autoregulatory circuits, one direct and one indirect via tra. Again the circuit that was weaker initially could have strengthened as the other weakened, producing the situation that exists today (Fig. 9F).
ACKNOWLEDGEMENTS

We thank Brant Peterson of the M. B. Eisen lab for help with the Sepsid $tra$ analysis; Satoru Uzawa of the B. J. Meyer lab for help with confocal microscopy; R. Lehmann for fly stocks; and B. J. Meyer and current members of the Cline lab for helpful discussions and comments on the manuscript. This work was supported by National Institutes of Health grant GM023468 to T.W.C. and a National Science Foundation graduate fellowship to S.G.S.
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<table>
<thead>
<tr>
<th>Cross&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Genotype</th>
<th>Genotype of snf&lt;sup&gt;a&lt;/sup&gt;?</th>
<th>tra&lt;sup&gt;F&lt;/sup&gt; transgene used</th>
<th>Mode of tra&lt;sup&gt;F&lt;/sup&gt; contribution</th>
<th>Oogenic ovaries per mutant female</th>
<th>Relative Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sxl</td>
<td>Extra copy</td>
<td></td>
<td>Maternal</td>
<td>Zygotic</td>
<td>Two (%) One (%) None (%) N females</td>
</tr>
<tr>
<td>1</td>
<td>f7,M1/f9</td>
<td>No</td>
<td>None</td>
<td>No</td>
<td>No</td>
<td>0     0  100  15</td>
</tr>
<tr>
<td>2</td>
<td>f7,M1/f9</td>
<td>No</td>
<td>U2af-tra&lt;sup&gt;F&lt;/sup&gt;</td>
<td>Yes</td>
<td>No</td>
<td>0     9  91   11</td>
</tr>
<tr>
<td>2</td>
<td>f7,M1/f9</td>
<td>No</td>
<td>U2af-tra&lt;sup&gt;F&lt;/sup&gt;</td>
<td>Yes</td>
<td>Yes</td>
<td>100   0  0    32</td>
</tr>
<tr>
<td>3</td>
<td>f7,M1/f9</td>
<td>Yes</td>
<td>None</td>
<td>No</td>
<td>No</td>
<td>0     5  95   65</td>
</tr>
<tr>
<td>4</td>
<td>f7,M1/f9</td>
<td>Yes</td>
<td>U2af-tra&lt;sup&gt;F&lt;/sup&gt;</td>
<td>Yes</td>
<td>No</td>
<td>26    37 37   65</td>
</tr>
<tr>
<td>4</td>
<td>f7,M1/f9</td>
<td>Yes</td>
<td>U2af-tra&lt;sup&gt;F&lt;/sup&gt;</td>
<td>Yes</td>
<td>Yes</td>
<td>95    5  0    25</td>
</tr>
<tr>
<td>5</td>
<td>f7,M1/f9</td>
<td>Yes</td>
<td>hsp83-tra&lt;sup&gt;F&lt;/sup&gt;</td>
<td>Yes</td>
<td>No</td>
<td>100   0  0    20</td>
</tr>
<tr>
<td>6</td>
<td>f7,M1/f9</td>
<td>Yes</td>
<td>UAS-tra&lt;sup&gt;F&lt;/sup&gt;; No GAL4</td>
<td>No</td>
<td>No</td>
<td>0     0  100  15</td>
</tr>
<tr>
<td>6</td>
<td>f7,M1/f9</td>
<td>Yes</td>
<td>UAS-tra&lt;sup&gt;F&lt;/sup&gt;; twi-GAL4</td>
<td>No</td>
<td>Yes</td>
<td>100   0  0    15</td>
</tr>
<tr>
<td>7</td>
<td>f7,M1/f9</td>
<td>Yes</td>
<td>UAS-tra&lt;sup&gt;F&lt;/sup&gt;; No GAL4</td>
<td>No</td>
<td>No</td>
<td>0     2  98   50</td>
</tr>
</tbody>
</table>
"Binsciny/Sxlf9" siblings with the same autosomal genotype as experimentals were the viability reference.

bCrosses:

1: w cm Sxlf7,M1 ct v/Binsciny, y w sn2B ♂ ♀ x ♂ ♀ y w cm Sxlf9/Y

2: w cm Sxlf7,M1 ct v/Binsciny, y w sn2B; P[U2af50-traF w*mW hs]2B/+ ♂ ♀ (sibs to females in A) x ♂ ♀ y w cm Sxlf9/Y.

3: w cm Sxlf7,M1 ct v/Binsciny, y w sn2B ♂ ♀ x ♂ ♀ y w cm Sxlf9/Y; P[snf* w*mC]19

4: w cm Sxlf7,M1 ct v/Binsciny, y w sn2B; P[U2af50-traF w*mW hs]2B/+ (sibs to females in C) ♂ ♀ x ♂ ♀ y w cm Sxlf9/Y; P[snf* w*mC]19

5: w cm Sxlf7,M1 ct v/Binsciny, y w sn2B; P[hsp83-traF w*mC]5.4/+ ♂ ♀ x ♂ ♀ y w cm Sxlf9/Y; P[snf* w*mC]19

6: w cm Sxlf7,M1 ct v/Binsciny, y w sn2B; CyO, P[GAL4-twist.G w*mC]2.2, P[UAS-2xEGFP]AH2.2/+ ♂ ♀ x ♂ ♀ y w cm Sxlf9/Y; P[snf* w*mC]19 P[UAS-traF w*mC]20J7

7: w cm Sxlf7,M1 ct v/Binsciny, y w sn2B; P[hsp70-GAL4 w*mW]how24B/+ ♂ ♀ x ♂ ♀ y w cm Sxlf9/Y; P[snf* w*mC]19 P[UAS-traF w*mC]20J7
### TABLE 2

Rescue of Sxl^{TM1}/Sxl^{9}; {+/P[snf^+] mutant gonads by somatic masculinization.}

<table>
<thead>
<tr>
<th>Cross</th>
<th>Relevant genotype</th>
<th>Sexual Phenotype</th>
<th>Number of gonads per chromosomal females</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(all also {+/P[snf^+]})</td>
<td></td>
<td>Two (%)</td>
<td>One (%)</td>
</tr>
<tr>
<td>1</td>
<td>Sxl^{M1,f7}/Sxl^{9}; tra^1 or v2/+</td>
<td>♀</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>1</td>
<td>Sxl^{M1,f7}/Sxl^{9}; tra^1/tra^v2</td>
<td>♂</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>+/Sxl^{9}; tra^1 or v2/+</td>
<td>♀</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>+/Sxl^{9}; tra^1/tra^v2</td>
<td>♂</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Sxl^{M1,f7}/Sxl^{9}; +/Df(dsx)</td>
<td>♀</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>+/Sxl^{9}; dsx^D/Df(dsx)</td>
<td>♂</td>
<td>60</td>
<td>28</td>
</tr>
<tr>
<td>2</td>
<td>Sxl^{M1,f7}/Sxl^{9}; dsx^D/Df(dsx)</td>
<td>♂</td>
<td>50</td>
<td>40</td>
</tr>
</tbody>
</table>

---

*Crosses:

1: w cm Sxl^{M1} ct w/Binsinscy, y w sn^{2}B; tra^v2 kar ry red/TM3,Sb Ser ♀ ♂ x ♀ ♂ y w cm Sxl^{9}/Y^{Ds}; P[snf^+]

w^{mC}19; tra^1/TM3,Sb Ser

2: w cm Sxl^{M1} ct/Binsinscy, y w sn^{2}B; Df(3R)dsx48, dsx^D/P[w^{mGT}1]CG7878 ♀ ♂ x ♀ ♂ y w cm Sxl^{9}/Y; P[snf^+]

w^{mC}19; dsx^D Sb sprd e l(3)e^1/+
### TABLE 3

**Effect of germ cells on Sxl^{f, M1}/Sxl^{f}; +/P{snf^*} ovary disintegration**

<table>
<thead>
<tr>
<th>Cross&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Relevant genotype</th>
<th>germ cells</th>
<th>Number of ovaries per female&lt;sup&gt;b&lt;/sup&gt;</th>
<th>N Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Two</td>
<td>One</td>
</tr>
<tr>
<td>1</td>
<td>+/- Sxl&lt;sup&gt;f&lt;/sup&gt;</td>
<td>No</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>Sxl&lt;sup&gt;M1,f7&lt;/sup&gt;/Sxl&lt;sup&gt;f9&lt;/sup&gt;</td>
<td>No</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>Sxl&lt;sup&gt;M1,f7&lt;/sup&gt;/Sxl&lt;sup&gt;f9&lt;/sup&gt;; +/-</td>
<td>Yes</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Sxl&lt;sup&gt;M1,f7&lt;/sup&gt;/Sxl&lt;sup&gt;f9&lt;/sup&gt;; P{otu-Sxlcfl}/+</td>
<td>Yes</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Crosses:

1: w cm Sxl<sup>f,M1</sup> ct v/Binsincy, y w sn<sup>a2</sup>B; Df(2R)tudl/ P(FRT(w<sup>h3</sup>))/G13 tud<sup>B45-6</sup> ♂ ♂ x ♀ ♀ y w cm Sxl<sup>f</sup> v/Y; P{snf^*} w<sup>SN160</sup> /19

2: w cm Sxl<sup>f,M1</sup> ct vFM7; P{otu-Sxlcfl w<sup>SN160</sup>}/+ ♀ ♀ x ♀ ♀ y w cm Sxl<sup>f</sup>/Y; P{snf^*} w<sup>SN160</sup> /19
### TABLE 4

**Rescue of SxI^{7,M1}/SxI^{p9}; +/P{snf^+} ovaries by a tra^F pulse as a function of developmental age**

<table>
<thead>
<tr>
<th>Developmental age when TRA-F induced</th>
<th>% of females with 2 ovaries</th>
<th>Differentiated ovaries as % of total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control^b</td>
<td>Experimental^c</td>
</tr>
<tr>
<td>No maternal or zygotic</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>tra^F</em> induced</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>maternal <em>tra^F</em> induced</td>
<td>0</td>
<td>36</td>
</tr>
<tr>
<td>zygotic <em>tra^F</em> induced at:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-6 hours</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>6-24 hours</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>1-2 days</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>2-3 days</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3-4 days</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4-5 days</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>5-6 days</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>6-7 days</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>7-8 days</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8-9 days</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Total 1-9 days</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

^a^Flies were administered two 20-min 37°C heat shocks 30 minutes apart at the developmental age shown (hAEL@25°C). No heat shock for females in first row. Progeny from the cross: w cm SxI^{7,M1} ct/Binsinscy, y w sn^B;
\(P\{w^{+mc} GAL4-Hsp70.PB\}/+ \♀♀ \times ♂♂\ y w cm Sxl^{0}/Y^{RS}; P\{snf^{+} w^{+mc}\}/19 P\{UAS-tra^{F} w^{+mc}\}/20 J7\) except for second row (see footnote d).

\(^b\) Controls carried \(UAS-tra^{F}\) but no maternal or zygotic source of GAL4.

\(^c\) Experimentals carried \(UAS-tra^{F}\) and maternal \(P\{w^{+mc} GAL4-nos.NGT\}/40\) or zygotic \(P\{w^{+mc} GAL4-Hsp70.PB\}/2\)

\(N=25\) each for experimentals and controls in first four rows. \(N=20\) each for all others.

\(^d\) Controls from the cross: \(w cm Sxl^{7,MI} ct/Binsinscy, y w sn^{12} B \♀♀ \times ♂♂\ y w cm Sxl^{0}/Y^{RS}; P\{snf^{+} w^{+mc}\}/19 P\{UAS-

\(tra^{F} w^{+mc}\}/20 J7\) and experimentals from the cross \(w cm Sxl^{7,MI} ct/Binsinscy, y w sn^{12} B; P\{w^{+mc} GAL4-

\(nos.NGT\}/40/\♀♀ \♀♀\) to the same males. Experimental and control females were sisters and males were brothers.
<table>
<thead>
<tr>
<th>Cross</th>
<th>Maternal tra genotype</th>
<th>Zygotic tra genotype</th>
<th>Oogenic mutant ovaries per female (n=100 females each)</th>
<th>Relative Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Two (%)</td>
<td>One (%)</td>
</tr>
<tr>
<td>1</td>
<td>+/+</td>
<td>+/+</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>tra\textsuperscript{v}/+</td>
<td>+/+</td>
<td>0</td>
<td>9</td>
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<tr>
<td>2</td>
<td>tra\textsuperscript{v}/+</td>
<td>tra\textsuperscript{v}/+</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Crosses:

1: \textit{w cm Sxl}\textsuperscript{f7,M1} ct/Binsinscy, \textit{y w sn\textsuperscript{v}B}; \textit{P\{EPgy2 w\textsuperscript{smC}\}EY04597/P\{EPgy2 w\textsuperscript{smC}\}EY04597} \♀ \♀ × \♂♂ \♀ y \textit{w cm Sxl}\textsuperscript{f9}/Y; \textit{P\{snf\textsuperscript{+} w\textsuperscript{smC}\}19}

2: \textit{w cm Sxl}\textsuperscript{f7,M1} ct/Binsinscy, \textit{y w sn\textsuperscript{v}B}; \textit{tra\textsuperscript{v}/+}\textit{tra\textsuperscript{v}/+}/; \textit{P\{EPgy2 w\textsuperscript{smC}\}EY04597} \♀ \♀ × \♂♂ \♀ \textit{y w cm Sxl}\textsuperscript{f9}/Y; \textit{P\{snf\textsuperscript{+} w\textsuperscript{smC}\}19}

Virgins for crosses 1 and 2 were from the cross: \textit{w cm Sxl}\textsuperscript{f7,M1} ct/Binsinscy, \textit{y w sn\textsuperscript{v}B}; \textit{tra\textsuperscript{v}/+ kar ry red/P\{EPgy2 w\textsuperscript{smC}\}EY04597} \♀ \♀ × \♂♂ \♀ \textit{y w cm Sxl}\textsuperscript{f9}/Y; \textit{P\{snf\textsuperscript{+} w\textsuperscript{smC}\}19}

\textit{tra\textsuperscript{v}/+ kar ry red/ P\{EPgy2 w\textsuperscript{smC}\}EY04597}. The marker \textit{P\{EPgy2 w\textsuperscript{smC}\}EY04597} is only 15 kB from \textit{tra}. 
## TABLE 6

**TRA-F can kill sensitized males by stimulating Sxl autoregulation**

<table>
<thead>
<tr>
<th>Cross&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% relative viability of Sxl&lt;sup&gt;f&lt;sup&gt;7,M1&lt;/sup&gt;/Y&lt;/sup&gt; males&lt;sup&gt;b&lt;/sup&gt;</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>no Dp(Sxl&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>with Dp(Sxl&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>no Dp(Snf&lt;sup&gt;+&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td>no Dp(snf&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>with Dp(snf&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>no Dp(snf&lt;sup&gt;+&lt;/sup&gt;)</td>
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<tr>
<td></td>
<td>no hsp::tra&lt;sup&gt;f&lt;/sup&gt;</td>
<td>with hsp::tra&lt;sup&gt;f&lt;/sup&gt;</td>
<td>no hsp::tra&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

1  
reference  
n(118)  
96%  103%  100%  88%  64%  16%  0.8%

2  
reference  
n(161)  
--  109%  --  96%  --  11%  --

<sup>a</sup>Crosses:

- cm Sxl<sup>f</sup>/Y; P[snf<sup>+</sup> w<sup>+MC</sup>]19/CyO, Cy △ △ X (1) ♀ w cm Sxl<sup>f<sup>7,M1</sup></sup> ct v/Binsncy, y w sn<sup>2</sup>B; P[hsp83-tra<sup>f</sup> w<sup>+MC</sup>]5.4/+; Dp(1;3)sn<sup>13a1</sup>, cm<sup>+</sup>Sxl<sup>+</sup>ct<sup>+</sup>/+ or (2) ♀

- cm Sxl<sup>f<sup>7,M1</sup></sup> ct v/Binsncy, y w sn<sup>2</sup>B; Dp(1;3)sn<sup>13a1</sup>, cm<sup>+</sup>Sxl<sup>+</sup>ct<sup>+</sup>/+.

<sup>b</sup>Sxl<sup>f<sup>7,M1</sup></sup> males used as the viability reference class were 89% (1) and 78% (2) as viable as their Sxl<sup>f<sup>7,M1</sup></sup>/+ sisters.
FIGURE LEGENDS

FIGURE 1.--- $Sxl^9$, a nonsense mutation in the $Sxl_{Pe}$-specific exon E1, generates epigenetic mosaicism in SXL-F expression in mutant female embryos.  (A) Diagram of the 5' end of $Sxl$ showing the molecular lesions in $Sxl^9$ and $Sxl^{7,M1}$.  (B) Wildtype stage 12 female embryo immunostained for SXL-F.  (C) $Sxl^9/Sxl^9$ stage 12 female embryo similarly immunostained to show the epigenetic mosaic pattern of SXL-F protein that develops at the level of single cells.  (C') Higher magnification of the region boxed in C.

FIGURE 2.---Imposing a uniform somatic sexual phenotype rescues $Sxl^{7,M1}/Sxl^9$ ovaries. Live whole mounts of diplo-X (chromosomally female) adult gonads.  (A) $Sxl^9/+\text{ ovary}$ illustrating the wildtype phenotype.  (B) Asymmetric $Sxl^{7,M1}/Sxl^9$ ovary.  (C) Typical $Sxl^{7,M1}/Sxl^9$ gonad lacking recognizable ovarian tissue.  (D) $Sxl^{7,M1}/Sxl^9$ gonad fully rescued by constitutive feminizing expression of TRA-F.  (E) $Sxl^9/+\text{ control gonad masculinized by } tra^1/tra^2$.  (F) $Sxl^{7,M1}/Sxl^9$ gonad rescued by $tra^1/tra^2$ masculinization.  (G) $Sxl^9/+\text{ control gonad masculinized by } dsxD/Df(dsx)^{48}$.  (H) $Sxl^{7,M1}/Sxl^9$ gonad rescued by $dsxD/Df(dsx)^{48}$ masculinization. A,B and D are pseudo-darkfield illumination, while C,E-H are phase contrast. Scale bar is 100 μm. All animals carried an extra copy of $snf^+$ and were progeny from the following crosses:

(A,B,C) $w\ cm\ Sxl^{7,M1}\ ct\ v/Binsiscy,\ y\ w\ sn^{+2}B\ ♀\ ♀\ \times\ ♂♂\ y\ w\ cm\ Sxl^9/Y;\ P\{snf^+\ w^{+mC}\}19$.  (D) $w\ cm\ Sxl^{7,M1}\ ct\ v/Binsiscy,\ y\ w\ sn^{+2}B;\ P\{U2af50-traF\ w^{+mW,hs}\}2B/+\ ♀\ ♀\ \times\ ♂♂\ y\ w\ cm\ Sxl^9/Y;\ P\{snf^+\ w^{+mC}\}19$.  (E,F) $w\ cm\ Sxl^{7,M1}\ ct\ v/Binsiscy,\ y\ w\ sn^{+2}B;\ tra^v2\ kar\ ry\ red/TM3,Sb\ Ser\ ♀\ ♀$
FIGURE 3.---Sxl$^{f7,M1}$/Sxl$^9$ ovaries become morphologically abnormal only during metamorphosis. Live whole mounts of gonads viewed under Nomarski (A-F,H) or pseudo-darkfield illumination (G), with germ cells tagged with GFP (green overlay). Larval gonads are late third instar, and pupal gonads are stage P5. Scale bar is 100 μm. (A) Sxl$^{M1,f7}$/Y larval testes illustrating normal male morphology. All other gonads in this figure are diplo-X (chromosomally female). (B) Sxl$^{M1,f7}$/Sxl$^{M1,f7}$ masculinized larval ovary. (C,E,G) $^+/Sxlf^9$; P{snf$^+$}/+ normal control ovaries of larvae, pupae, and adults respectively. (D,F,H); Sxl$^{M1,f7}$/Sxl$^9$; P{snf$^+$}/+ abnormal mutant ovaries of larvae, pupae, and adults respectively. Progeny were from the following crosses:

(A&B): y cm Sxl$^{f7,M1}$ ct/Binscny, y w sn$^{x2}B$, let P{w$^{+mC,hs-hid}$}♀♀ x ♂♂ w cm Sxl$^{f7,M1}$ ct v/Y; P{w$^{+mC}$ GAL4::VP16-nos.UTR}MVD1, P{w$^{+mC}$ UASp-GFPS65C-alphaTub84B}3. Progeny were administered a 1 h 37° heat shock 4-28 h after egg laying). (D-H) w cm Sxl$^{f7,M1}$ ct v/Binscny, y w sn$^{x2}B$; P{w$^{+mC}$ GAL4::VP16-nos.UTR}MVD1, P{w$^{+mC}$ UASp-GFPS65C-alphaTub84B}3 ♀♀ x ♂♂ y w cm Sxl$^9$/Y; P{snf$^+$ w$^{+mC}$}19

FIGURE 4.---Molecular analysis of dsx regulation in Sxl$^{f7,M1}$/Sxl$^9$ females reveals a maternal effect of tra. Sex-specific splicing of dsx transcripts was assessed by rtPCR of RNA from wandering (late) third instar larvae. Primer pairs used are shown on the partial schematic of the dsx gene in which female-specific exons are black and male-specific exons are grey.
Panels A and B reflect separate experiments. Male and female *dsx* amplification products were run on the same gel but in different lanes. Whole larvae were used for all lanes except A6 and A7, in which case ovaries were separated from the fat body in which they were embedded and RNA was extracted separately from the two tissues. Mothers for the larvae in lanes A3 and B3, but not the larvae themselves, carried a constitutively feminizing *traF* transgene. Mothers for the other larvae in each experiment were sisters of those *traF* mothers.

Progeny were from the following crosses:

A1-7 except A3: w cm *Sxl^{7,M1} ct v/Binsinscy, y w *sn^{2}B ♀ ♂ x ♂ ♂ y w cm *Sxl^{9}/Y; P{snf^{+} w^{+mc}}_{19}.

A3: w cm *Sxl^{7,M1} ct v/Binsinscy, y w *sn^{2}B; P{U2af50-traF w^{+m,W,ko}2B/+ ♀ ♀ x ♂ ♂ y w cm *Sxl^{9}/Y; P{snf^{+} w^{+mc}}_{19}.

B1-2: w cm *Sxl^{7,M1} ct v/Binsinscy, y w *sn^{2}B; +/+CyO, P{GAL4-Kr.C}DC3, P{UAS-GFP.S65T}DC7 ♀ ♂ x ♂ ♂ y w cm *Sxl^{9}/Y; P{snf^{+} w^{+mc}}_{19}.

B3: w cm *Sxl^{7,M1} ct v/Binsinscy, y w *sn^{2}B; P{hsp83-traF w^{+mc}5.4/CyO, P{w^{+mc} GAL4-Kr.C}DC3, P{w^{+mc} UAS-GFP.S65T}DC7 ♀ ♀ x ♂ ♂ y w cm *Sxl^{9}/Y; P{snf^{+} w^{+mc}}_{19}.

**FIGURE 5.**---*Sxl^{7,M1}/Sxl^{9}* adult ovaries are grossly disorganized and intersexual.

Gonads from wildtype (A) and *Sxl* mutant (B; B’) adult females were immunostained for EYA protein (red), which marks gonadal soma, while nuclei were marked by DAPI (blue) and germ cells by GFP (green). Panels C and D show gonads from wildtype and *Sxl* mutant adult females respectively, also marked by DAPI and GFP, but immunostained for EN (red) to mark female cells. Arrows indicate differentiated terminal filament and cap cells. Panel E shows a normal testis with its pigmented sheath, the distinctive color of which reflects the male sexual identity of the underlying gonadal mesoderm. Panel F shows a typical disorganized gonad from a *Sxl* mutant adult female with islands of pigmented testes sheath. Scale bar throughout is 100μm.
A-D are progeny from the cross $w \text{cm} \text{Sxl}^{7,M1} \text{ct v/Binsinscy}, y w \text{sn}^{X2}B; P\{w^{+mC} \text{GAL4}:\text{VP16-nos.UTR}\}\text{MVD1}, P\{w^{+mC} \text{UASp-GFPS65C-alphaTub84B}\}3\♀\♀ \text{x }\♂\♂ y w \text{cm} \text{Sxl}^{9}/Y P\{\text{snf}^{+} w^{+mC}\}19$ with the following zygotic genotypes: (A,C) +/Sxl$^{9}; P\{\text{snf}^{+} w^{+mC}\}19/+; P\{w^{+mC} \text{GAL4}:\text{VP16-nos.UTR}\}\text{MVD1}, P\{w^{+mC} \text{UASp-GFPS65C-alphaTub84B}\}3/+; (B,D) Sxl$^{7,M1}$/Sxl$^{9}; P\{\text{snf}^{+} w^{+mC}\}19/+ P\{w^{+mC} \text{GAL4}:\text{VP16-nos.UTR}\}\text{MVD1}, P\{w^{+mC} \text{UASp-GFPS65C-alphaTub84B}\}3/+$. E and F are Sxl$^{7,M1}$/Y; P\{\text{snf}^{+} w^{+mC}\}19/+ and Sxl$^{7,M1}$/Sxl$^{9}; P\{\text{snf}^{+} w^{+mC}\}19/+ progeny respectively from the cross $y \text{cm} \text{Sxl}^{7,M1} \text{ct v/Binsinscy}, y w \text{sn}^{X2}B \♀\♀ \text{x }\♂\♂ w \text{Sxl}^{9}\text{ctl/Y}; P\{\text{snf}^{+} w^{+mC}\}19$.

**Figure 6.**---Lack of compensatory growth in Sxl$^{7,M1}$/Sxl$^{9}$ mutant ovaries indicates cell-nonautonomous stimulation of Sxl positive feedback loop engagement. Histogram indicating the approximate size and frequency of embryonically-induced FLP-out clones in the somatic portion of 30 3-day old adult Sxl$^{7,M1}$/Sxl$^{9}$ mutant ovaries (black bars) and 30 matched ovaries of their Sxl$^{+/Sxl^{9}}$ control sisters (white bars). The somewhat smaller size of the mutant ovaries is reflected in the fact that they had fewer ovarioles (297 versus 505 in the control). Embryonic precursors of the follicle stem cells were genetically tagged at 3-27 h after egg laying in an experiment in which only 3% of the adult mutant females had recognizable ovaries, 93% of which occurred singly, rather than in pairs. Most tagged ovarioles were mosaic like the example in the inset. Ovaries were from daughters from the cross: $w \text{cm} \text{Sxl}^{7,M1} \text{ct v/Binsinscy}, y w \text{sn}^{X2}B; P\{w^{+mC} \text{GAL4-Act5C(FRT.CD2).P}\}\text{S}\ P\{\text{UAS-GFP}\}♀♀ \text{x }\♂\♂ y w \text{cm} \text{Sxl}^{9} P\{\text{ry}^{+7.2}\text{hsFLP}\}22/Y; P\{\text{snf}^{+} w^{+mC}\}19$.

**Figure 7.**---Molecular analysis of tra transcripts in Sxl$^{7,M1}$/Sxl$^{9}$ mutants.
Sex-specific splicing of \textit{tra} transcripts was assessed by rtPCR of RNA using the primer pair shown in the schematic, with \textit{Actin5c} as a loading control. Female-specific \textit{tra} transcripts yielded a 256 bp band versus 432 bp for the non-sex-specific (nonfunctional) species. Panel A lanes 1 and 2 assay wild-type adult male and females, respectively. Panel A lane 3 assays unfertilized eggs from mothers that did not carry the constitutively-feminizing \textit{u2af-tra} \textsuperscript{F} transgene, while lane 4 assays eggs from mothers that did. Panel B assays whole third instar larvae, including males of the genotypes +/Y (lane 1), and \textit{Sxl} \textsuperscript{7,M1}/Y (lanes 2-3). The mothers of the males in B3 carried the \textit{u2af-tra} \textsuperscript{F} transgene, but the males themselves did not. Panel B lanes 4 and 5 assay females of the genotype +/\textit{Sxl} \textsuperscript{9} and \textit{Sxl} \textsuperscript{7,M1}/\textit{Sxl} \textsuperscript{9}, respectively. Eggs and progeny were from the following crosses (the \textit{sam} \textsuperscript{2} sterile male mates stimulated laying of unfertilized eggs): Lane A3: \textit{w cm \textit{Sxl} \textsuperscript{7,M1} ct v/Binsincy, y w sn\textsuperscript{2}B ♀ ♂ x ♀ ♀ sam\textsuperscript{2} ec/Y}. Lane A4: \textit{w cm \textit{Sxl} \textsuperscript{7,M1} ct v/Binsincy, y w sn\textsuperscript{2}B; \textit{P{U2af50-traF w\textsuperscript{+mw,hs}}2B/+ ♀ ♀ x ♀ ♀ sam\textsuperscript{2} ec/Y}. Lanes B1-2, B4-5: \textit{w cm \textit{Sxl} \textsuperscript{7,M1} ct v/Binsincy, y w sn\textsuperscript{2}B ♀ ♀ x ♀ ♀ y w cm \textit{Sxl} \textsuperscript{9}/Y; \textit{P{snf\textsuperscript{+ w\textsuperscript{+mc}}19}}}. Lane B3: \textit{w cm \textit{Sxl} \textsuperscript{7,M1} ct v/Binsincy, y w sn\textsuperscript{2}B; \textit{P{U2af50-traF w\textsuperscript{+mw,hs}}2B/+ ♀ ♀ x ♀ ♀ y w cm \textit{Sxl} \textsuperscript{9}/Y; \textit{P{snf\textsuperscript{+ w\textsuperscript{+mc}}19}}}.  

\textbf{FIGURE 8.---}Highly conserved putative TRA-F protein binding sites are present exclusively in the sex-specifically regulated region of \textit{Sxl}. The region between the non-sex-specific \textit{Sxl} exon 2 and the male-specific, translation terminating exon 3 is shown to scale for twelve species from the genus Drosophila, and one from the closely related genus Scaptodrosophila. Females skip exon 3, while males utilize it.
FIGURE 9.—How regulatory functional redundancy might have facilitated an evolutionary transition from \textit{tra} to \textit{Sxl} as the autoregulating master sex-determination switch gene. A hypothetical stepwise scenario is illustrated beginning with the current situation in \textit{Tephritid} flies where \textit{tra} is the master sex switch and positively autoregulates directly while \textit{Sxl} seems not to be involved in sex determination. The scenario ends with the current situation in \textit{D. melanogaster} where \textit{Sxl} is the master sex switch and positively autoregulates directly, but a vestige of \textit{tra} autoregulation via \textit{Sxl} remains.