Transposon Insertions Causing Constitutive Sex-Lethal Activity in Drosophila melanogaster Affect Sxl Sex-Specific Transcript Splicing

Mitchell Bernstein,* Robert A. Lersch,† Lakshman Subrahmanyan‡ and Thomas W. Cline*‡

*Department of Biology, Princeton University, Princeton, New Jersey 08544, †Department of Biology, Yale University, New Haven, Connecticut 06520-8103, and ‡Division of Genetics, Department of Molecular and Cell Biology, University of California, Berkeley, California 94720-3204

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ABSTRACT

Sex-lethal (Sxl) gene products induce female development in Drosophila melanogaster and suppress the transcriptional hyperactivation of X-linked genes responsible for male X-chromosome dosage compensation. Control of Sxl functioning by the dose of X-chromosomes normally ensures that the female-specific functions of this developmental switch gene are only expressed in diplo-X individuals. Although the immediate effect of X-chromosome dose is on Sxl transcription, during most of the life cycle “on” vs. “off” reflects alternative Sxl RNA splicing, with the female (productive) splicing mode maintained by a positive feedback activity of SXL protein on Sxl pre-mRNA splicing. “Male-lethal” (SxlM) gain-of-function alleles subvert Sxl control by X-chromosome dose, allowing female Sxl functions to be expressed independent of the positive regulators upstream of Sxl. As a consequence, SxlM haplo-X animals (chromosomal males) die because of improper dosage compensation, and SxlM chromosomal females survive the otherwise lethal effects of mutations in upstream positive regulators. Five independent spontaneous SxlM alleles were shown previously to be transposon insertions into what was subsequently found to be the region of regulated sex-specific Sxl RNA splicing. We show that these five alleles represent three different mutant types: SxlM1, SxlM3, and SxlM4. SxlM1 is an insertion of a roo element 674 bp downstream of the translation-terminating male-specific exon. SxlM3 is an insertion of a hobo transposon (not 297 as previously reported) into the 3' splice site of the male exon, and SxlM4 is an insertion of a novel transposon into the male-specific exon itself. We show that these three gain-of-function mutants differ considerably in their ability to bypass the sex determination signal, with SxlM4 being the strongest and SxlM1 the weakest. This difference is also reflected in effects of these mutations on sex-specific RNA splicing and on the rate of appearance of SXL protein in male embryos. Transcript analysis of double-mutant male-viable SxlM derivatives in which the SxlM insertion is cis to loss-of-function mutations, combined with other results reported here, indicates that the constitutive character of these SxlM alleles is a consequence of an alteration of the structure of the pre-mRNA that allows some level of female splicing to occur even in the absence of functional SXL protein. Surprisingly, however, most of the constitutive character of SxlM alleles appears to depend on the mutant alleles' responsiveness, perhaps greater than wild-type, to the autoregulatory splicing activity of the wild-type SXL proteins they produce.

Gain-of-Function (g-o-f) alleles of the X-linked regulatory gene, Sex-lethal, partially bypass the primary sex-determination signal of Drosophila melanogaster. These alleles have played an important part in the elucidation of the mechanism by which the sex of D. melanogaster is determined (Cline 1978, 1979, 1983, 1984), and they continue to be useful in such studies (Steinmann-Zwicky et al. 1989; Oliver et al. 1990, 1993; Salz 1992; Pauli et al. 1993; Steinmann-Zwicky 1993). However, little is known about the specific molecular nature of their mutant lesions, the mechanistic basis for their constitutive expression, or the functional differences that might exist among alleles of this class.

Genetic and molecular characterization presented here addresses these questions. It should enhance the usefulness of these alleles, as well as provide insights into mechanisms leading to ectopic gene expression in higher eukaryotes.

Sex determination in Drosophila is controlled by the activity of Sxl, which, in response to the primary sex-determinant, X-chromosome dose, is active in females (XX) and inactive in males (XY) (recently reviewed by Belote 1992; Cline 1993). Sxl in turn determines the functional states of more developmentally specialized regulatory genes downstream. Loss-of-function mutations in Sxl are specifically lethal to chromosomal females because of dosage compensation upsets (Lucchesi and Skrisky 1981; Cline 1983; Gergen 1987; Bernstein and Cline 1994). This lethality generally obscures the masculinizing effect of such mutations; however, effects on female sexual differentiation can
be observed readily in genetic mosaics and triploid intersexes (Cline 1979; Sanchez and Nöthiger 1982; Cline 1983).

G-of alleles in Sxl were recovered as mutations that rescued chromosomal females that otherwise would have died because of the lethal maternal effect of mutations in daughtercless (Cline 1978). Although the maternal activity of da is normally required for the activation of Sxl in the zygote, Sxl g-of mutations partially bypass this requirement (Cline 1980, 1983). In bypassing the normal mechanism that activates Sxl in response to X-chromosome dose, these mutations also behave as dominant male-specific lethals due to their inappropriate expression of female-specific Sxl activities in haplo-X individuals (Cline 1984). For this reason, they are known as SxlM alleles, in contrast to the female-lethal Sxl loss-of-function alleles. The feminizing effect of these male-specific lethals on adult differentiation is apparent in genetic mosaics and triploid intersexes (Cline 1979, 1983; Apart from this regulatory defect, SxlM alleles are functionally wild-type, as evidenced by their ability to support wild-type female development even when hemizygous.

The initial control of Sxl by X-chromosome dose is at the level of transcription and reflects effects on the activity of a "sexual pathway establishment" promoter, Sxl,g (Keyes et al. 1992). A short burst of SXL protein is generated very early in development in females as a result of the transitory expression of Sxl in response to their double dose of X-linked "XA numerator element" genes. The single dose of these same genes in males is not sufficient to activate Sxl,g. Throughout most of development, however, Sxl,g is silent in both sexes, and the activity state of Sxl reflects alternative RNA splicing and the operation of a positive feedback loop for transcripts derived from a "sexual pathway maintenance" promoter, Sxl,m, located 5 kb upstream of Sxl,g (Bell et al. 1988, 1991; Samuels et al. 1991). Only females splice Sxl,m transcripts into mRNAs encoding full-length active SXL proteins. This is because SXL proteins exhibit an RNA binding activity (Sosnowski et al. 1989; Inoue et al. 1990; Sakamoto et al. 1992; Valcarcel et al. 1993; Samuels et al. 1994) that inhibits inclusion of a male-specific translation-terminating exon that would otherwise be present in Sxl,m-derived mRNAs and prevent the production of functional Sxl products (Bell et al. 1988, 1991). This positive feedback on RNA splicing is responsible for maintaining cells' commitment to the female developmental pathway after X-chromosome dose has been assessed. Transcripts from Sxl,m, unlike those from Sxl,g, are productively spliced even in the absence of SXL protein. Thus, it is the early burst of SXL protein from transcripts originating at Sxl,g that initiates the female commitment by setting the autoregulatory feedback loop in operation for Sxl,m transcripts. Although Sxl is nonfunctional in males, Sxl,m is active in this sex, beginning at the blastoderm stage and continuing through adulthood (Salz et al. 1989; Samuels et al. 1991); however, because males lack the initial burst of SXL protein derived from Sxl,m expression, they never generate functional protein products because the translation-terminating male-specific exon is always present in their Sxl mRNA. In this way, the nonfunctional state of Sxl, the committed male developmental pathway, is maintained by default.

When Sxl was first cloned, it was shown that the DNA alterations associated with five spontaneously generated SxlM alleles are transposon insertions within a 1-kb region of the gene (Maine et al. 1985) that was subsequently shown to contain the male-specific exon (Bell et al. 1988; Salz et al. 1989). Given our current understanding of Sxl regulation, there are at least three ways that the transposon insertions present in the SxlM alleles might bypass the normal sex-determination signal: (1) the transposon might activate Sxl,g in the absence of the positive regulators normally required for its expression, (2) promoters in the transposon itself might generate novel transcripts that could encode functional SXL proteins, or (3) the transposons might disrupt the structure of the Sxl,M-derived RNA so that SXL protein is no longer required to promote the productive transcript splicing mode. The finding that all five transposon insertions in the SxlM alleles inserted in the vicinity of the male-specific exon, rather than in the vicinity of Sxl,g, would seem to favor a direct effect on Sxl splicing, but by itself this finding does not eliminate other alternatives. Because transcriptional enhancers can work at considerable distances, enhancers carried by SxlM transposons might be able to activate Sxl,g. Also, the discovery that translation of Sxl mRNA seems to reinitiate in adult males, albeit at very low efficiency downstream of the male-specific exon (Bopp et al. 1991), raises the possibility that transcripts initiating from transposons in the vicinity of the male-specific exon might be able to generate significant levels of functional SXL protein (it should be noted, however, that there was no indication of activity for the truncated adult male proteins mentioned). Independent of the mechanism leading to the initial burst of ectopic Sxl expression, the autoregulatory activity of SXL proteins generated could be expected to influence the splicing and hence functioning of subsequent transcripts; therefore, it is of interest to know the extent to which the constitutive functioning of SxlM alleles depends on Sxl autoregulation.

Here we address the question of what is responsible for the constitutive behavior of SxlM alleles by defining more precisely the specific molecular nature of the SxlM lesions and by examining their effects on the kinds of Sxl mRNAs that are generated in the presence and absence of SXL autoregulatory activity. Our results indicate that at least one SxlM allele directly affects Sxl splicing [alternative (3) above] but also indicate that the level of female transcripts generated in males—the
level of constitutive expression—is remarkably dependent on the autoregulatory activity of the SXL proteins produced. Moreover, those levels may reflect an increased sensitivity of the mutant alleles to such autoregulatory activity.

MATERIALS AND METHODS

Fly culture: Flies were raised at 25°C on a standard Drosophila medium (Cline 1978). Sxl alleles are conveniently maintained as stocks in a genetically balanced condition in which females are Sxl+/Sxl1 and males are Sxl+/Y, where Sxl is Sxl<sup>F<inf>B</inf>}, a male-viable deletion of the entire Sxl locus (Salz et al. 1987, 1989). Unless otherwise referenced in the text, mutations are described in Lindsley and Zimm (1992). The criterion for survival was eclosion.

Sxl genetic nomenclature: As described in Lindsley and Zimm (1992), an attempt has been made to standardize the allele designations for Sxl. Hence, the doubly mutant alleles designated Sxl<sup>5</sup>M3 and Sxl<sup>3</sup>M3 designate the alleles described in Main et al. (1985) that are referred to as Sxl<sup>5</sup>M3 and Sxl<sup>3</sup>M3 pending further genetic and/or molecular characterization of the newly induced Sxl allele that it must carry. In connection with the updating of the Flybase gene list by R. Drysdale and M. Ashburner, a decision has been made to minimize the use of dashes in gene names; hence, sis-a and sis-b become sisx and sisY.

Origin of Sxl<sup>5</sup>M3 alleles: Sxl<sup>5</sup>M3 arose spontaneously on a pn chromosome in a stock homozygous for da<sup>1</sup> kept at 18°C. Sxl<sup>5</sup>M3, Sxl<sup>5</sup>M7, and Sxl<sup>5</sup>M8 arose spontaneously in the course of a mapping experiment at 25°C in which Sxl, Sxl<sup>5</sup>B, and Sxl<sup>5</sup>C were kept at y<sup>1</sup>-/pn females crossed to y<sup>1</sup> da<sup>1</sup> females. After electron microscopy was used to cross to y<sup>1</sup> cm<sup>1</sup> mothers, males were crossed to y<sup>1</sup> cm<sup>1</sup> sn<sup>1</sup> females. Both src and cramped (cr) alleles were crossed to y<sup>1</sup> cm<sup>1</sup> sn<sup>1</sup> males. Sxl<sup>5</sup>M2 arose on the Sxl<sup>5</sup>M2 chromosome, whereas Sxl<sup>5</sup>M1 and Sxl<sup>5</sup>M3 arose on the Sxl<sup>5</sup> homologue. The three mutants arose on 5.9 x 10<sup>6</sup> female zygotes. Sxl<sup>5</sup>M3 arose on the Sxl<sup>5</sup> chromosome in a mapping cross for Sxl<sup>11</sup>M1 of the same design as that described above for Sxl<sup>5</sup>M1.

DNA sequence analysis of Sxl<sup>5</sup>M alleles: Standard procedures were used for isolation and manipulation of DNA clones (Sambrook et al. 1989). The Sxl<sup>5</sup>M alleles were isolated from libraries of Mob partial restriction digests constructed in phage EMBl3 and screened with a 4.8-kb XhoI fragment (coordinates 6.9-11.7 of the Sxl genomic map; see Samuel et al. 1991) and Figure 1) known to include the Sxl<sup>5</sup>M insertion sites (Maine et al. 1985a,b). Phage inserts were cloned into Bluescript SK (+) (Stratagene, San Diego, CA) for further analysis. For DNA sequencing, fragments were isolated, ligated, and sequenced as described (Barker and Barrell 1983) and then ligated into Bluescript SK (+). Random clones were sequenced by the dideoxy-nucleotide chain-termination method using Sequenase (U.S. Biochemicals, Cleveland, OH). Sequence assembly and analysis were done with the programs of Staden (1986). Both strands of the XhoI genomic DNA fragments containing Sxl<sup>5</sup>M<sup>5</sup>M<sup>5</sup> and Sxl<sup>5</sup>M<sup>1</sup> were sequenced in their entirety. Primers were used to confirm the positions of the Sxl<sup>5</sup>M and Sxl<sup>5</sup>M<sup>1</sup> insertions. The position of the Sxl<sup>5</sup>M<sup>1</sup> insertion was obtained by sequencing ~500 bp of DNA from both of the Sxl-hobo junctions present in this allele.

DNA sequence analysis of loss-of-function lesions in Sxl<sup>M5</sup> male-viable derivatives: Fine-structure recombination mapping had placed Sd<sup>5</sup>F on the 5' side of the roo insertion in Sxl<sup>M5</sup> and had placed Sd<sup>5</sup>F<sup>7</sup> and Sd<sup>5</sup>F<sup>10</sup> on the 3' side (Cline et al. 1984). Because all three double-mutant alleles (and indeed several more whose DNA changes will be described elsewhere) had been derived from the same parental allele, extraneous sequence polymorphisms were not a concern in this study. For Sd<sup>5</sup>F, the position of the lesions was identified by scanning 5 kb of Sd sequence upstream of the roo insertion (including all of exon 2) for single base mismatches using a mismatch detection technique described and referenced further in Bopp et al. (1993). Single-stranded template DNA from the region of mismatch was produced using asymmetric PCR (McCabe 1990) and sequenced by the dideoxy-nucleotide chain-termination method. For Sd<sup>5</sup>F<sup>7</sup> and Sd<sup>5</sup>F<sup>10</sup>, all exons (4-10) 3' to the roo insertion were sequenced. PCR fragments containing these exons from the mutant alleles were generated in which one member of the primer pair was biotinylated. That strand was then purified using streptavidin-coated magnetic beads as described in Hultman et al. (1989) and sequenced. Primer sequences used in these experiments are available from the authors upon request.

RNA analysis: RNA from staged embryos and adult flies was isolated by suspending samples in a 1:1 mixture of buffer (0.2 M Tris/HCl, pH 7.5, 0.5 M NaCl, 0.01 M EDTA, 1% SDS) and organic (phenol, chloroform, isomyl alcohol, 25:24:1), followed by disruption for 1 min with a Brinkman tissue grinder and separation of phases by centrifugation at room temperature. The aqueous phase was extracted repeatedly with organic until no further material accumulated at the phase boundary during centrifugation. Poly(A) mRNA was isolated using oligo dT cellulose as described (Sambrook et al. 1989). For Northern analysis and RNAse protection experiments, each lane or hybridization contained 10 μg of poly(A) mRNA.

For Northern analysis, RNA was fractionated on 10% agarose gels containing formaldehyde (Sambrook et al. 1989). After electrophoresis, RNAs were transferred to nylon membranes and hybridized with probes generated by the random priming method as described (Samuels et al. 1991). All probes for Northern analysis were made from the 1.3-kb XhoI-SalI fragment between coordinates 11.9 and 13.1 of the Sxl genomic map. This fragment includes exons 5-7, which are present in all Sxl transcripts.

 Fragments used to generate cDNA probes for RNAse protection were converted to blunt end molecules and subcloned into the EcoRV site of Bluescript SK (+). The female cDNA probe was generated by subcloning a 229-bp NdeI-BalI restriction fragment from a female cDNA (cDNA MS3 of Samuels et al. 1991). This fragment contains a portion of exons 2, 4, and 5. The more 3' acceptor site of exon 5 was used in the transcript that generated the cDNA, that is, the female protection probe contains the “long form” of exon 5. The male cDNA probe was generated by using PCR to subclone a 315-bp fragment from a male cDNA [cDNA CM1 of Bell et al. (1988)]. The male cDNA probe contains a portion of exon 2, the long form of exon 3, exon 4, and a portion of exon 5 (“short form”). Sequencing confirmed that subcloned fragments had identical sequences to the original cDNAs. In vitro transcription reactions used to produce labeled antisense RNA and RNAse protections were carried out as described by Promega (Madison, WI), except that final RNAse concentrations for digestion of hybrids were 4 μg/ml RNaseA and 70 U/ml RNaseT1 (Bethesda Research Labs, Gaithersburg, MD). RNAse protection products were analyzed...
lyzed on 6% polyacrylamide sequencing gels with end-labeled MspI-cut pBR322 fragments as size standards.

**Immunostaining of embryos**: Anti-SXL antibody was raised as mouse ascites tumors against a 183-residue female-specific fragment of SXL protein (plus an N-terminal methionine) starting at MSGSD and ending at EHEGK, which is common to all SXL isoforms and contains both RRM domains of the SXL protein. This Sxl polypeptide was generated using a standard T7 expression system in *Escherichia coli* strain BL21 [DE3] pLysS as described by Studier et al. (1990). Immunostaining was performed as described in Bopp et al. (1991), except that all blocking steps and antibody incubations were done in 0.1 M malate, 0.15 M NaCl, 0.1% Triton X-100, pH 7.5, containing 1% (v/v) Boehringer Mannheim block reagent (BMB 1096176). Anti-SXL antibody (α serum) and HRP-conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch) were used at 1:1,000 and 1:300 dilutions, respectively. DAB staining was enhanced by adding NiCl₂ (0.4% final concentration).

**Isolation and characterization of a SxlM° male-viable derivative**: The approach was similar to that reported in CLINE (1984), which generated SxlM° and SxlM°1 (including genetic “virginating” steps). Two- to five-day-old virgin females were exposed to 5,200–5,800 rad of γ rays and mated to Ore-R males. In the absence of further mutation, all matroclinous males from this cross will die. Surviving F1 males were collected as candidates for chromosomes carrying new loss-of-function Sxl mutations that suppressed in *cis* the dominant male-specific lethality of the parental SxlM° lesion. Approximately 75,000 chromosomes were screened, yielding only a single male-viable derivative, SxlM°1. Genomic Southern blot analysis showed that SxlM°1 contained no gross alterations in the region of Sxl (ca. 500 bp), indicating that the original transposon insertion associated with SxlM° had remained intact. The allele was determined to be a null based on its failure to complement a variety of partial-loss-of-function Sxl alleles, including the homozygous-viable allele, SxlM°/°, and a tandem duplication of SxlM°/°, which is semiviable but intersexual in compound with established null alleles in XX animals.

The specific molecular nature of the loss-of-function lesion associated with SxlM°/° is not yet known. In the course of investigating the source of the low viability and recombination rate of the SxlM°/° X chromosome, we discovered that the parental SxlM°/° sock had become fixed for an inversion with breakpoints in cytological regions 4 and 9. This inversion is unrelated to the SxlM° phenotype of this line and arose ≥2 years after SxlM°. Reduced male viability of SxlM°/°° was attributed entirely to the region 4 breakpoint, because full male viability was restored when that breakpoint was covered by the Dp(1;2) 4FR of *Salz* (1992). In contrast to the parental allele SxlM°° (see discussion of Table 3 below), neither female nor male SxlM°/°° embryos appear to produce SXL cross-reacting material. When stage 13–15 embryos from the cross SxlM°/°°° × FM3, Sxl°° × SxlM°/°°° were stained with anti-SXL antibody, only 27% of 161 embryos were positive, consistent with expectations if only SxlM°/°°° × FM3, Sxl°° daughters express SXL protein.

**RESULTS**

**DNA sequence analysis of SxlM° mutant lesions—five independent alleles comprise three different but related types**: Five spontaneous SxlM° alleles were known to be associated with transposon insertions within the region of Sxl responsible for SXL-dependent sex-specific Sxl RNA splicing. To define more precisely the location of these insertions and the identity of the transposons involved, we cloned the Sxl gene from each of the SxlM° mutants and sequenced the junctions of the insertion sites. The sites of insertion relative to the Sxl transcription unit are shown in Figure 1. Descriptions of the SxlM° insertions are summarized in Table 1. Because the five mutations proved to represent only three different allele types, throughout most of our analysis we characterized only three representative g-of alleles.

The first type is represented by alleles SxlM°1 and SxlM°2. These mutants were previously shown to be insertions of a roo (B104) element at or very near the same site (Maine et al. 1985). SxlM°2 arose on a chromosome carrying SxlM°°°°, a γ-ray induced derivative of SxlM° that is functionally indistinguishable from the wild-type allele but that retains what appeared to be a small fragment of the original SxlM° roo insertion. We sequenced the roo fragment (position 6.9–11.7 of the Sxl genomic map; see Figure 1) that contained the SxlM°°°° insertion. The insertion proved to be a single full-length (428 bp) roo LTR, indicating that SxlM°°°° arose from SxlM° by recombination loop-out between the two roo LTRs of the original SxlM° insertion. The insertion was located 674 bp downstream of the male-specific exon (exon 3) and 249 bp upstream of exon 4. (In wild-type Sxl, this intron is 918 bp long; however, the transposon generates a 5-bp duplication at its insertion site.) Sequencing across the Sxl-roo junctions of SxlM° and SxlM°2 confirmed that both of these full-length roo elements were present at the identical site and in
the same orientation as the $Sxl^{M1\text{-}ed1}$ insertion. Hence, the event that converted the single $roo$ LTR of $Sxl^{M1\text{-}ed1}$ to the full-length $roo$ element of $Sxl^{M2}$ seems likely to have been recombination between a double-stranded DNA $roo$ transposition intermediate and the $Sxl^{M1\text{-}ed1}$ LTR (see Boeke and Chapman 1991; Ji et al. 1993). The fact that the restriction maps of the $Sxl^{M1}$ and $Sxl^{M2}$ $roo$ elements are different (Maine et al. 1985) indicates that a different $roo$ element was the source of the insertion in these two cases.

The second type is represented by $Sxl^{M3}$ and $Sxl^{M5}$. These two mutations arose as independent events on two differently marked chromosomes, yet previous work indicated that they contained insertions of transposons with identical restriction maps at or near the same site in $Sxl$. Analysis of the insert junctions showed that the insertion site is indeed identical for these two independent alleles. That site is 6 bp upstream of the promoter-proximal 3′ splice site of the male-specific exon (Figure 2). There are two nearby 3′ splice sites to this exon. Although the transposon previously had been reported to be a 297 element, the sequence of the $Sxl^{M3}$ insert junction revealed that the transposon is a $hobo$ element, oriented so that it is transcribed in the same direction as $Sxl$ (see Finnegan and Fawcett 1986).

The third type is represented by $Sxl^{M4}$. Because the identity of this insertion was not clear from previous work, the 4.8-kb $XhoI$ fragment that contained the $Sxl^{M4}$ insertion was sequenced in its entirety. The $Sxl^{M4}$ insertion element, which we have called $hopper$, proved to be 1,435 bp in length (see Figure 3) and located within the male-specific exon 3 of $Sxl$, splitting it into a 135-bp 5′ fragment and a 60-bp 3′ fragment. $hopper$ contained perfect 33 bp inverted repeats at its ends and generated a 5-bp duplication of $Sxl$ sequences at its site of insertion. Both of these characteristics are typical of transposable elements; however, the sequence did not correspond to any in current databases. Using $hopper$ to probe Southern blots of genomic DNA that had been digested with EcoRI (which does not cut within the transposon), we found that element was present in ~20–30 copies per genome (data not shown). When genomic DNA was digested with $Ssp1$ and PvuII, both of which cut within $hopper$, a prominent band of 1200 bp was detected (data not shown), as predicted from the $Sxl^{M4}$ insertion sequence. This indicates that most copies of $hopper$ are similar in structure to $Sxl^{M4}$ over this region. The longest open reading frame within the $hopper$ sequence could encode a 141 amino acid peptide. However, because the codon usage of this putative peptide,

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**TABLE 1**

Insertions associated with $Sxl^M$ constitutive alleles

<table>
<thead>
<tr>
<th>$Sxl$ allele</th>
<th>Insertion element</th>
<th>Size (kb)</th>
<th>Site of insertion</th>
<th>Direction of transcription relative to $Sxl$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M1$ and $M2$</td>
<td>$roo$</td>
<td>9.5</td>
<td>674 bp downstream of male-specific exon 3; 249 bp upstream of exon 4</td>
<td>Opposite</td>
</tr>
<tr>
<td>$M1$-ed1, a male-viable derivative of $M1$</td>
<td>$roo$ LTR</td>
<td>0.43</td>
<td>Same site as $M1$ and $M2$</td>
<td>Opposite</td>
</tr>
<tr>
<td>$M3$, $M5$</td>
<td>$hobo$</td>
<td>3.5</td>
<td>At the 3′ splice site of male-specific exon 3 (see Figure 2)</td>
<td>Same</td>
</tr>
<tr>
<td>$M4$</td>
<td>novel ($hopper$); 33-bp inverted terminal repeats; 5-bp target site duplication</td>
<td>1.4</td>
<td>Within the male-specific exon 3; splits exon 3 into 135 and 60 bp segments</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

*For a description of the $roo$ and $hobo$ elements, see Finnegan and Fawcett (1986).
like that of the other smaller open reading frames within hopper, showed a poor match to preferred Droso-
phila codon usage, it is unclear whether the mobile
sequences required for hopper transposition. There has
failed to hybridize to mRNA from embryos or adults
that otherwise would have died as a conse-
sequence of the other smaller open reading frames
as a probe of Northern blots, the cloned hopper
sequence may be a fragment of a larger full-length element and not itself encode
ka and hence had only the
hypotheses of many that were examined are shown in Table 2. The experimental females were hemizygous for hypo-
alleles of sisA and sisB. Females from crosses B, D, and F were also heterozygous for a null Sxl allele and hence had only the Sxl allele to provide Sxl function. Clearly, Sxl was more effective at suppressing
female lethality (45-73%) than Sxl (1-38%), which
which was more effective than the original allele Sxl, which
male lethal to males, they do differ significantly in their ability
to rescue females.

Data for the two most extreme female-lethal situations of many that were examined are shown in Table 2. The experimental females were hemizygous for hypo-
the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under the accession

The three classes of Sxl alleles differ in their ability to bypass the primary sex determination signal: The Sxl alleles were isolated based on their ability to rescue daughters that would otherwise have died as a conse-
sequent position numberX80025,

| 1 | CACCATGCGG CATTGCGCT GTTTTTTTTA CAAAATTAAT TACCTTCAA |
| 51 | ACTATGCGAG ATATAAGTTT TATACCGTGA TACATCGCCT CAGATGCCCT |
| 101 | CAGGAAATTT TTGGAAGAAT CACCTGCCGC CACCTGCCGC CAGATTTCCT |
| 151 | TTTTTTTTTT TTTTTTTTT TAAATAATTT TTTAAAGATT TATTTTTAAT |
| 201 | TCTTCAATTT TTATATATTT TAAACATAT TAAATTAACAT TATTTATTGA |
| 251 | CTGAGAAATT TTGAGAACGT TCAAAATGCC AAGCTACCT TTCAATGACT |
| 301 | CAGTGATGAGA GAGATAATGC CTTATTATT TCAACTTCTA AAATTATTTG |
| 351 | TGATTTATAT GGTCCCGAG TACCCATTTT TATGTAATAAG GACATCGGCA |
| 401 | AGCTTTAAGG GTTCTTCAGG TAAAATATTG TTTTCTATTT AACATTAAA |
| 451 | ATGGCTGCAA GATCCCGCAG GCAGCACTTC CGTGCAGCTT GAACCAAAA |
| 501 | TCTGGTGGAA GAGATAGTCG CTTATGTTTT CGCCTCTTTA AATTTATTGA |
| 551 | AGCTACTAAT AGTTTTTGCC GTGGCTGGCT TCAACAAAAG AATTTTAAGT |
| 601 | TCTCTTCTCT ATATAATTT ACTCTCAAAC TTAACACTAC TACATTATTT |
| 651 | TACCAAAATT GAAATTAAAT TTTAATATTT ATATATTTTT CAGATTGATT |
| 701 | TCTTTCAATT TCGTAAGAAG TGTACCGTAT CCTATAGAGA CAGTAAGATG |
| 751 | TATTTGAAAT TTATATATTT TAAACATAT TAAATTAACAT TATTTATTGA |
| 801 | ATATAATTTT GTAATTTTT TTATGAACGT TCAAAATGCC AAGCTACCTT |
| 851 | CCACACTTCC AGAGGGCAG CTTGGAAGAT CATGATATGG TTCAAAATTT |
| 901 | ATGTGAAATT TCTCAAAACCG CGCAAAAAC GACATAGAG ACTACCTGAT |
| 951 | ATGAGTTTGG ATGAGTTTGG CTTATGTTTT CGCCTCTTTA AATTTATTGA |
| 1001 | CAGGAATATT TTGGAAAAGT GGGCGTGCCC CAACTCCGCC CCATTTTTTT |
| 1051 | ACTATTGGAG ATATTTGGAT GAATTTTTTT TTATGCGTTA CACATGCCTC |
| 1101 | ACTAAAATAC ACTTTAACAG ACTTTATATC ACTTTATATC ACTTTATATC |
| 1151 | ACTAAAATAC ACTTTAACAG ACTTTATATC ACTTTATATC ACTTTATATC |
| 1201 | ACTAAAATAC ACTTTAACAG ACTTTATATC ACTTTATATC ACTTTATATC |
| 1251 | ACTAAAATAC ACTTTAACAG ACTTTATATC ACTTTATATC ACTTTATATC |
| 1301 | ACTAAAATAC ACTTTAACAG ACTTTATATC ACTTTATATC ACTTTATATC |
| 1351 | ACTAAAATAC ACTTTAACAG ACTTTATATC ACTTTATATC ACTTTATATC |
| 1401 | ACTAAAATAC ACTTTAACAG ACTTTATATC ACTTTATATC ACTTTATATC |

Figure 3.—Restriction map and sequence of the novel Sxl insertion element, hopper. This sequence will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under the accession number X80025.
the magnitude of such genetic background differences on female rescue. The fact that the relative viability of the +/+ Sxl allele was 73% for a female genotype with SxlM that had three fewer doses of the female-vital six' alleles and no SXL allele, illustrates just how effective the strongest SxlM allele is in suppressing extremely powerful female-lethal effects.

SxlM transcripts in adult females: As a first step in analyzing the effects of the transposon insertions carried by SxlM alleles on Sxl mRNAs, we examined transcripts produced in adult females of the genotype SxlM/SxlP780. Because SxlP780 is a male-viable deletion of the entire Sxl transcription unit, all Sxl transcripts in such females must be derived from the SxlM allele. Such hemizygous females are fully viable (data not shown). SxlM generates three size classes of transcripts in adults: a female series ca. 4.1, 3.1, and 2.0 kb in length and a corresponding male series each ca. 200 bp longer, reflecting the inclusion of the male-specific exon 3 (Salz et al. 1989; Samuels et al. 1991). Because only the middle-size class of male mRNA is very abundant, it is the species that is most useful for sex-specific splicing comparisons on Northern blots. The Northern blot data of Figure 4 show that in adult females, each SxlM allele was capable of producing the same three size classes of mRNAs found in wild-type females (OR-R). No male transcripts were observed in these animals. Most important, no aberrant transcripts were detected, indicating that the insertions did not appear to promote transcription of novel mRNAs that had originated from within the repetitive elements.

These conclusions were supported by RNase protection data shown in Figure 5. Figure 5 describes the probes used for this study. A labeled antisense RNA that contained a portion of a Sxl female cDNA extending from exon 2 (upstream of the SxlM insertions) down into the long form of alternatively spliced exon 5 (not a sex-specific alternative splice) was hybridized to mRNA from Sxl+ and SxlM females. In all cases the same three protection products were produced (Figure 6A). These products corresponded to the fragment predicted for full protection of the probe (229 nucleotides, Figure 5A, a) by “long exon 5” wild-type female transcripts, as well as the two protection fragments expected (136 and 69 nucleotides, Figure 5A, b) from hybridization to “short exon 5” wild-type female transcripts. Most importantly, we did not detect a protection product of 206 nucleotides (Figure 5A, c) that would have been present if Sxl transcripts had originated from within the elements.

This RNase protection experiment tested for novel transcripts from the SxlM alleles, but the probe used would not have detected male transcripts produced in these strains if the resulting looped out region of the cold male RNAs failed to induce nicking of the hot probe. Therefore, to test more directly for male tran-

### TABLE 2

<table>
<thead>
<tr>
<th>Cross</th>
<th>SxlM allele</th>
<th>Sxl+ allele</th>
<th>Temperature (°C)</th>
<th>Number recovered</th>
<th>Viability relative to control sibs</th>
<th>Number recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>M1</td>
<td>Wild-type</td>
<td>25</td>
<td>13</td>
<td>6%</td>
<td>217</td>
</tr>
<tr>
<td>A</td>
<td>M1</td>
<td>Wild-type</td>
<td>29</td>
<td>0</td>
<td>0%</td>
<td>90</td>
</tr>
<tr>
<td>B</td>
<td>M1</td>
<td>Null (f1)</td>
<td>25</td>
<td>76</td>
<td>21%</td>
<td>356</td>
</tr>
<tr>
<td>C</td>
<td>M3</td>
<td>Wild-type</td>
<td>25</td>
<td>57</td>
<td>19%</td>
<td>299</td>
</tr>
<tr>
<td>C</td>
<td>M3</td>
<td>Wild-type</td>
<td>29</td>
<td>1</td>
<td>1%</td>
<td>119</td>
</tr>
<tr>
<td>D</td>
<td>M3</td>
<td>Null (f1)</td>
<td>25</td>
<td>135</td>
<td>38%</td>
<td>358</td>
</tr>
<tr>
<td>E</td>
<td>M4</td>
<td>Wild-type</td>
<td>25</td>
<td>118</td>
<td>45%</td>
<td>261</td>
</tr>
<tr>
<td>E</td>
<td>M4</td>
<td>Wild-type</td>
<td>29</td>
<td>26</td>
<td>48%</td>
<td>54</td>
</tr>
<tr>
<td>F</td>
<td>M4</td>
<td>Null (f1)</td>
<td>25</td>
<td>277</td>
<td>73%</td>
<td>382</td>
</tr>
</tbody>
</table>

Note: No SxlM males were recovered in any of the crosses.

1 Balancer, Dp(1;1)sisB+, Sxl+/Df(sisB); Df(sisA); Dp(1;2)sisB+sisA+/+. Full genotypes of crosses (females × males): A, C, and E, sc's<sup>ab</sup> w cm SxlM v sisA m/Binsinney, γ w sn B × Df(1)sc<sup>+</sup> and Df(1)N71, yrsisB+sisA/-; γ X2P(1;2)Hw<sup>+66</sup> and Dp(1;2)v<sup>56</sup>, y+Hw sisB+sisA+/+. Note: sc's<sup>ab</sup> is a new designation for sis<sup>b<sup>53</sup></sup> (a.k.a. sc<sup>b6</sup>). See MATERIALS AND METHODS. B, D, and F, same females as above × Df(1)sc<sup>+</sup> and Df(1)N71, yrsisB+cm Sxl<sup>P780</sup>; Xs sisA+/Y; X2P(1;2)Hw<sup>+66</sup> and Dp(1;2)v<sup>56</sup>, y+Hw sisB+sisA+/+.
script species, we used a probe derived from a portion of a Sxl male cDNA (Figure 5B). When hybridized to mRNA from SxlM adult females, only the predicted female protection product of 88 nucleotides was produced (Figure 6B). No male protection products were produced, which indicated that even with this very sensitive assay, usage of the male-specific exon 3' splice site seems to be completely blocked in SxlM females. We did note a very low but detectable level of male-spliced forms in RNA from wild-type females and female-spliced forms in RNA from wild-type males, but we estimate that these protection products were never present at >5% of the level of the expected splice forms.

**SxlM transcripts in mixed-sex embryos:** The experiments described above showed that the SxlM transposon insertions allow for a wild-type transcript pattern in adult females, with perhaps some lowering of the already low background of male-spliced forms. More meaningful for understanding the mode of action of the SxlM alleles, however, is the effect of these insertions during development, particularly in males. Such analysis is more complicated, however, because of the domi-
A Northern blot analysis of Sxl transcripts generated by such Sxl\(^{M}\) balanced lines during the embryonic period is shown in Figure 7. The wild-type control (OR-R) shows that the same size classes of male and female RNAs are present throughout most of the embryonic period as in adults. In addition, female embryos between 2 and 4 h postfertilization produce a transient set of somewhat lower molecular weight transcripts (labeled “initiation” in Figure 7) that initiate Sxl expression (Salz et al. 1989; Keyes et al. 1992). Figure 7 shows that throughout embryogenesis, the weakest g-o-f allele, Sxl\(^{M}\), produced a pattern of Sxl transcripts very similar to that of the wild-type, including male species as well as female. In contrast, the stronger alleles, Sxl\(^{M3}\) and Sxl\(^{M4}\), are clearly deficient with respect to male Sxl transcripts in these mixed-sex populations. Generation of Sxl\(^{M}\) (initiation) transcripts appears wild-type for all three Sxl\(^{M}\) mutants. There is no indication of an elevation or persistence of this species of transcript to suggest any effect of these g-o-f mutations on Sxl\(^{M}\) expression in male embryos.

As a more sensitive assay for production of transcripts that included the male-specific exon, we used RNase protection to measure the relative levels of female vs.
male transcripts during embryogenesis (Figure 8).

(See Figure 5B for a description of the predicted protection fragments.) The results from this assay were consistent with the Northern data. Throughout embryogenesis, SxlM embryos appeared to synthesize male and female transcripts at the same level and in the same ratio as wild-type embryos. By this more sensitive assay, SxlM and SxlP embryos were found to be capable of producing some male transcripts, but the level was much below that for the wild-type. SxlM embryos produced low levels of both forms of male RNAs, indicating that sequences near the end of the hobo element must be capable of functioning as a branch site (s) for both of the alternative male exon acceptor sites. In addition to the normal male protection products, RNA from 8 to 12 h SxlM embryos generated a prominent protection product of 145–150 nucleotides, as well as other minor products that were not produced in other embryos. The sizes of these products were consistent with cleavage of the cDNA probe opposite the loop that would be formed by the SxlM insert if it remained in the processed RNA (See Figure 5B, d and e). The level of such an aberrant mRNA was not sufficiently high to be detected on Northern blots.

The appearance of SXL protein in SxlM male embryos: As an indirect assessment of female-specific Sxl transcript splicing in SxlM males, we stained populations of embryos at various developmental stages with Female-specific anti-SXL antibodies (Table 3). The crosses used were the same as for the embryo RNA analysis described above; hence, any SXL protein detected had to have been derived from the mutant SxlM alleles. Half of the embryos had no Sxl allele and served to establish the background level of staining in the absence of SXL protein, whereas one quarter were females with a single copy of SxlM and served as the standard for full female staining at each developmental stage. Of interest was the level of anti-SXL staining among the remaining one quarter of embryos that were male and that carried a single copy of SxlM, just like their sisters. The data in Table 3, group I, show that early in development, soon after expression of Sxl(m) has begun, the proportion of SXL-positive embryos was near 25%, indicating that most SxlM male embryos were splicing little if any of their Sxl transcripts in the female mode. However, as development proceeded (group II), the fraction of SXL-positive embryos rose toward the 50% level expected if both male and female SxlM-bearing embryos produced female SXL protein. When staining first rose above the 25% level, two distinct classes of stained embryos were observed: a lighter (“intermediate”) class as expected if both male and female SxlM-bearing embryos produced female SXL protein. When staining first rose above the 25% level, two distinct classes of stained embryos were observed: a lighter (“intermediate”) class presumably representing the males and a darker (“full staining”) class expected to be their female sibs. As development continued (group III), however, male and female staining eventually became indistinguishable for embryos carrying the stronger alleles, SxlM and SxlP, indicating that the level of SXL protein in those mutant male embryos had eventually reached the threshold required to fully engage the Sxl RNA splicing feedback loop. Two distinct staining classes remained at the later stages for the weaker allele, SxlM, presumably reflecting the failure of SxlM males to fully engage the splicing feedback loop by developmental stage 15; however, some of the SxlM animals at later stages did display a salt-and-pepper pattern of staining, with some dark cells arrayed against a lighter-staining background. This staining pattern suggested that although the mutant males as a whole had not fully engaged the RNA splicing feedback loop, some cells within some mutant males had reached the required threshold. Similar salt-and-pepper staining patterns have been seen to develop at
later stages for many partial-loss-of-function Sxl alleles in females, an important difference being (as expected) that the darkly staining cells are eventually present against an unstained background (data not shown).

**Transcript analysis for SxlM male-viable derivative alleles:** We wanted to be able to distinguish primary cis-acting effects of SxlM insertions on Sxl transcript splicing in males from secondary effects that could be expected to arise as a consequence of the autoregulatory activity of any female SXL proteins that might be made. We were also anxious to examine the effects of SxlM insertions on Sxl transcript splicing in pure populations of fully viable males. To achieve these ends, we turned to the analysis of male-viable double-mutant derivatives of SxlM alleles. The male viability of such derivatives results from the generation of loss-of-function mutations that, in cis, suppress the effects of the g-o-f insertion present in the same allele (CLINE 1984). SxlM/F3, Sxl/F2,M, and Sxl/0,M are examples of such male-viable derivatives (Table 4). The loss-of-function mutations SxlF3 and SxlF2 reduce Sxl activity, whereas SxlF0 eliminates it. In all three cases the effect on Sxl activity is due to changes in the proteins encoded. Although SxlM/F3 and Sxl/F2,M have a residual level of constitutive activity that can be assayed in females, males tolerate these double-mutant alleles without ill effects, even in the presence of a duplication of Sxl+ (CLINE 1984, 1986). Whatever the residual level of constitutive activity is in males, it is insufficient to activate a Sxl+ allele in trans in the absence of additional copies of other regions of the X that are now known to be involved in autoregulation (see DISCUSSION).

Figure 9A shows that, as judged by RNase protection analysis, SxlM/F3 and Sxl/F2,M adult males exhibited significantly elevated levels of female splicing, consistent with their retaining some low level of constitutive Sxl expression in this sex. However, as expected from the previous analysis of SxlM in embryos, a substantial
amount of RNA was still spliced in the male mode. Interestingly, the \textit{Sxl}^{M1.3} derivative produced only a single class of male-protection product (260 bp), indicating that this allele was only capable of using the downstream male exon 3' splice site. This is a consequence of the transversion mutation associated with \textit{Sxl}^{M3} at the 5' splice site of exon 2 and is not a feature of \textit{Sxl}^{M4}. Although these results would appear to suggest that \textit{Sxl}^{M1} can generate significant levels of female \textit{Sxl} RNA in the absence of \textit{Sxl} autoregulation, results with the null derivative, \textit{Sxl}^{10, M4}, argue otherwise. By both Northern analysis (Figure 9B) and RNase protection (Figure 9A), this double mutant appeared to generate only the normal male splice forms of \textit{Sxl} RNA. As a control to exclude the possibility that the \textit{Sxl}^{10} mutation might be helping suppress \textit{Sxl} male lethality by acting in \textit{cis} directly to inhibit the joining of exon 2 to exon 4 (the female-specific splice), \textit{Sxl} transcripts in heterozygous \textit{Sxl}^{+} / \textit{Sxl}^{10, M4} females were examined. In this case only normal female splice forms were observed, indicating that transcripts from \textit{Sxl}^{10, M4} could be spliced fully in the female mode in the presence of \textit{Sxl} activity supplied in \textit{trans}.

We used the same male-viable derivative approach to determine whether the stronger allele, \textit{Sxl}^{M2}, was capable of generating female splice forms in the absence of \textit{Sxl} autoregulatory activity. Northern analysis of a functionally null male-viable derivative, \textit{Sxl}^{M3/d} (Figure 10A), showed that this allele produces only a very low level of \textit{Sxl} mRNA in males, but what little transcript is produced is spliced in the female mode. The absence of a significant level of male spliced forms was consistent with the behavior described earlier for \textit{Sxl}^{M3} in embryos. The conclusions from Northern analysis were confirmed by RNase protection (Figure 10B). Although the absolute level of the female splice form in \textit{Sxl}^{M3/d} adult males was only moderately higher than that for wild-type males, it was far greater than that of the male splice forms. Hence, for \textit{Sxl}^{M3}, the ratio of \textit{Sxl} mRNAs is greatly skewed toward the female forms even in the absence of autoregulatory activity.

### DISCUSSION

The ability of the \textit{Sxl}^{M} alleles to partially bypass the normal control of \textit{Sxl} by X-chromosome dose is responsible both for their dominant lethality in males and their ability to rescue females from mutations affecting the primary sex determination signal. The work reported here is an investigation at the molecular level of how this bypass of the sex determination signal occurs. Analysis of the bypass mechanism is complicated both

<table>
<thead>
<tr>
<th>Developmental stage*</th>
<th>Antibody staining level</th>
<th>From mothers carrying \textit{Sxl}^{M1}</th>
<th>From mothers carrying \textit{Sxl}^{M3}</th>
<th>From mothers carrying \textit{Sxl}^{M4}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I. Late stage 5 through 7</td>
<td>No staining</td>
<td>98</td>
<td>78</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>Intermediate staining</td>
<td>4</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Full staining for stage</td>
<td>24</td>
<td>19</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Total embryos collected</td>
<td>126</td>
<td>83</td>
<td>115</td>
</tr>
<tr>
<td>Group II. Stages 8 through 12</td>
<td>No staining</td>
<td>187</td>
<td>58</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td>Intermediate staining</td>
<td>76</td>
<td>23</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Full staining for stage</td>
<td>61</td>
<td>19</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>Total embryos collected</td>
<td>324</td>
<td>307</td>
<td>587</td>
</tr>
<tr>
<td>Group III. Stages 13 through 15</td>
<td>No staining</td>
<td>70</td>
<td>42</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Intermediate staining</td>
<td>53</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Full staining for stage</td>
<td>44</td>
<td>26</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>Total embryos collected</td>
<td>167</td>
<td>174</td>
<td>213</td>
</tr>
</tbody>
</table>
by the dominant lethality of the SxlM alleles and by the positive autoregulatory activity of SXL protein. In wild-type chromosomal females, it is positive autoregulation that translates the transient very early transcriptional effect of the sex determination signal on SxZpe into a long-lasting effect on RNA splicing of transcripts from SxZpr, committing cells to the male or female mode of development during the remainder of the life cycle. SxlM mutant lesions must somehow generate SXL proteins in the absence of the SXL protein normally generated early in development as a consequence of SxZpe expression in response to the primary sex-determining signal, the X:Y balance.

One possibility that seems ruled out by the data presented here is that the SxlM transposon insertions induce expression of SxZpr in the absence of the regulatory factors normally required for the functioning of this promoter. The anti-SXL antibody staining of young embryos presented in Table 3 indicates that SXL proteins appear in SxlM males significantly later than they appear in females and well after the point at which the switchover to SxZpr has taken place. Therefore, if induction of SxZpr expression were the basis for SxlM effects in males, this abnormal SxZpr expression would have to occur significantly later in SxlM mutant males than in wild-type females. But our RNA analysis of mixed-sex embryos and of adult females shows that SxlM mutations appear to have no effect on the timing of SxZpr expression. Our results pointing to an effect of the SxlM mutations only after the switchover to SxZpr has occurred are consistent as well with earlier work on dosage compensation showing that the initial sex-specific functioning of Sxl, which we now know reflects the expression of SxZpe (BERNSTEIN and CLINE 1994), occurs normally in SxlM embryos (GERGEN 1987).

It seems far more likely that SxlM lesions partially bypass the requirement for autoregulation by changing the splicing rules for SxZpr-derived transcripts. However this bypass is accomplished initially, once a significant level of SXL protein has been generated by this primary effect, operation of the normal mechanism of autoregulation can lead to a far higher self-maintained level of expression of Sxl’s feminizing activities. This feedback effect can potentially obscure the primary effect of the mutant lesions. Anti-SXL antibody staining of young embryos showed that although the synthesis of SXL protein in mutant SxlM males initially lags behind that in their sisters, who had the benefit of a head start provided by SxZpe expression, most of the SxlM3 and SxlM4 males catch up to their sisters by the time of dorsal closure. Males that carry the weaker g-o-f mutation, Sxl", are much slower to approach the female level of SXL protein expression, with only a minority of cells within a minority of mutant male embryos reaching the female level by the time of dorsal closure.

The location of the SxlM lesions suggests that the primary effect of these mutations is likely to be disruption of the normal splicing rules for SxZpr transcripts.
so that female splice forms are generated even in the absence of preexisting SXL protein. Horabin and Schedl (1993b) showed that the major determinant of the sex-specific splicing of Sxl-pre-derived RNA is the ability to make the splice between the 5' donor site of male-specific exon 3 and the 3' acceptor site of exon 4. In males, which normally lack SXL protein, this splice occurs efficiently and is subsequently followed by the joining of exon 2 and the male-specific exon, producing a nonfunctional mRNA. In females, inhibition of the splice between the male-specific exon and exon 4 by SXL protein strongly favors the joining of exon 2 directly to exon 4, skipping the male-specific exon and leading to the production of mRNA that encodes active SXL protein. The SXL binding sites that allow SXL protein to block the joining of exon 3 and exon 4 have yet to be defined, but there is likely to be a large number of them on both sides of the male-specific exon (Horabin and Schedl 1993b; Samuels et al. 1994). It is important to appreciate that although the male mode of Sxl RNA splicing is the “default” state for wild-type Sxl pre-mRNA, transgene splicing reporter constructs that do not leave the entire exon 2–3–4 region intact produce high levels of female splice forms even in the absence of SXL protein (Horabin and Schedl 1993a,b). Thus, it appears that the default mode of splicing is the male mode rather than the female as consequence of the specific structure of the entire exon 2–3–4 region of Sxl rather than being an intrinsic property of the particular splice donors and acceptors themselves. As a consequence, insertions into any part of the exon 2–3–4 region might have the potential to directly effect the male/female splicing choice.

We show here that the strongest of the three SxlM mutant types represented among this group of five spontaneous mutations is due to a transposon insertion into the male-specific exon 3 itself. The next strongest is an insertion into one of the two alternative 3' splice sites of that same exon. Even the weakest of the alleles, the original g-0-f mutant, SxlM, is due to an insertion in the intron between exons 3 and 4, the region of the key regulated splice. However, we found that splicing is not grossly disrupted even with such insertions: all three SxlM alleles are still capable of generating some of the normal male RNA splice forms in the absence of SXL protein (at least during the embryonic period) and a fully wild-type level of the normal female mRNAs in its presence.

Analysis of the transcripts generated in adult males
by the null double-mutant allele, \( Sxl^{M3/d} \), showed that \( Sxl^M \) does have a significant direct effect on the default \( Sxl \) splicing pattern, skewing the ratio toward the female alternative. But in view of the fact that disruptions of the region between exons 2 and 4 are known to increase the level of female-specific splice forms (see above) and that \( Sxl^M \) alleles are invariably male lethal, the level of female splice forms generated by \( Sxl^{M3/d} \) in the absence of autoregulatory activity was remarkably low. Interpretation of the results with the male-viable derivative, \( Sxl^{M3/d} \), is complicated somewhat by the fact that the overall \( Sxl \) transcript level in adult mutant males was low. It is possible that the second-site loss-of-function mutation in \( Sxl^{M3/d} \) destabilizes \( Sxl \) transcripts. Alternatively, some transcripts initiated at \( Sdl_{rp} \) may splice into the \( hobo \) element of \( Sxl^{M3/d} \) in males, yielding aberrant mRNAs that are degraded. This lowered level of \( Sxl \) transcript may account for the fact that \( Sxl^{M3/d}/Y \) males were seen only to produce female transcripts, yet a mixed sex population of \( Sxl^{M3} \) embryos exhibited some male splicing. Only very low levels of male splice forms were found in populations of \( Sxl^{M3} \) embryos, suggesting that even in \( Sxl^{M3} \) males, the female \( Sxl \) splice forms predominate. If the level of male transcripts in \( Sxl^{M3/d} \) was a similarly small fraction of the already low level of female transcripts, it is likely that they would have been below the level of detection, even of the sensitive RNase protection assay.

The situation with the weaker g-o-f allele, \( Sxl^M \), was even more surprising. Splicing of the transcripts from a null double-mutant male-viable derivative of \( Sxl^M \) in adult males was essentially indistinguishable from that for wild-type males, with no measurable level of female splice forms generated. Moreover, in male embryos, \( Sxl^M \) produced nearly wild-type levels of male transcripts, in contrast to the other two types of \( Sxl^M \) alleles.
The low level of female transcripts observed in males harboring the null male-viable derivatives is somewhat difficult to reconcile with the tight dominant male-lethal phenotypes of the parental Sxl" alleles. Where do the initial female splice forms come from in the absence of SxI\textsubscript{up} expression? Could dominant male lethality be caused by a significant level of female splicing in only a few vital cells of the organism, making the transcripts difficult to detect by standard bulk RNA analysis? The embryo anti-SXL antibody staining results reported here argue against this possibility, especially for Sxl\textsuperscript{M1} and Sxl\textsuperscript{M2}. Even for Sxl\textsuperscript{M1}, substantial amounts of SXL protein were observed throughout the mutant male embryos; moreover, earlier analysis of genetic mosaics and SxlM4. Even for Sxl" alleles to autoregulate by SXL protein. By this hypothesis, the level of SXL protein required to establish the positive feedback loop for Sxl" alleles might be considerably lower than that required for Sxl" alleles. The f 3 and f 7 partial-loss-of-function mutations in these two male-viable derivatives greatly reduce but do not eliminate constitutive autoregulatory activity; however, the level of autoregulation achieved by a single dose of either of these double mutants in males is below the level that will activate an Sxl" allele in trans, as evidenced by the full viability and normal phenotype of males carrying Sxl" and either double-mutant allele (CLINE 1984). [Note, however, that Sxl" can be trans-activated in males at low frequency during the larval stage by a single dose of Sxl\textsuperscript{M1,7} if the dose of cytological region 4 of the X chromosome is increased; see the footnote to Table 17 in CLINE (1988).] Nevertheless, we show here that both of these double-mutant alleles have significantly higher levels of female Sxl splice forms in adult males than the null double mutant, Sxl\textsuperscript{f,10,11}. Hence, it appears that the residual autoregulatory activity of the mutant f 3 and f 7 proteins that is not sufficient to activate a wild-type Sxl allele must be sufficient to influence the splicing of transcripts from the two double-mutant alleles. A significantly increased sensitivity to autoregulation for the Sxl\textsuperscript{M1} alleles relative to Sxl" would also account for the observation that when SxI\textsubscript{up} expression is completely blocked by mutations in the six genes (Table 2), the ability of Sxl\textsuperscript{M} alleles to rescue females was not increased by the presence of an Sxl" allele. In such a situation, the rate-limiting levels of SXL protein that would determine whether the autoregulatory splicing loop would or would not engage for Sxl\textsuperscript{M} alleles could be far below the levels that would be needed to engage the loop for Sxl" alleles; hence, under these conditions the presence of an Sxl" allele would be of little consequence. Even with such increased sensitivity to autoregulation, however, it is clear both from RNA and SXL protein analysis reported here that most of the cells of Sxl\textsuperscript{M} male embryos have not fully engaged the female-splicing feedback loop. Such engagement might not occur for them until the larval stage in development, and indeed some mutant cells may never engage. It was shown years ago that some chromosomally male cells can occasionally escape the deleterious effects of this weakest of the g-o-f alleles (CLINE 1979), presumably because they never reach the threshold for stable autoregulation.

We found no evidence in the present study for any direct source of constitutive behavior for the Sxl\textsuperscript{M} alleles other than from effects on Sxl\textsubscript{up}-transcript splicing. Formally, however, other mechanisms could also lead to activation of Sxl\textsuperscript{M} alleles. For example, enhancer elements within the Sxl\textsuperscript{M1} insertion could promote transcription initiation between exon 3 and exon 4, and translation initiation of such transcripts at start codons within exon 4 could produce SXL proteins, truncated by either 38 or 48 amino acids, that contained the RNA binding domain of SXL. Arguing against this idea is the fact that we saw no novel transcripts or splice forms originating from Sxl\textsuperscript{M1}, whose translation might generate a truncated SXL product or a fusion protein with autoregulatory activity. Moreover, there is no evidence to suggest that a SXL protein missing its normal N terminus would be functional, particularly at low levels. Indeed, the indication that low levels of truncated SXL protein are present in male heads with no adverse effect on sexual behavior argues against such activity (Bopp et al. 1991). In the present paper we also show that a point mutation, Sxl\textsuperscript{M13}, in exon 2, far upstream of the roo insertion into Sxl\textsuperscript{M1}, can suppress the dominant male-lethal phenotype of this g-o-f lesion—one more indication that whatever the primary effect of the Sxl\textsuperscript{M1} insertion might be, its phenotype must depend to a large extent on the productive splicing and translation of standard Sxl\textsubscript{up}-derived transcripts.

The RNase protection experiments with Sxl\textsuperscript{M} embryos indicated that this allele produces at least some Sxl-hopper fusion transcripts (Figure 8), but the functional implications of this are unclear. It is interesting to note that clipping opposite the hopper insertion generated easily detectable protection products from exons downstream of the insertion (148/153 bp, corresponding to the 3' end of exons 3–5) but only a low yield of protection products from upstream of the insertion
exon 2 had been spliced directly to the hopper element. We note, however, that at least a proportion of the SxlM transcripts initiated within the hopper element will be needed to determine if either of these possibilities is correct.

Although these spontaneous SxlM alleles have been useful for the genetic analysis of the sex determination pathway and of Sxl itself, the characterization we present here reinforces the point that these alleles only partially bypass the primary sex-determination signal. Moreover, their level of constitutive expression of female functions depends strongly on their response to Sxl RNA splicing autoregulation, a response that may differ in sensitivity from that of wild-type alleles. These complexities need to be considered when conclusions are made based on phenotypic interactions between SxlM alleles and mutations in other elements of the sex determination pathway. In discussing regulatory relationships between Sxl and other genes in this pathway and in discussing the sex-determination signal itself, it might be prudent to avoid use of the somewhat ambiguous term “upstream” unless the distinction between effects on Sxlp vs. Sxl autoregulation is clearly made.

Of the members of this set of spontaneous SxlM alleles, we show here that SxlM is the strongest, judged by its ability to rescue females from mutations in the sex determination signal and by its low level of male splicing in embryos. For that reason it would seem to be the allele of choice in epistasis studies. The constitutive character of even this strong allele, however, may still depend on autoregulation and hence be sensitive to mutations in genes required for that process. Only many hours after the activation of Sxlp does the level of SXL protein in SxlM/Y male embryos appear to reach that of their SxlM/Sxl sisters. Moreover, this point seems not to be reached much earlier for SxlM than for SxlM, an allele shown here to be sensitive to autoregulation. This result could stem from some significant early difference in the level of expression of Sxlp between haplo-X and diplo-X embryos that eventually disappears; alternatively, it could reflect autoregulation steadily ramping up the level of productive splicing of Sxlp-derived transcripts generated by the g-of allele.

Because the primary transcripts of these SxlM alleles will contain large stretches of transposon RNA, it is difficult to predict—or indeed to determine—the many ways in which their processing might differ from that of the wild-type. It will be interesting to contrast the molecular effects of these transposon insertion SxlM alleles with the effects of deletion SxlM alleles that are now accumulating as the byproduct of screens to identify genes of the primary sex-determination signal (D. A. BARRASH, S. J. FEIST, and T. W. CLINE, unpublished data). Such deletion SxlM alleles are likely to be of particular value for elucidating the RNA structure-function relationships that govern sex-specific Sxl splicing. Most useful in this connection will be lesions whose constitutive activity is less dependent on Sxl RNA splicing positive feedback than is the case for the transposon-insertion alleles described here.

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