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

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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" (Sxl^M) 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, Sxl^M haplo-*X* animals (chromosomal males) die because of improper dosage compensation, and Sxl^M chromosomal females survive the otherwise lethal effects of mutations in upstream positive regulators. Five independent spontaneous Sxl^M 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: Sxl^{M1}, Sxl^{M3}, and Sxl^{M4}. Sxl^{M1} is an insertion of a *roo* element 674 bp downstream of the translation-terminating male-specific exon. Sxl^{M3} is an insertion of a *hobo* transposon (not *297* as previously reported) into the 3′ splice site of the male exon, and Sxl^{M4} 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 Sxl^{M4} being the strongest and Sxl^{M1} 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 Sxl^M derivatives in which the Sxl^M insertion is *cis* to loss-of-function mutations, combined with other results reported here, indicates that the constitutive character of these Sxl^M 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 Sxl^M 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 SKRIPSKY 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

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be observed readily in genetic mosaics and triploid intersexes (CLINE 1979; SANCHEZ and NÖTHIGER 1982; CLINE 1983).

G-o-f 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 *daughterless* (CLINE 1978). Although the maternal activity of *da* normally is required for the activation of Sxl^+ in the zygote, *Sxl* g-o-f 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 Sxl^M alleles, in contrast to the female-lethal Sxl^f 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, Sxl^M 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_{Pe} (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_{Pe} in response to their double dose of *X*-linked "*X*:A numerator element" genes. The single dose of these same genes in males is not sufficient to activate Sxl_{Pe}. Throughout most of development, however, Sxl_{Pe} 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_{Pm}, located 5 kb upstream of Sxl_{Pe} (BELL *et al.* 1988, 1991; SAMUELS *et al.* 1991). Only females splice Sxl_{Pm} 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_{Pm}-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_{Pe}, unlike those from Sxl_{Pm}, are productively spliced even in the absence of SXL protein. Thus, it is the early burst of SXL protein from transcripts originating at Sxl_{Pe} that initiates the female commitment by setting the autoregulatory feedback loop in operation for Sxl_{Pm} transcripts. Although *Sxl* is nonfunctional in males, Sxl_{Pm} 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_{Pe} 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 Sxl^M 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 Sxl^M alleles might bypass the normal sex-determination signal: (1) the transposon might activate Sxl_{Pe} 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_{Pm}-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 Sxl^M alleles inserted in the vicinity of the male-specific exon, rather than in the vicinity of Sxl_{Pe}, 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 Sxl^M transposons might be able to activate Sxl_{Pe}. 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 Sxl^M alleles depends on *Sxl* autoregulation.

Here we address the question of what is responsible for the constitutive behavior of Sxl^M alleles by defining more precisely the specific molecular nature of the Sxl^M 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 Sxl^M 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° on a standard Drosophila medium (CLINE 1978). Sxl^M alleles are conveniently maintained as stocks in a genetically balanced condition in which females are Sxl^M/Sxl^- and males are Sxl^-/Y, where Sxl^- is Sxl^{fP7BO}, 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 $Sxl^{M1,fm3}$, $Sxl^{fm7,M1}$, $Sxl^{fm4,M1}$, and $Sxl^{fm5,M1}$ in MAINE *et al.* (1985) are now referred to as $Sxl^{M1,f3}$, $Sxl^{f7,M1}$, $Sxl^{f10,M1}$, and $Sxl^{f11,M1}$. The male-viable derivative of Sxl^{M3} is referred to as $Sxl^{M3/d1}$ pending further genetic and/or molecular characterization of the newly induced Sxl^f 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 *sisA* and *sisB*. In addition, an attempt has been made to standardize designations for alleles differentially impaired in the sex-determination (*sisB*) and proneural (*scute*) functions of transcription unit T4. It is proposed that all alleles be designated *scute*, with the superscript designation, *sisB*, reserved for alleles impaired primarily or exclusively in the sex-determination function. Hence, sc^{3-1} (a.k.a. *sis-b*$^{vg3-1}$) is renamed sc^{sisB-1}. When refering to gene function rather than to specific mutant alleles, we believe that it is useful to maintain the *sc vs. sisB* terminology to avoid ambiguity.

Origin of Sxl^M alleles: Sxl^{M1} arose spontaneously on a *pn* chromosome in a stock homozygous for da^1 kept at 18°. Sxl^{M2}, Sxl^{M3}, and Sxl^{M4} arose spontaneously in the course of a mapping experiment at 25° in which $Sxl^{M1\text{-}vd1}$ sn^3 (+/-v)/*pn cm* Sxl^r shi^{ts}; da^1/da^1 *pr cn*; *Kpn ca*/+ females were crossed to *y cm* ct^6 sn^3 *v*/*Y* males. Sxl^{M2} arose on the $Sxl^{M1\text{-}vd1}$ chromosome, whereas Sxl^{M3} and Sxl^{M4} arose on the Sxl^+ homologue. The three mutants arose among 5.9×10^5 female zygotes. Sxl^{M5} arose on the Sxl^+ chromosome in a mapping cross for $Sxl^{f11,M1}$ of the same design as that described above for $Sxl^{M1\text{-}vd1}$.

DNA sequence analysis of Sxl^M alleles: Standard procedures were used for isolation and manipulation of DNA clones (SAMBROOK *et al.* 1989). The Sxl^M insertions were isolated from libraries of *Mbo*I partial restriction digests constructed in phage EMBL3 and screened with a 4.8-kb *Xho*I fragment [coordinates 6.9–11.7 of the *Sxl* genomic map; see SAMUELS *et al.* (1991) and Figure 1] known to include the Sxl^M insertion sites (MAINE *et al.* 1985a,b). Phage inserts were subcloned into Bluescript SK (+) (Stratagene, San Diego, CA) for further analysis. For DNA sequencing, fragments were isolated, ligated, and sheared by sonication as described (BANKIER and BARRELL 1983) and then ligated into Bluescript SK(+). Random clones were sequenced by the dideoxynucleotide 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 *Xho*I genomic DNA fragments containing $Sxl^{M1\text{-}vd1}$ and Sxl^{M4} were sequenced in their entirety. Primers were used to confirm the positions of the Sxl^{M1} and Sxl^{M2} insertions. The position of the Sxl^{M3} 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^{M1} male-viable derivatives: Fine-structure recombination mapping had placed Sxl^{f3} on the 5′ side of the *roo* insertion in Sxl^{M1} and had placed Sxl^{f7} and Sxl^{f10} on the 3′ side (CLINE 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 Sxl^{f3}, the position of the lesions was identified by scanning 5 kb of *Sxl* 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 dideoxynucleotide chain-termination method. For Sxl^{f7} and Sxl^{f10}, 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, isoamyl 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. PolyA 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 polyA mRNA.

For Northern analysis, RNA was fractionated on 1.0 % 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 1.2-kb *Xho*I-*Sal*I 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 *Eco*RV site of Bluescript SK(+). The female cDNA probe was generated by subcloning a 229-bp *Nde*I-*Bal*I 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 5′ 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 ana-

lyzed on 6% polyacrylamide sequencing gels with end-labeled *Msp*I-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 MGSDD and ending at EEHGK, 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% (w/v) Boeringer Mannheim block reagent (BMB 1096176). Anti-SXL antibody (as 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 NiCl (0.4% final concentration).

Isolation and characterization of a Sxl^{M3} male-viable derivative: The approach was similar to that reported in CLINE (1984), which generated $Sxl^{f\,3,M1}$ and $Sxl^{M1,f\,7}$ (including genetic "virginating" steps). Two- to five-day-old virgin *y pn cm* Sxl^{M3}*/y w Df(1) Sxl-ra* sn^3*; Kpn ca/+* females were exposed to 3,200–3,800 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 Sxl^{M3} lesion. Approximately 75,000 chromosomes were screened, yielding only a single male-viable derivative, $Sxl^{M3/d1}$. Genomic Southern blot analysis showed that $Sxl^{M3/d1}$ contained no gross alterations in the region of *Sxl* (ca. <50 bp), indicating that the original transposon insertion associated with Sxl^{M3} 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, Sxl^{fhv1}, and a tandem duplication of $Sxl^{f\,2593}$, 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 $Sxl^{M3/d1}$ is not yet known. In the course of investigating the source of the low viability and recombination rate of the $Sxl^{M3/d1}$ *X* chromosome, we discovered that the parental Sxl^{M3} stock had become fixed for an inversion with breakpoints in cytological regions 4 and 9. This inversion is unrelated to the Sxl^{M} phenotype of this line and arose ≥2 years after Sxl^{M3}. Reduced male viability of $Sxl^{M3/d1}$ 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 Sxl^{M3} (see discussion of Table 3 below), neither female nor male $Sxl^{M3/d1}$ embryos appear to produce SXL cross-reacting material. When stage 13–15 embryos from the cross $Sxl^{M3/d1}$*/FM3,* Sxl^{+} × $Sxl^{M3/d1}$*/Y* were stained with anti-SXL antibody, only 27% of 161 embryos were positive, consistent with expectations if only $Sxl^{M3/d1}$*/FM3,* Sxl^{+} daughters express SXL protein.

RESULTS

DNA sequence analysis of Sxl^{M} mutant lesions—five independent alleles comprise three different but related types: Five spontaneous Sxl^{M} 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 Sxl^{M} 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 Sxl^{M} 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-o-f alleles.

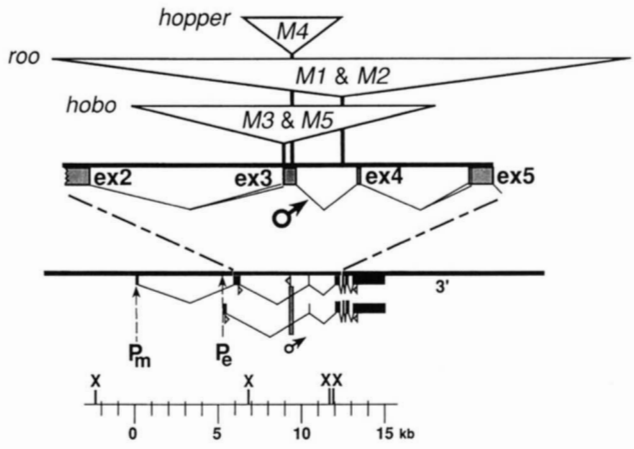


FIGURE 1.—Molecular map of the *Sex-lethal* gene with the five spontaneous Sxl^{M} insertions studied here shown to scale. The region surrounding male-specific exon three (ex3) is expanded for clarity (dashed horizontal lines). Two representative transcripts are diagrammed with exons indicated to scale by filled bars. Both are female transcripts of the "intermediate" size class, one originating at the "maintenance" promoter, Sxl_{Pm}, and the other at the "establishment" promoter, Sxl_{Pe}. The locations of translation start and stop sites are indicated by the small right- and left-facing triangles, respectively. Molecular coordinates and restriction sites (X, *Xho*I) referred to in MATERIALS AND METHODS are indicated on the scale bar.

The first type is represented by alleles Sxl^{M1} and Sxl^{M2}. These mutants were previously shown to be insertions of a *roo* (*B104*) element at or very near the same site (MAINE *et al.* 1985). Sxl^{M2} arose on a chromosome carrying $Sxl^{M1\text{-}vd1}$, a γ-ray induced derivative of Sxl^{M1} that is functionally indistinguishable from the wild-type allele but that retains what appeared to be a small fragment of the original Sxl^{M1} *roo* insertion. We sequenced the *Xho*I fragment (position 6.9–11.7 of the *Sxl* genomic map; see Figure 1) that contained the $Sxl^{M1\text{-}vd1}$ insertion. The insertion proved to be a single full-length (428 bp) *roo* LTR, indicating that $Sxl^{M1\text{-}vd1}$ arose from Sxl^{M1} by recombination loop-out between the two *roo* LTRs of the original Sxl^{M1} 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 Sxl^{M1} and Sxl^{M2} confirmed that both of these full-length *roo* elements were present at the identical site and in

TABLE 1

Insertions associated with Sxl^M constitutive alleles

Sxl allele	Insertion element[a]	Size (kb)	Site of insertion	Direction of transcription relative to *Sxl*
M1 and *M2*	*roo*	9.5	674 bp downstream of male-specific exon 3; 249 bp upstream of exon 4	Opposite
M1-vd1, a male-viable derivative of *M1*	*roo* LTR	0.43	Same site as *M1* and *M2*	Opposite
M3, *M5*	*hobo*	3.5	At the 3′ splice site of male-specific exon 3 (see Figure 2)	Same
M4	novel (*hopper*); 33-bp inverted terminal repeats; 5-bp target site duplication	1.4	Within the male-specific exon 3; splits exon 3 into 135 and 60 bp segments	Unknown

[a] For a description of the *roo* and *hobo* elements, see FINNEGAN and FAWCETT (1986).

the same orientation as the $Sxl^{M1\text{-}vd1}$ insertion. Hence, the event that converted the single *roo* LTR of $Sxl^{M1\text{-}vd1}$ 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 $Sxl^{M1\text{-}vd1}$ 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 *Xho*I 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 *Eco*RI (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 *Ssp*I and *Pvu*II, 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,

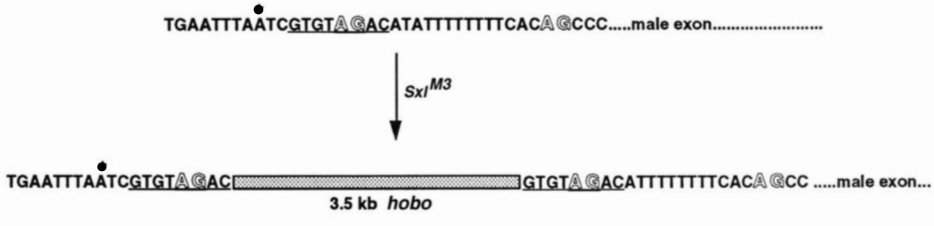
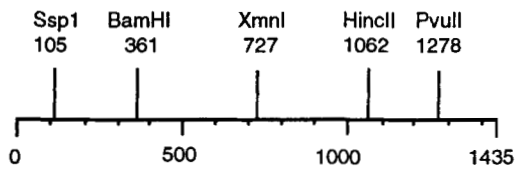


FIGURE 2.—The Sxl^{M3} insertion. Sxl^{M3} contains a 3.5-kb *hobo* insertion adjacent to the 3′ splice site of exon 3, the male-specific exon. An 8-bp genomic region duplicated due to the Sxl^{M3} insertion is underlined; highlighted AG nucleotides indicate the alternative 3′ splice sites used by exon 3; asterisk indicates a potential branch point, deduced from sequence, that may be used in splicing.

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   1  CACTATGGGG CATTTGGCCT GTTTTTTTTA CAAAAATTAA TACCTCCTAA
  51  ACTATTGGAG ATATTTGGAT GAATTTTTTT TTATGCGTTA CACATGCCTC
 101  CAGGAATATT TTGGAAAAGT GGGCGTGCCC CAACTCCGCC CCATTTTTTT
 151  TTTTTTTTTT TTTTTTTTTT AATAATATAT TTTTAAAGTT TATTTTTAAT
 201  TTCAATAATG TATAATTCAT AACCGTCTTC CTCTTCACAA TCAGTAGAGT
 251  CTGAAGAATT TTTATCAGGT TCAAATTCGC AAGCTAACAT TTCAATGACT
 301  TCTGGTGGAA GAGATAGTCG CTTATGTTTT CGCCTCTTTA AATTTATTGA
 351  TGATATTATG GGATCCGAAG TATCCATTGC TCTGTAAAAG ACATCTGCGA
 401  AGCTACTAAT AGTTTTTGCC GTGGCTGGCT TCAACAAAAG AATTTTAAGT
 451  ATGGCTGCAA GATCCCGCAG GCAGCACTTC CGTGCAGCTT GAACCAAAAG
 501  ACGTTCGTTG TGTTTCTGCG CCCTTACGAG TTCATCTGCT TGCTGTCTTG
 551  GGCCACTCAA ATTTTTTAAA TAATATGACG TTTTCGGGAG TCCAACTAAT
 601  TTCCTTTCCT ATTTATTTTT CTCCTTTACC TTCAGGACTA GGTGTTCTTC
 651  TAACCAATTT GAAAAAAATT TTAAAAATTC ATATATTTTT CGATTGCATT
 701  TTCTCCAATT TCGTAAAAGA TTGACTGAAA TCATTCGTTA TTATTATTAT
 751  TAGTTAATCG TTTATTAAAG TCTAGCTTGC TATCAGAAAA ATGCCCACTG
 801  ATAAAAGTGC AAATAGAATT TTCCTTTTGA CGAACACCCT TTTGCGTGCG
 851  CCACACTTCC AGCAGGGCAG CATTGGAAAT CGAGATATTG CTCCCTAAAA
 901  AATGAAATTT CTCAAAAAAC CGCAAAAAAC GCACATAGAG ACTACCTGAT
 951  ATGAGTTAGG AATTGAACAC ACTACAACAT GGATATAAAC ACTTACTGAA
1001  CAAATTTGAA CAAATTGTTG TAGCTCTATT CAAAGTTGAA AATTTTTTCA
1051  AACAACTACA TCTTGACACC ACTTGTTAAA TGTACAAATT GTTAGAAATA
1101  GGCGCACACA ATAAACAATA TATTAATAAC AACACATAAT AAGAACCTAA
1151  AGATTGATTA TCCATTTCAA ATTATACTCT CCTTCTTCTT CTTTTTAAAT
1201  TTTAACACTT TGAAAGTTAA GCTAAATTTT GTGCGCAAAG CAGCCACGTG
1251  GTATATGCTC GCAACAGCCG ACTTTAACAG CTGTTATTAT AACAGTGCAT
1301  TGTTAAATTA ACTTATGCGG GCTATATCAT AACAGTTTAA CGTATTTCCA
1351  ATGTATTAAT ACTAAAATAC TTCAAATTTG CATACTTGTG AAAAACACAT
1401  TATTGTAAAA AAAACAGGCC AAATGCCCCA TAGTG
```

FIGURE 3.—Restriction map and sequence of the novel Sxl^{M4} insertion element, *hopper*. This sequence will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under the accession number X80025.

like that of the other smaller open reading frames within *hopper*, showed a poor match to preferred Drosophila codon usage, it is unclear whether the mobile element in Sxl^{M4} actually encodes a protein. When used as a probe of Northern blots, the cloned *hopper* element failed to hybridize to mRNA from embryos or adults (data not shown). Taken together, these results suggest that the cloned *hopper* sequence may be a fragment of a larger full-length element and not itself encode functions required for *hopper* transposition. There has been no indication of genetic instability for any of the Sxl^{M} alleles.

The three classes of Sxl^{M} alleles differ in their ability to bypass the primary sex determination signal: The Sxl^{M} alleles were isolated based on their ability to rescue daughters that otherwise would have died as a consequence of the da^{1} female-lethal maternal effect. As expected, they also rescue females from the female-specific *zygotic* lethal effect of mutations in *sisA* and *sisB*, two *X*-linked "numerator elements" of the *X*:A sex-determination signal that function in an additive fashion as dose-dependent positive regulators of *Sxl* (CLINE 1988). Because it was known that Sxl^{M1} is not fully constitutive in the sense of being able to fully bypass the requirement for either maternal da^{+} or a double dose of *X* chromosomes (CLINE 1980, 1984), we investigated whether the different Sxl^{M} alleles might also differ in their degree of constitutive functioning as reflected in their ability to suppress the female-lethal phenotype of loss-of-function mutations in specific *X*-linked regulatory genes responsible for the *X*-chromosome dose effect on sexual fate. The results reported in Table 2 show that although each of the Sxl^{M} alleles is completely 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 hypomorphic 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^{M} allele to provide *Sxl* function. Clearly, Sxl^{M4} was more effective at suppressing female lethality (45–73%) than Sxl^{M3} (1–38%), which in turn was more effective than the original allele Sxl^{M1} (0–21%). Rescue by Sxl^{M} alleles did not increase with the addition of an Sxl^{+} allele, probably because the disruption of Sxl_{Pe} expression from the Sxl^{+} allele by the *sis* mutations was so strong. The fact that rescue was actually somewhat less in the presence of an Sxl^{+} allele is undoubtedly a consequence of genetic background differences between the two types of cross. Indeed, one reason for presenting the two sets of data is to illustrate

TABLE 2

***Sxl*^{M} alleles differ in their ability to bypass the X:A signal**

Cross	*Sxl*^{M} allele	*Sxl*^{x} allele	Temperature (°)	Females from the crosses indicated[a]: Experimental females $\frac{sc^{sisB\text{-}1}}{Df(sisB)}$ Number recovered	Experimental females $\frac{Sxl^{M}}{Sxl^{x}}$ Viability relative to control sibs	Experimental females $\frac{sisA}{Df(sisA)}$	Control female sibs as viability reference[b] $\frac{Sxl^{+}}{Sxl^{x}}$ Number recovered
A	*M1*	Wild-type	25	13	6%		217
A	*M1*	Wild-type	29	0	0%		90
B	*M1*	Null (*f1*)	25	76	21%		356
C	*M3*	Wild-type	25	57	19%		299
C	*M3*	Wild-type	29	1	1%		119
D	*M3*	Null (*f1*)	25	135	38%		358
E	*M4*	Wild-type	25	118	45%		261
E	*M4*	Wild-type	29	26	48%		54
F	*M4*	Null (*f1*)	25	277	73%		382

Because the B, D, and F crosses were done more than a year after the A, C, and E crosses, the genetic background even of the mothers in these two sets of crosses is likely to have been different. Female viability is sensitive to a variety of factors, including maternal age.

[a] No *Sxl*^{M} males were recovered in any of the crosses.

[b] *Balancer, Dp(1;1)sisB*$^{+}$, *Sxl*$^{+}$*/Df(sisB)Sxl*^{x} *Df(sisA); Dp(1;2)sisB*$^{+}$ *sisA*$^{+}$/+. Full genotypes of crosses (females × males): A, C, and E, $sc^{sisB\text{-}1}$ *w cm* Sxl^{M} *v sisA m/Binsinscy, y w sn B* × *Df(1)sc*^{19} and *Df(1)N71, y sisB*$^{-}$ *sisA*$^{-}$*/Y;* $X^{d}2^{p}T(1;2)Hw^{bap}$ and *Dp(1;2)v*^{65}, y^{+}*Hw sisB*$^{+}$*sisA*$^{+}$/+. Note: $sc^{sisB\text{-}1}$ is a new designation for $sis\text{-}b^{sc3\text{-}1}$ (a.k.a. $sc^{3\text{-}1}$). See MATERIALS AND METHODS. B, D, and F, same females as above × *Df(1)sc*^{19} and *Df(1)N71, y sisB*$^{-}$ *cm* Sxl^{f1} *ct*^{6} *sisA*$^{-}$*/Y;* $X^{d}2^{p}T(1;2)Hw^{bap}$ and *Dp(1;2)v*^{65}, y^{+}*Hw sisB*$^{+}$ *sisA*$^{+}$/+.

the magnitude of such genetic background differences on female rescue. The fact that the relative viability of the + + / Sxl^{f1} *sisA* female sibling control class in these crosses (not shown) was only 0.09% (1/1,096), compared with 73% for a female genotype with Sxl^{M4} that had three *fewer* doses of the female-vital *sis*$^{+}$ alleles and no *Sxl*$^{+}$ allele, illustrates just how effective the strongest Sxl^{M} allele is in suppressing extremely powerful female-lethal effects.

***Sxl*^{M} transcripts in adult females:** As a first step in analyzing the effects of the transposon insertions carried by Sxl^{M} alleles on *Sxl* mRNAs, we examined transcripts produced in adult females of the genotype Sxl^{M}/Sxl^{fP7BO}. Because Sxl^{fP7BO} is a male-viable deletion of the entire *Sxl* transcription unit, all *Sxl* transcripts in such females must be derived from the Sxl^{M} allele. Such hemizygous females are fully viable (data not shown). *Sxl*$^{+}$ 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 Sxl^{M} 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 6. 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 Sxl^{M} insertions) down into the long form of alternatively spliced exon 5 (*not* a sex-specific alternative splice) was hybridized to mRNA from *Sxl*$^{+}$ and Sxl^{M} 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 within the elements.

This RNase protection experiment tested for novel transcripts from the Sxl^{M} 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-

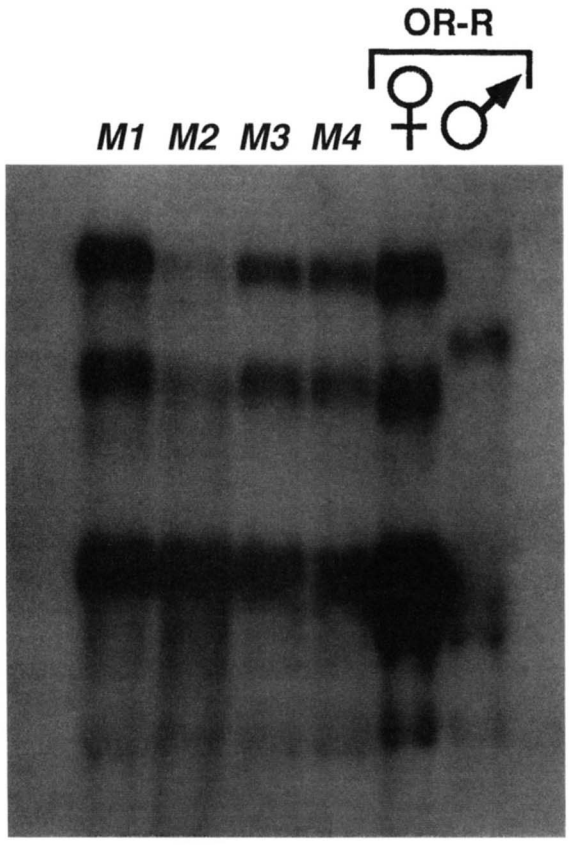

FIGURE 4.—Northern analysis of Sxl^M female adults. RNA from Sxl^M/Sxl^{f7BO} females was subjected to Northern analysis and probed with *Sxl* sequences [note that Sxl^{f7BO} = *Df(Sxl)*]. The three classes of *Sxl* female transcripts are ca. 4,100, 3,100, and 2,000 nucleotides in length. RNA samples from wild-type Oregon-R (OR-R) males and females are included as standards. The somewhat different relative levels of the three transcript size classes for Sxl^{M2} compared with those for the essentially identical allele, Sxl^{M1}, reflect the fact that transcript proportions depend on factors such as age of the females and genetic background.

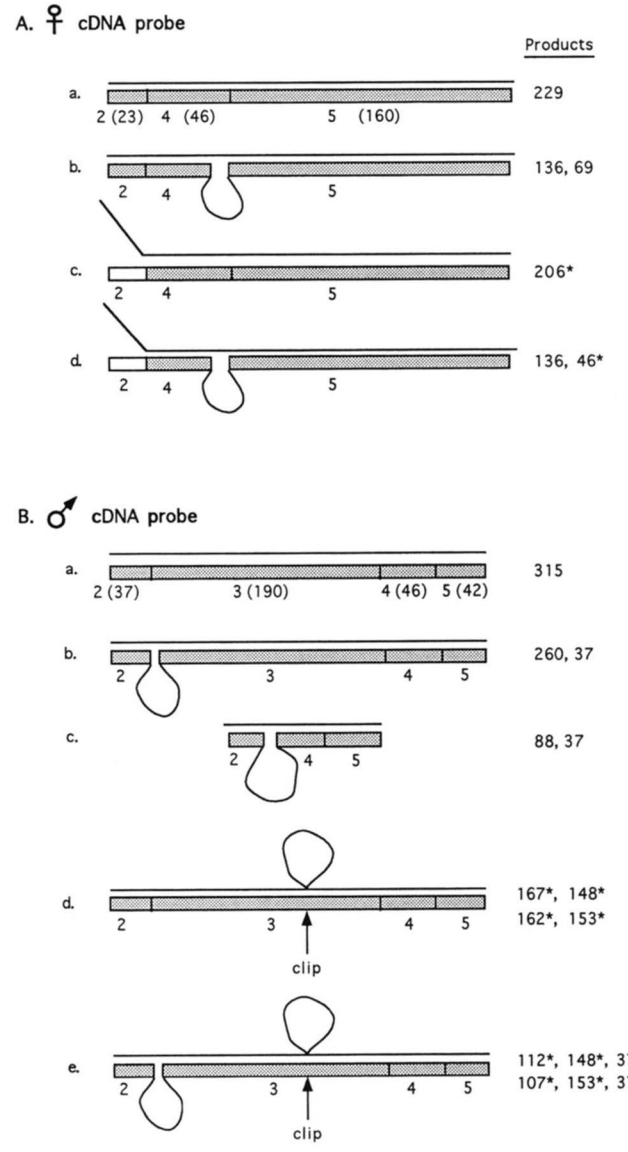

FIGURE 5.—Predicted products from RNase protection experiments. (A) Labeled female cDNA containing the long form of exon 5 as probe hybridized to (a) complementary female mRNA; (b) female mRNA containing the short form of exon 5; (c) putative message initiated from the sites of Sxl^M insertions, assuming the long form of exon 5; and (d) putative message initiated from the sites of Sxl^M insertions, assuming the short form of exon 5. (B) Labeled male cDNA containing the long form of exon 3 and short form of exon 5 hybridized to (a) complementary male mRNA; (b) male mRNA containing the short form of exon 3; (c) female mRNA; and (d and e) male mRNA containing the Sxl^{M4} insertion with the long (d) and short (e) forms of exon 3. Thin lines indicate mRNA hybridized to probes; boxed lines indicate labeled probes from *Sxl* cDNAs. For simplicity, the alternate forms of splicing possible at the 3′ splice site of exon 5 are not depicted in part B; both forms protect the labeled cDNA probe in this region. *, predicted protection products specific to messages initiated from the sites of Sxl^M insertions.

script species, we used a probe derived from a portion of a *Sxl* male cDNA (Figure 5B). When hybridized to mRNA from Sxl^M 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 Sxl^M 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.

***Sxl*^{M} transcripts in mixed-sex embryos:** The experiments described above showed that the Sxl^M 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 Sxl^M alleles, however, is the effect of these insertions during development, particularly in males. Such analysis is more complicated, however, because of the domi-

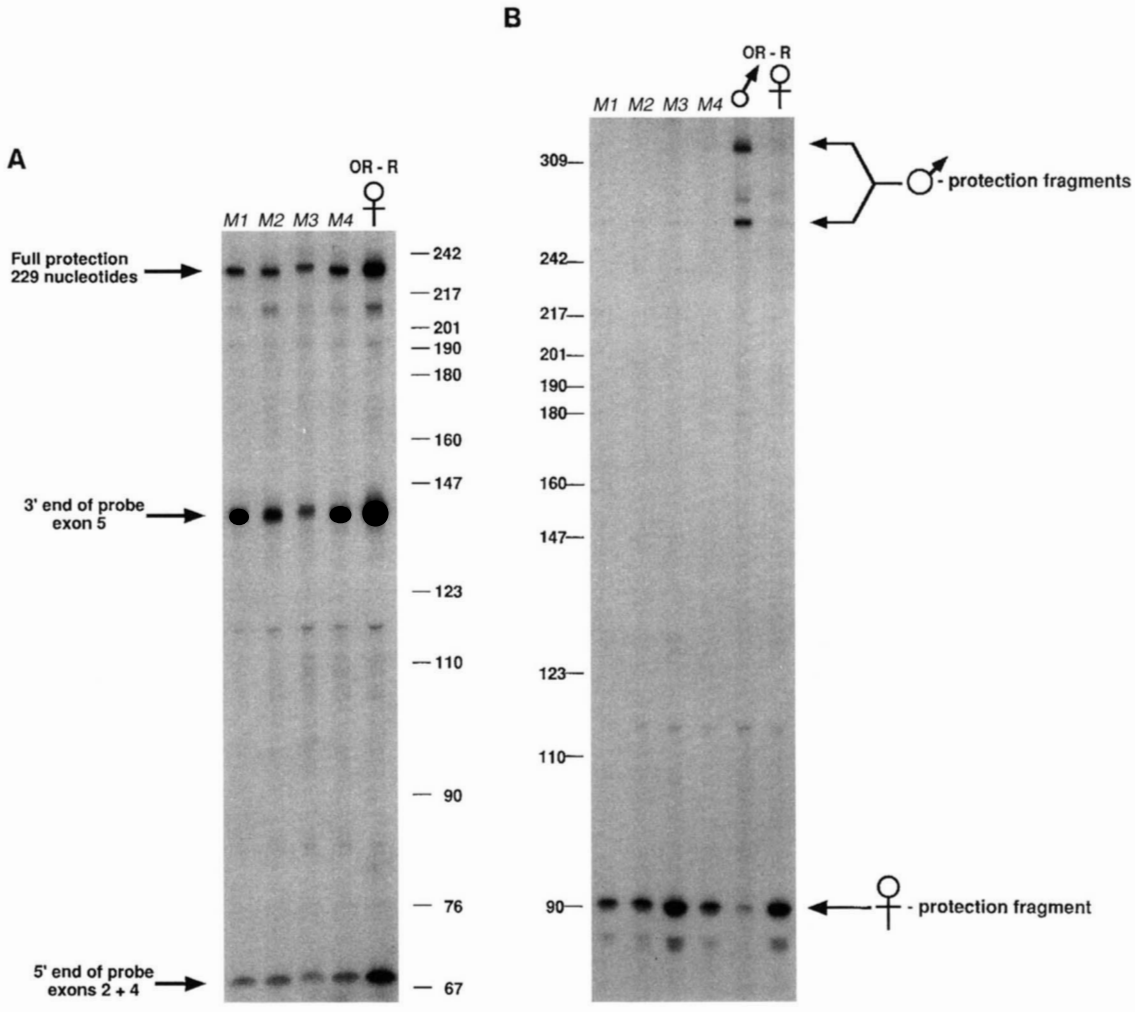

FIGURE 6.—RNase protection of RNA from Sxl^M female adults. RNA from Sxl^M/Sxl^{fP7BO} females was subjected to RNase protection analysis with labeled probes derived from a female specific cDNA (A) and male-specific cDNA (B). RNA samples from wild type Oregon-R (OR-R) males and females are included as standards. Size markers are *Msp*I restriction fragments of pBR322.

nant male-specific lethality of the Sxl^M alleles and the difficulty of separating male and female animals at early stages for RNA analysis. Moreover, the transcript pattern during the very earliest stages of embryogenesis is complicated by additional size classes of mRNAs that are generated from expression of Sxl_{Pe} and maternal female-specific mRNAs from Sxl_{Pm} that persist in both sexes of embryos for the first 2 hours after fertilization. However, the availability of the male-viable deletion, Sxl^{fP7BO}, used in the previous section facilitated the analysis of Sxl^M RNA at earlier stages by allowing for the construction of balanced genetic lines in which *Sxl* transcripts were generated only from the Sxl^M alleles, regardless of the stage or sex. The cross is of Sxl^M/Sxl^{fP7BO} females to Sxl^{fP7BO}/Y males. It remains balanced due to the death of Sxl^M/Y males and Sxl^{fP7BO}/Sxl^{fP7BO} females.

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^{M1}, 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_{Pe} (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_{Pe} 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.*

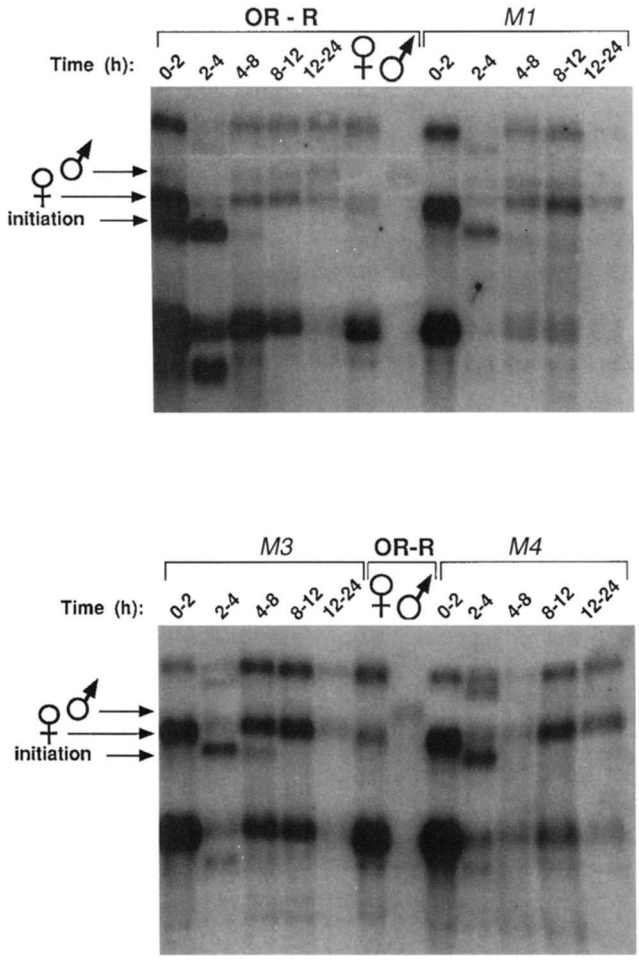

FIGURE 7.—Northern analysis of Sxl^M expression in embryos. RNA from embryos at different stages of development was subjected to Northern analysis and probed with *Sxl* sequences. Embryos were collected from stocks containing only Sxl^M and Sxl^{fP7BO} chromosomes; therefore, all hybridizing transcripts were from either Sxl^M/Sxl^{fP7BO} females or Sxl^M/Y males. Arrows point to middle size class of male, female, and initiation (Sxl_{Pe}-derived) transcripts. All three size classes of mRNAs are evident for female and initiation transcripts; for males, only the highly expressed midsized transcript is obvious. OR-R, *M1*, *M3*, and *M4* are mRNAs from stocks of wild-type, Sxl^{M1}, Sxl^{M3}, and Sxl^{M4}, respectively. Male and female lane labels indicate wild-type adult (OR-R) RNA samples.

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, Sxl^{M1} 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, Sxl^{M3} and Sxl^{M4} embryos were found to be capable of producing some male transcripts, but the level was much below that for the wild-type. Sxl^{M3} 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 Sxl^{M4} 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 Sxl^{M4} 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 Sxl^M male embryos: As an indirect assessment of female-specific *Sxl* transcript splicing in Sxl^M 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 Sxl^M alleles. Half 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 Sxl^M 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 Sxl^M, just like their sisters. The data in Table 3, group I, show that early in development, soon after expression of Sxl_{Pm} has begun, the proportion of SXL-positive embryos was near 25%, indicating that most Sxl^M 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 Sxl^M-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, Sxl^{M3} and Sxl^{M4}, 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, Sxl^{M1}, presumably reflecting the failure of Sxl^{M1} males to fully engage the splicing feedback loop by developmental stage 15; however, some of the Sxl^{M1} 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

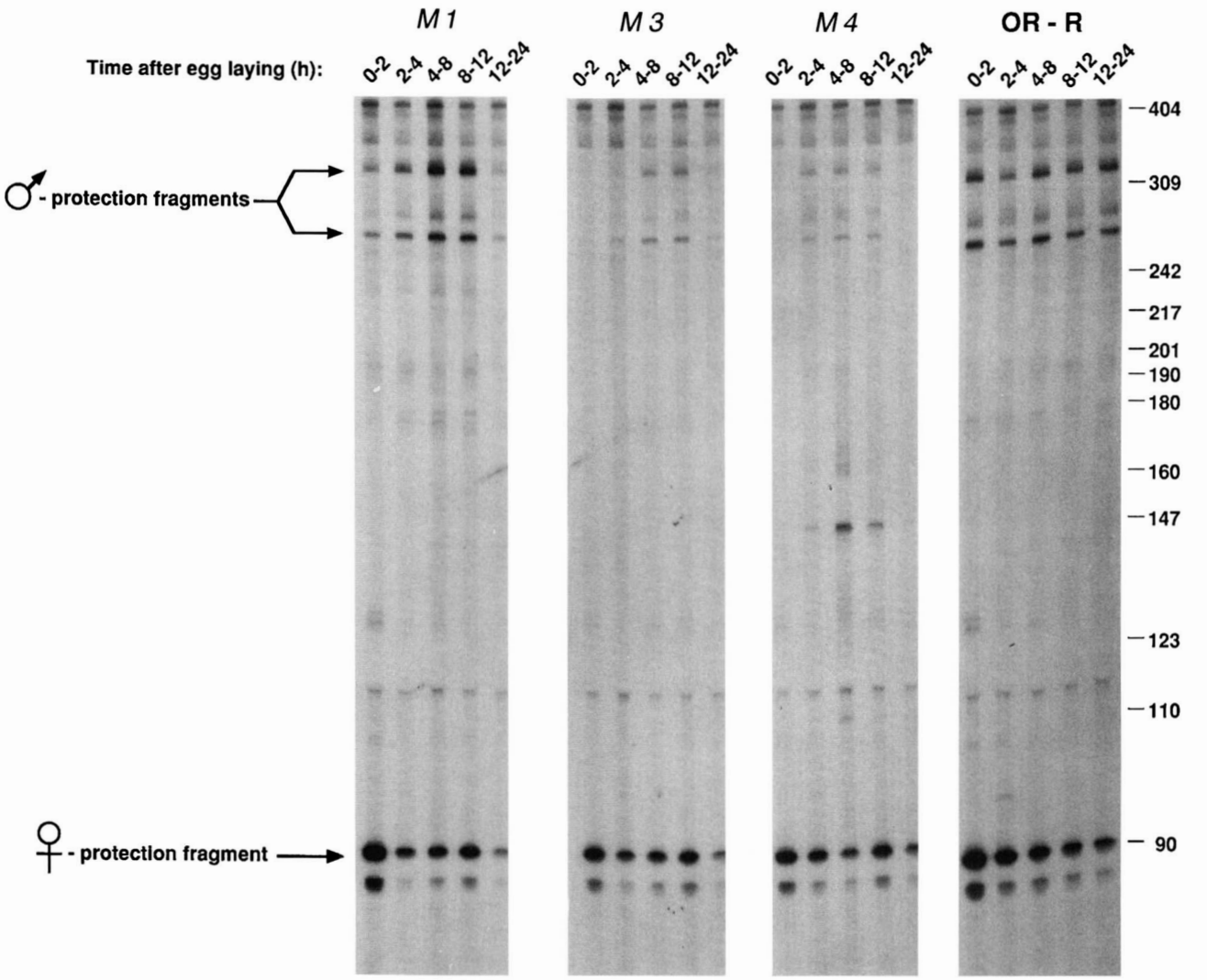

FIGURE 8.—RNase protection by RNA from Sxl^M embryos. RNA from embryos at different stages of development was subjected to RNase protection analysis using a probe derived from a male-specific cDNA. *M1*, *M3*, *M4*, and OR-R are mRNAs from stocks of Sxl^{M1}, Sxl^{M3}, Sxl^{M4}, and wild-type, respectively.

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 Sxl^M male-viable derivative alleles: We wanted to be able to distinguish primary *cis*-acting effects of Sxl^M 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 Sxl^M 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 Sxl^M 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). $Sxl^{M1,f3}$, $Sxl^{f7,M1}$, and $Sxl^{f10,M1}$ are examples of such male-viable derivatives (Table 4). The loss-of function mutations Sxl^{f3} and Sxl^{f7} reduce *Sxl* activity, whereas Sxl^{f10} eliminates it. In all three cases the effect on *Sxl* activity is due to changes in the proteins encoded. Although $Sxl^{M1,f3}$ and $Sxl^{f7,M1}$ 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, $Sxl^{M1,f3}$ and $Sxl^{f7,M1}$ 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 Sxl^{M1} in embryos, a substantial

TABLE 3

Anti-SXL antibody staining of Sxl^M mutant embryos from crosses of the form: $Sxl^M/Df(Sxl)$ females × $Df(Sxl)/Y$ males

Developmental stage[a]	Antibody staining level	From mothers carrying Sxl^{M1} Number	% of total	From mothers carrying Sxl^{M3} Number	% of total	From mothers carrying Sxl^{M4} Number	% of total
Group I. Late stage 5 through 7	No staining	98	78	55	66	85	74
	Intermediate staining	4	3	6	7	1	1
	Full staining for stage	24	19	22	27	29	25
	Total embryos collected	126		83		115	
Group II. Stages 8 through 12	No staining	187	58	152	50	344	59
	Intermediate staining	76	23	40	13	132	23
	Full staining for stage	61	19	115	37	111	19
	Total embryos collected	324		307		587	
Group III. Stages 13 through 15	No staining	70	42	70	40	112	53
	Intermediate staining	53[b]	32	2	1	5	2
	Full staining for stage	44	26	102	59	96	45
	Total embryos collected	167		174		213	

Expected progeny ratios: 50% no *Sxl* allele [no staining expected]:25% Sxl^M/Y [staining of interest]:25% $Sxl^M/Df(Sxl)$ [full staining expected].

Full genotype of crosses: *y pn cm* Sxl^M/*y pn cm* Sxl^{f7BO} females × males *y pn cm* Sxl^{f7BO}/Y @ 25°, where Sxl^M = *M1*, *M3*, and *M4*, respectively.

[a] Stages are according to CAMPOS-ORTEGA and HARTENSTEIN (1985). Developmental staging for the purposes of deducing the appropriate full staining value was determined more precisely than indicated above, with the stages then being grouped in the table for ease of presentation. Group I includes late blastoderm to early gastrulation (ca. 2:50–3:25 h After Egg Laying); group II includes germ band extension through germ band retraction (ca. 3:25–9:20 h AEL); group III includes head involution through dorsal closure (ca. 9:20–13:00 h AEL).

[b] Four of these animals showed salt-and-pepper full staining superimposed on an intermediate staining background.

amount of RNA was still spliced in the male mode. Interestingly, the $Sxl^{M1,f3}$ 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 Sxl^{f3} at the 5′ splice site of exon 2 and is not a feature of Sxl^{M1}. Although these results would appear to suggest that Sxl^{M1} can generate significant levels of female *Sxl* RNA in the absence of *Sxl* autoregulation, results with the null derivative, $Sxl^{f10,M1}$, 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 *Sxl* RNA. As a control to exclude the possibility that the Sxl^{f10} mutation might be helping suppress Sxl^{M1} male lethality by acting in *cis* directly to inhibit the joining of exon 2 to exon 4 (the female-specific splice), *Sxl* transcripts in heterozygous $Sxl^+/Sxl^{f10,M1}$ females were examined. In this case only normal female splice forms were observed, indicating that transcripts from $Sxl^{f10,M1}$ could be spliced fully in the female mode in the presence of Sxl^+ activity supplied in *trans*.

We used the same male-viable derivative approach to determine whether the stronger allele, Sxl^{M3}, was capable of generating female splice forms in the absence of *Sxl* autoregulatory activity. Northern analysis of a functionally null male-viable derivative, $Sxl^{M3/d1}$ (Figure 10A), showed that this allele produces only a very low level of *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 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 $Sxl^{M3/d1}$ 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 Sxl^{M3}, the ratio of *Sxl* mRNAs is greatly skewed toward the female forms even in the absence of autoregulatory activity.

DISCUSSION

The ability of the Sxl^M alleles to partially bypass the normal control of *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 4

Nature of the mutant *Sxl* alleles used to study the primary effects of Sxl^{M1} on *Sxl* RNA splicing

Male-viable Sxl^{M1} derivative	Molecular nature of the loss-of-function lesion: At the DNA level	Molecular nature of the loss-of-function lesion: At the protein level	Location of the lesion	Functional consequences of the lesion
$Sxl^{M1,f3}$	G → C	S → T and potential reduction in protein levels due to inhibition of exon 2 to 4 splicing	Last nucleotide in exon 2 (upstream of Sxl^{M1})	Hypomorph for somatic dosage compensation: severe hypomorph for autoregulation; null for somatic sex determination; germline functions near wild-type
$Sxl^{f7,M1}$	G → T	D → Y	Exon 5; first residue of putative alpha helix 1 in RRM#1[a] (downstream of Sxl^{M1})	Moderate hypomorph for somatic dosage compensation and autoregulation; null for somatic sex determination; germline functions wild-type
$Sxl^{f10,M1}$	A → Δ	Frameshift terminating translation after 14 scrambled residues	Exon 6; sixth residue of putative beta sheet 3 in RRM#1[a]	Null for all somatic and germline functions

[a] According to the structure of the SXL protein predicted by KENAN *et al.* (1991).

by the dominant lethality of the Sxl^{M} 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 Sxl_{Pe} into a long-lasting effect on RNA splicing of transcripts from Sxl_{Pm}, committing cells to the male or female mode of development during the remainder of the life cycle. Sxl^{M} mutant lesions must somehow generate SXL proteins in the absence of the SXL protein normally generated early in development as a consequence of Sxl_{Pe} expression in response to the primary sex-determination signal, the *X*:A balance.

One possibility that seems ruled out by the data presented here is that the Sxl^{M} transposon insertions induce expression of Sxl_{Pe} 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 Sxl^{M} males significantly later than they appear in females and well after the point at which the switchover to Sxl_{Pm} has taken place. Therefore, if induction of Sxl_{Pe} expression were the basis for Sxl^{M} effects in males, this abnormal Sxl_{Pe} expression would have to occur significantly later in Sxl^{M} mutant males than in wild-type females. But our RNA analysis of mixed-sex embryos and of adult females shows that Sxl^{M} mutations appear to have no effect on the timing of Sxl_{Pe} expression. Our results pointing to an effect of the Sxl^{M} mutations only after the switchover to Sxl_{Pm} 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 Sxl_{Pe} (BERNSTEIN and CLINE 1994), occurs normally in Sxl^{M1} embryos (GERGEN 1987).

It seems far more likely that Sxl^{M} lesions partially bypass the requirement for autoregulation by changing the splicing rules for Sxl_{Pm}-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 Sxl^{M} males initially lags behind that in their sisters, who had the benefit of a head start provided by Sxl_{Pe} expression, most of the Sxl^{M3} and Sxl^{M4} males catch up to their sisters by the time of dorsal closure. Males that carry the weaker g-o-f mutation, Sxl^{M1}, 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 Sxl^{M} lesions suggests that the primary effect of these mutations is likely to be disruption of the normal splicing rules for Sxl_{Pm} transcripts

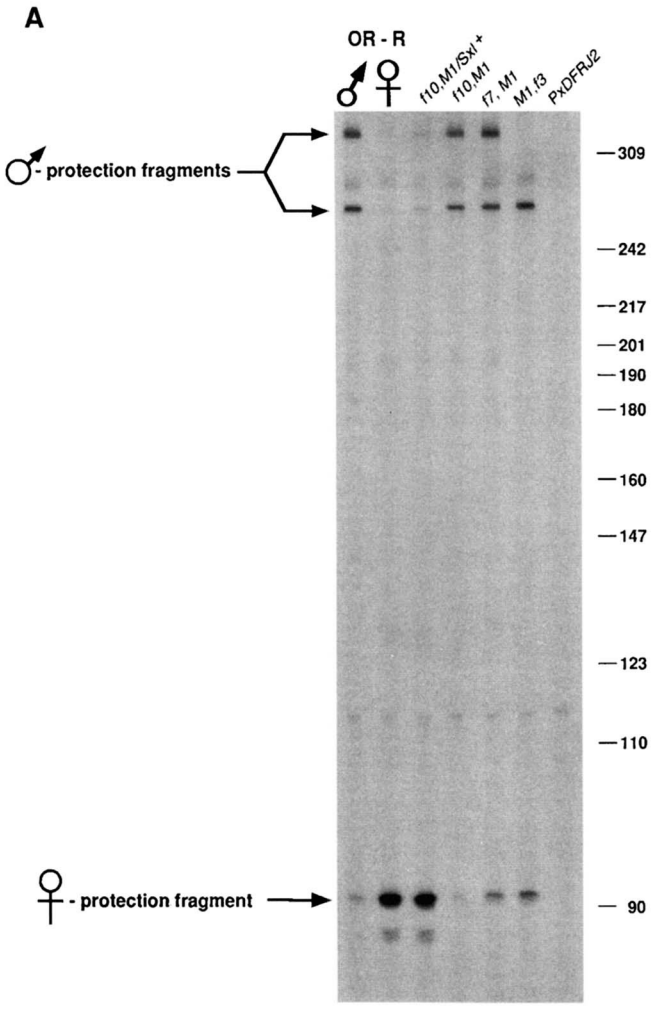

FIGURE 9.—RNA analysis of Sxl^{M1} male-viable derivatives. (A) RNase protection using a labeled male-specific cDNA as probe. Size markers are *Msp*I restriction fragments of pBR322. (B) Northern analysis of $Sxl^{f10,M1}$ adult females (heterozygous with Sxl^{+}) and males and wild-type (OR-R) males and females, probed with *Sxl* sequences.

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_{Pm}-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 Sxl^{M} 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-o-f mutant, Sxl^{M1}, 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 Sxl^{M} 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

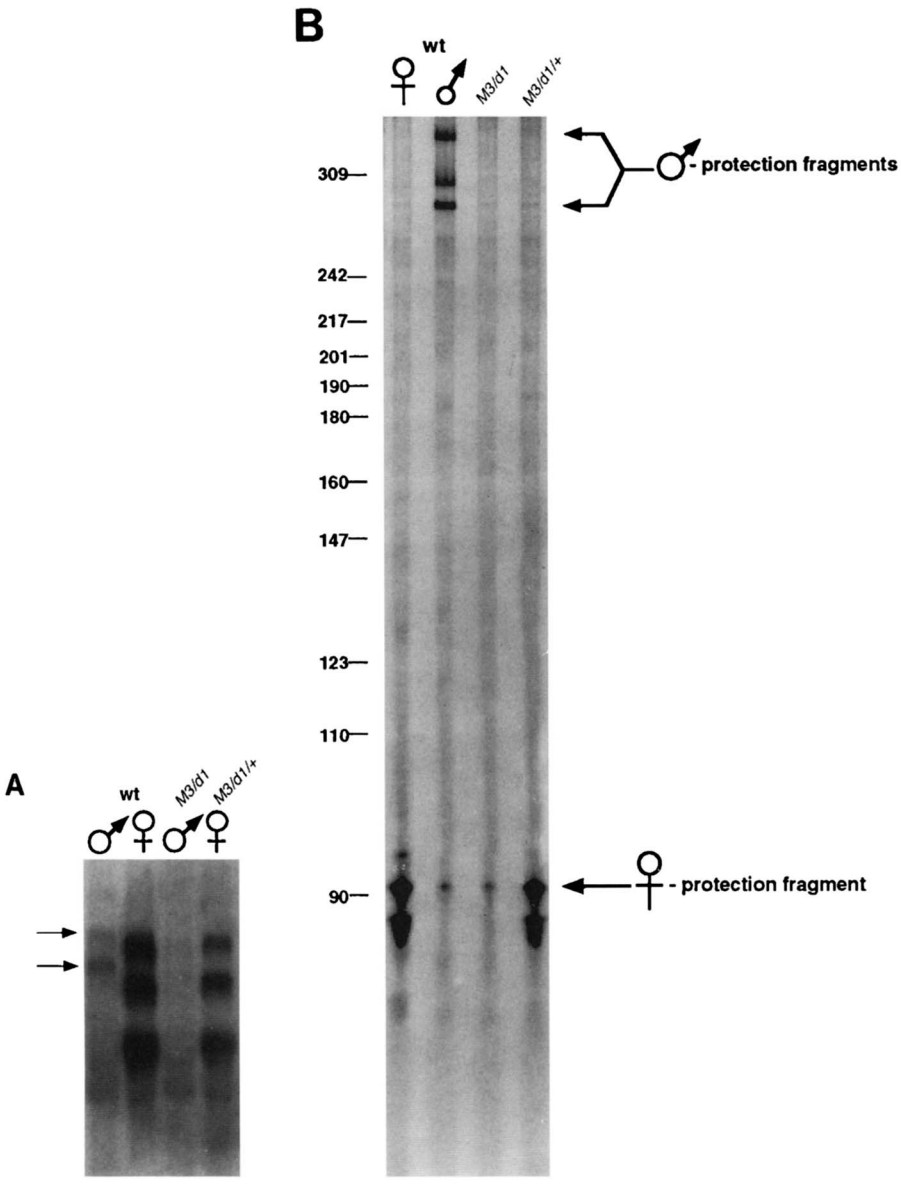

FIGURE 10.—RNA analysis of the $Sxl^{M3/d1}$ male-viable derivative. (A) Northern analysis of $Sxl^{M3/d1}/Sxl^{+}$ adult females, $Sxl^{M3/d1}$ males, and wild-type males and females, probed with *Sxl* sequences. Arrows indicate position of wild-type male transcripts. The same filter rehybridized with a probe against ribosomal protein rp1 established that three of the four lanes contained equivalent amounts of mRNA but the $Sxl^{M3/d1}$ lane contained approximately half as much (data not shown). (B) RNase protection using a labeled male-specific cDNA as probe. All males and half the females contained *Dp(1;2)4FR* [3C2–5A1,2 minus 3F-4A], $w^{+}N^{+}$. Size markers are *Msp*I restriction fragments of pBR322.

by the null double-mutant allele, $Sxl^{M3/d1}$, showed that Sxl^{M3} 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} in the absence of autoregulatory activity was remarkably low. Interpretation of the results with the male-viable derivative, $Sxl^{M3/d1}$, 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 $Sxl^{M3/d1}$ destabilizes *Sxl* transcripts. Alternatively, some transcripts initiated at Sxl_{Pm} may splice into the *hobo* element of $Sxl^{M3/d1}$ in males, yielding aberrant mRNAs that are degraded. This lowered level of *Sxl* transcript may account for the fact that $Sxl^{M3/d1}/Y$ 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 $Sxl^{M3/d1}$ 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^{M1}, was even more surprising. Splicing of the transcripts from a null double-mutant male-viable derivative of Sxl^{M1} 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^{M1} 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^M alleles. Where do the initial female splice forms come from in the absence of Sxl_{Pe} 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^{M3} and Sxl^{M4}. Even for Sxl^{M1}, substantial amounts of SXL protein were observed throughout the mutant male embryos; moreover, earlier analysis of genetic mosaics showed that the development of male cells throughout the organism could be affected (CLINE 1979). A more reasonable way of reconciling the low level of female splicing observed in male-viable derivatives at the adult stage to the strict male-specific lethality of the parental Sxl^M alleles is to propose that the basal level of female splicing for these mutant alleles in the absence of *Sxl* autoregulation is higher at preadult stages in the life cycle than at the adult stage.

But that level may not have to be very much higher to account for the dominant male-specific lethality. Transcript analysis reported here for the male-viable derivatives, $Sxl^{f3,M1}$ and $Sxl^{M1,f7}$, suggests that a second factor may contribute to the mutant phenotype of Sxl^M alleles: the insertions may hypersensitize the mutant alleles to autoregulation by SXL protein. By this hypothesis, the level of SXL protein required to establish the positive feedback loop for Sxl^M 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 transactivated in males at low frequency during the larval stage by a single dose of $Sxl^{M1,f7}$ 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, $Sxl^{f10,M1}$. 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^M alleles relative to Sxl^+ would also account for the observation that when Sxl_{Pe} expression is completely blocked by mutations in the *sis* genes (Table 2), the ability of Sxl^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^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^{M1} 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^M alleles other than from effects on Sxl_{Pm}-transcript splicing. Formally, however, other mechanisms could also lead to activation of Sxl^M alleles. For example, enhancer elements within the Sxl^{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^{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^{f3}, in exon 2, far upstream of the *roo* insertion into Sxl^{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^{M1} insertion might be, its phenotype must depend to a large extent on the productive splicing and translation of standard Sxl_{Pm}-derived transcripts.

The RNase protection experiments with Sxl^{M4} embryos indicated that this allele produced 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

(162/167, 107/112, and 73 bp, corresponding to exon 2 and the 5′ end of exon 3), suggesting either that mRNAs initiated within the *hopper* element or that *Sxl* exon 2 had been spliced directly to the the *hopper* element. We note, however, that at least a proportion of the Sxl^{M4} transcripts include all of exon 3, as judged by the presence of normal male protection fragments. Further experiments on the nature of the transcripts produced in Sxl^{M4} and the transcriptional potential of the *hopper* element will be needed to determine if either of these possibilities is correct.

Although these spontaneous Sxl^{M} 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 Sxl^{M} 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 Sxl_{Pe} *vs. Sxl* autoregulation is clearly made.

Of the members of this set of spontaneous Sxl^{M} alleles, we show here that Sxl^{M4} 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 Sxl_{Pm} does the level of SXL protein in Sxl^{M4}/Y male embryos appear to reach that of their Sxl^{M4}/Sxl^{-} sisters. Moreover, this point seems not to be reached much earlier for Sxl^{M4} than for Sxl^{M3}, an allele shown here to be sensitive to autoregulation. This result could stem from some significant early difference in the level of expression of Sxl_{Pm} between haplo-*X* and diplo-*X* embryos that eventually disappears; alternatively, it could reflect autoregulation steadily ramping up the level of productive splicing of Sxl_{Pm}-derived transcripts generated by the g-o-f allele.

Because the primary transcripts of these Sxl^{M} 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 Sxl^{M} alleles with the effects of deletion Sxl^{M} alleles that are now accumulating as the byproduct of screens to identify genes of the primary sex-determination signal (D. A. BARBASH, S. J. FEIST, and T. W. CLINE, unpublished data). Such deletion Sxl^{M} 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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