

The Regulation of the *Drosophila msl-2* Gene Reveals a Function for *Sex-lethal* in Translational Control

Greg J. Bashaw and Bruce S. Baker

Department of Biological Sciences
Stanford University
Stanford, California 94305

Summary

In *Drosophila*, dosage compensation occurs by increasing the transcription of the single male X chromosome. Four *trans*-acting factors encoded by the *male-specific lethal* genes are required for this process. Dosage compensation is restricted to males by the splicing regulator *Sex-lethal*, which functions to prevent the production of the MSL-2 protein in females by an unknown mechanism. In this report, we provide evidence that *Sex-lethal* acts synergistically through sequences in both the 5' and 3' untranslated regions of MSL-2 to mediate repression. We also provide evidence that the repression of MSL-2 is directly regulated by *Sex-lethal* at the level of translation.

Introduction

Dosage compensation equalizes the imbalance in X-linked gene products between the sexes that would arise if the single male X and each of the two female X chromosomes were expressed equivalently. In *Drosophila melanogaster*, dosage compensation is achieved by doubling the transcription of the single male X chromosome (reviewed by Lucchesi and Manning, 1987; Baker et al., 1994; Kelley and Kuroda, 1995). The products of four *male-specific lethal* (*msl*) loci (*msl-1*, *msl-2*, *msl-3*, and *mle*) genes are required for dosage compensation. These proteins bind to a large number of sites along the male X chromosome as a complex and are thought to mediate the changes in chromatin structure and transcriptional activity associated with dosage compensation.

Dosage compensation is controlled by the *Sex-lethal* gene (*Sxl*), which acts to prevent the association of the MSL proteins with the female's X chromosomes (Gorman et al., 1993; Hilfiker et al., 1994). *Sxl* also controls sex determination (reviewed by Cline, 1993; Cline and Meyer, 1996). *Sxl* encodes an RNA-binding protein that regulates its own expression by regulating splicing (Bell et al., 1991). In females, *Sxl* protein-directed splicing of *Sxl* pre-mRNA generates an open reading frame (ORF). In males, default splicing results in an mRNA with premature stop codons. *Sxl* also regulates *transformer* (*tra*) (Boggs et al., 1987; Nagoshi et al., 1988; Sosnowski et al., 1989): in females, the *Sxl*-directed splice generates a protein coding mRNA, while in males, default splicing results in an mRNA with premature stop codons. It thus seemed likely that *Sxl* would regulate dosage compensation similarly, by controlling the splicing of the pre-mRNA of one or more of the *msl*s, such that a functional product was not made in females.

Characterizations of *mle*, *msl-1*, and *msl-3* revealed no differences in transcript structure between males and females, suggesting that they are not targets of *Sxl* (reviewed by Baker et al., 1994). Analysis of *msl-2* revealed that it is the target of *Sxl* regulation (Bashaw and Baker, 1995; Kelley et al., 1995; Zhou et al., 1995). However, the data with respect to *msl-2* suggested that its regulation is not achieved by restricting mRNAs with a functional ORF to males. In both sexes, *msl-2* transcripts are present and have the same ORF, but MSL-2 protein is only detected in males (Bashaw and Baker, 1995; Kelley et al., 1995; Zhou et al., 1995). Two features of the *msl-2* transcripts suggested possible bases for male-specific translation. First, there is a small intron in the 5' UTR of *msl-2* that is retained in females and removed in males (Bashaw and Baker, 1995; Kelley et al., 1995; Zhou et al., 1995). Second, there are poly(U) runs in both the 5' and 3' UTRs that resemble the SXL-binding sites in *Sxl* and *tra* (hereafter called SXL-binding sites). The SXL-binding sites in the *msl-2* 5' UTR are within the intron, and thus they are only retained in the female message (Bashaw and Baker, 1995; Kelley et al., 1995; Zhou et al., 1995). How these features of *msl-2* mRNA may prevent translation in females is considered below.

Removal of the 3' or 3' and 5' UTRs results in expression of MSL-2 protein in females. Expression of MSL-2 also results in the X chromosome association of the other MSLs, suggesting that *msl-2* is the primary sex-specific target of *Sxl* (Bashaw and Baker, 1995; Kelley et al., 1995). One proposal for how the UTRs repress protein production is that SXL binds to the UTRs of *msl-2* mRNA and blocks the export of these mRNAs from the nucleus. Alternatively, SXL may more directly interfere with *msl-2* translation.

Two features of the male-specific intron suggest how it may prevent translation. First, there is a small upstream ORF (uORF) in the intron; regulation of translation by upstream ORFs is well established in other systems (reviewed by Hinnebusch, 1994). Second, possible secondary structures involving the intron may block translation. These above possibilities are not mutually exclusive, and regulation may require multiple elements.

We used two approaches to address the roles of these features for the 3' and 5' UTRs. The effects of mutations in the potential regulatory elements of the *msl-2* UTRs were assayed in transgenic flies and also in SL-2 cells. We found that the SXL-binding sites in the 5' and 3' UTRs function together to confer SXL repression. Neither the 5' nor the 3' ends alone strongly repressed *msl-2* translation; only when both were present was there a substantial repression of translation. Consistent with these findings, we show that SXL protein can directly interact with both *msl-2* UTRs. *msl-2* RNA is found in the cytoplasm in both males and females, suggesting that SXL acts cytoplasmically to repress MSL-2 translation. Furthermore, in cells cotransfected with *msl-2*- β -gal reporters and *Sxl*, there is a 7-fold decrease in β -gal activity relative to cells that do not receive *Sxl*; however, there is no significant difference in the amount of *msl-2*- β -gal message present in the cytoplasm of the two

populations of cells. We conclude that in addition to acting as a splicing regulator, *Sxl* also has a direct role in translational control.

Results

Transgenic Flies

To examine the role of the *msl-2* UTRs in conferring female-specific repression, a series of *msl-2* cDNA constructs with mutations in the potential regulatory elements were introduced into flies by P element-mediated germline transformation. These include mutations in the 3' and 5' putative SXL-binding sites, both singly and together, the splice junctions of the intron in the 5' UTR, and the initiation codon of the uORF in the 5' UTR's intron. All constructs were driven by the *Actin-5C* promoter in the CaSpeR-*Actin* vector, and they are diagrammed in Figure 1. Our goal was to compare the effects of these mutations in the UTRs of *msl-2* on translation. To make such comparisons straightforward, we sought lines carrying the different constructs that made equivalent amounts of *msl-2* mRNAs. Two criteria were used to ensure that lines with equivalent levels of *msl-2* gene expression were compared. First, lines with similar levels of expression of the mini-*w⁺* gene carried on the transposon were selected, since mini-*w⁺* expression is a sensitive indicator of position effects. Second, and most importantly, the expression levels of the selected inserts were directly compared by RNase protection (see below).

That males could be obtained in which these transgenes represented the only functional *msl-2* gene indicates that all sequences essential for *msl-2* function are present in these constructs. Females carrying single copies of the transgenes were assayed for ectopic MSL-2 expression by immunostaining salivary polytene chromosomes with MSL-1 or MSL-2 antibodies. Females carrying a wild-type construct showed low expression of MSL-2 (Figures 2A and 2B). This expression is likely due to leaky splicing of the transgene RNA (Figure 2, legend). *msl-2* males that carry the wild-type construct express MSL-2 at much higher levels and show normal MSL X chromosome staining (Figures 2C and 2D). Thus, wild-type *msl-2* cDNA driven by the *Actin-5c* promoter can be effectively regulated.

The SXL-Binding Sites in the 5' and 3' UTRs Function Together to Prevent MSL-2 Expression in Females

Mutation of the SXL-binding sites in the 3' or the 5' UTR results in defective regulation of *msl-2*, as evidenced by a dramatic increase in MSL-2 expression in females. For the 3' UTR, two deletion constructs were assayed; one removed a substantial portion of the 3' UTR (data not shown), while the second removed only the 115 most distal nucleotides of the 3' UTR, including the four SXL-binding sites (Figures 3A and 3B). Both deletions resulted in similar staining levels. Two mutations in the SXL-binding sites in the 5' UTR were also assayed. One replaces the normal female 5' UTR with the form present in males (i.e., intron removed; data not shown), and the

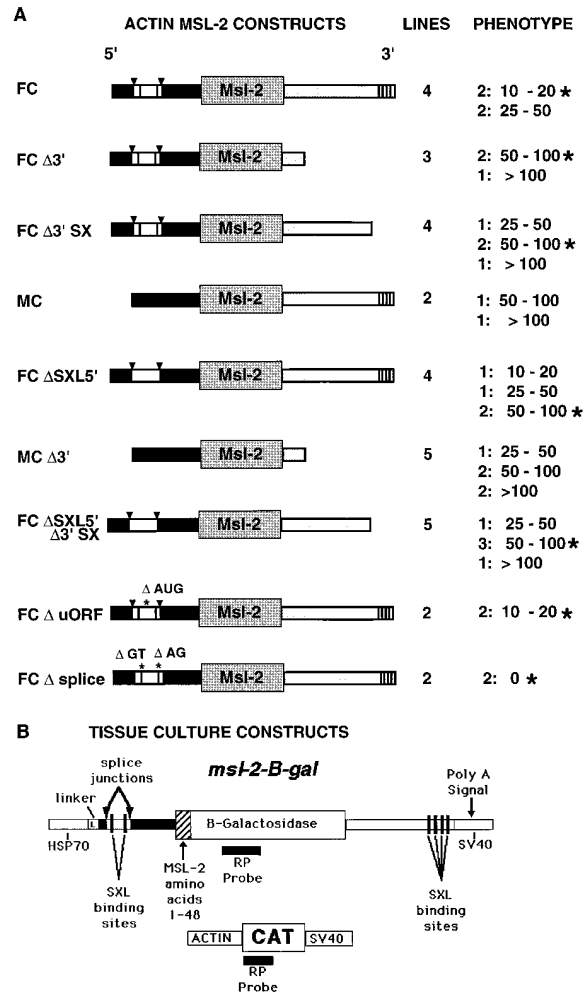


Figure 1. Constructs Used in This Study

(A) *Actin-*msl-2** constructs. The *Actin-*msl-2** constructs used in this study are diagrammed. Constructs are driven by the *Actin-5c* promoter, which is joined to the 5' UTR of *msl-2* by a common linker region. Arrowheads indicate the splice junctions of the male-specific intron. Intron sequences are indicated by the open portion of the 5' UTR. The closed portions of the 5' UTR are regions that male and female forms of *msl-2* share in common. The SXL-binding sites in the UTRs are indicated by closed lines. The constructs all contain *Actin* polyadenylation signals. Structures of the various mutant versions of *msl-2* are indicated. To the right of each construct, the number of lines examined for expression of MSL-2 in females is indicated. The range of phenotypes observed for each construct in terms of number of MSL bands detected in females is indicated at the far right: these data show that there was significant variation in MSL-2 protein expression levels between lines with the same construct inserted at different locations. The lines used for comparison of MSL binding and RNA levels are indicated by asterisks. Not all of the lines could be assayed in the *msl-2* mutants; however, since wild-type females do not express any MSL-2, all detected protein can be assumed to be derived from the transgene. To test this assumption, the female expression levels of several inserts were examined in the presence or absence of endogenous *msl-2* and were found to be the same (data not shown).

(B) Tissue-culture constructs: schematic diagram of *msl-2-β-gal* and *cat* reporter constructs. Features of the *msl-2-β-gal* UTRs are the same as in (A), except the *hsp-70* promoter drives expression and the polyadenylation signals are provided by SV40 sequences. In the case of the *cat* reporter, expression is driven by the *Actin* promoter. The positions of the probes used for RNase protection are indicated beneath each construct (RP probe).

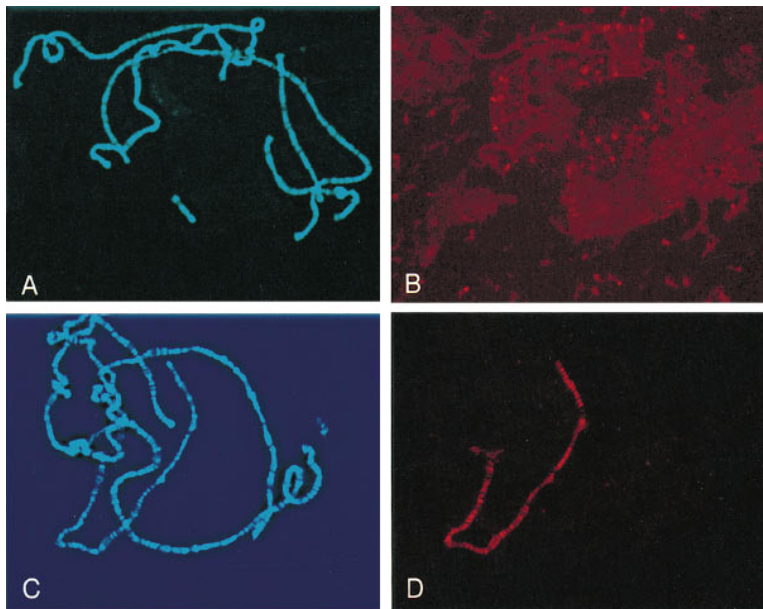


Figure 2. Anti-MSL-2 Antibody Staining of Males and Females Carrying the *Actin-msl-2* Transgene

Polytene chromosomes of *msl-2* males and females carrying a single copy of the *FC* transgene were stained with anti-MSL-2 antibodies.

(A) DAPI staining of a female nucleus.

(B) Cy3 staining of female nuclei reveals a low level of MSL-2 expression (note small number of MSL-2 bands on the X chromosome). This low level of staining is likely due to leaky splicing of the transgene RNA, as no staining is ever observed in females carrying the Δspl (splice junction mutant) transgene (data not shown).

(C) DAPI staining of a male nucleus.

(D) Cy3 staining of male nucleus. Note the strong staining of the male X chromosome. As a control for fixation conditions, preparations were stained simultaneously with an RBP1 sex-nonspecific antibody. No significant differences between preparations were observed with the control antibody.

second substitutes numerous U residues in the two SXL-binding sites with other residues (Figures 3C and 3D). Females carrying either transgene produce equivalent levels of MSL-2, and these levels are similar to those observed for the 3' UTR mutants. Thus, the effect on

MSL-2 expression of mutating the SXL-binding sites in the two UTRs appears equivalent.

To ask if the roles of the two ends in conferring repression were additive or synergistic, the SXL sites at the two ends of the *msl-2* mRNA were mutated simultaneously. If

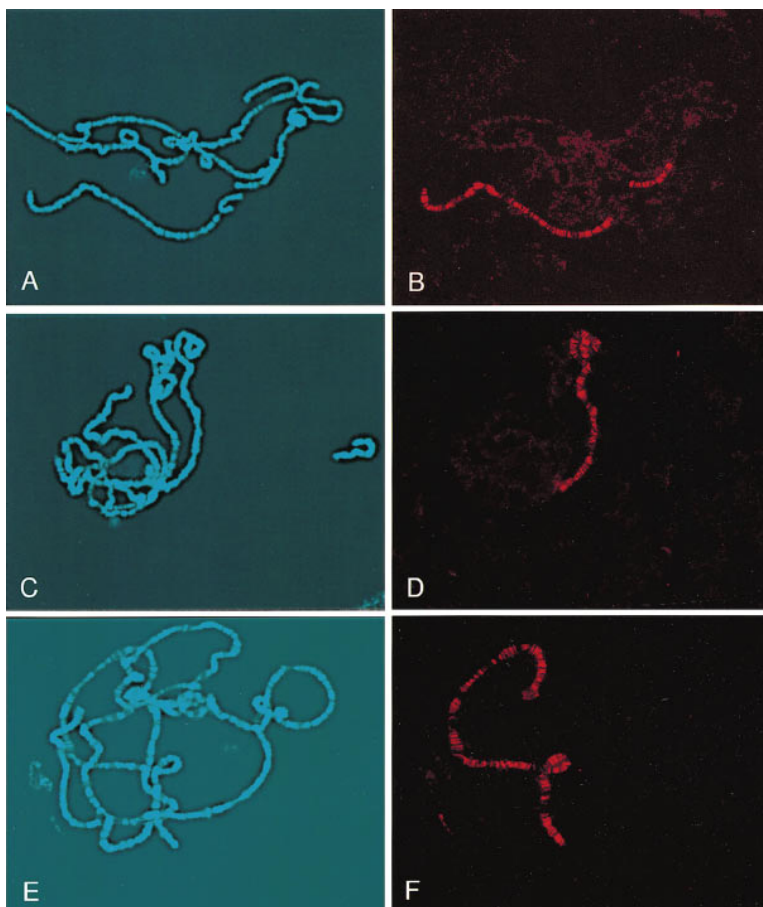


Figure 3. MSL-2 Expression in Females Carrying Mutant Forms of the *Actin-msl-2* Transgene

Anti-MSL-1 staining of polytene chromosomes of females carrying single copies of either the $FC\Delta 3'SX$ transgene (A and B), the $FC\Delta Sx15'$ transgene (C and D), or the $FC\Delta Sx15'+\Delta 3'SX$ (E and F). Nuclei were stained with DAPI to reveal DNA in blue (A, C, and E) or Cy3 to reveal MSL staining (B, D, and F). The staining of the double mutant (E and F) is only slightly more intense than that of the single mutants (compare D and F). As a control for fixation conditions, preparations were stained simultaneously with an RBP1 sex-nonspecific antibody. No significant differences between preparations were observed with the control antibody.

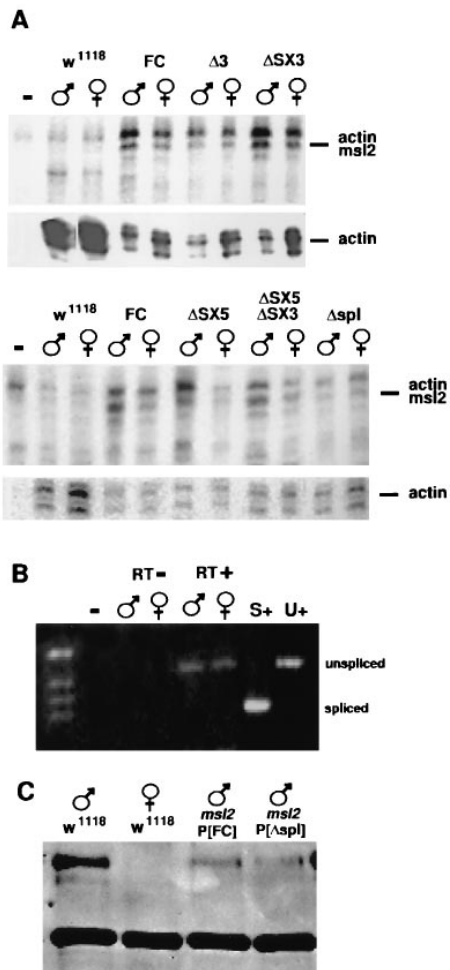


Figure 4. Comparison of Expression Levels of *Actin-msl-2* Transgenes

(A) RNase protection analysis of the *Actin-msl-2* transgenes was performed using antisense RNA probes that protect different-sized fragments from the transgenes and the endogenous *Actin* gene. Genotypes are indicated above the gels. Signal from the transgene RNA is labeled *Actin-msl-2* (note the absence of this signal in the tRNA negative control [–] and in RNA prepared from *w¹¹¹⁸* males and females). The RNase protections shown for the transgenes were performed on the same inserts as shown in Figures 2 and 3. Protection of the endogenous *Actin* gene serves as an internal control for RNA loading. In the top gel, *Actin-msl-2* signal is from a 60 hr exposure; *Actin* signal is from a 12 hr exposure of the same gel. In the bottom gel, *Actin-msl-2* signal is from a 72 hr exposure; *Actin* signal is from a 6 hr exposure. The *FC* transgene is included in both gels to correct for differences in electrophoretic conditions and exposure times. Signals were quantified using the Bio-Rad Phosphorimager and Molecular Analyst software. Relative signals normalized to the *Actin* control are given below for each genotype. The values are given in percent, and the signal from *FC* males was arbitrarily chosen as 100%: *FC* male, 100%, female, 64%; *FC* Δ 3 male, 59%, female, 40%; *FC* Δ SX3 male, 120%, female, 69%; *FC* Δ SX5 male, 119%, female, 69%; *FC* Δ SX5/ Δ SX3 male, 120%, female, 65%; *FC* Δ spl male, 47%, female, 31%.

(B) RT-PCR analysis of splice-junction mutant males and females. RT-PCR was performed on RNA from splice-junction mutant males and females using one primer specific to *msl-2* and one primer specific to the *Actin-5c* leader (see Experimental Procedures). Lanes are indicated above the gel: (–), negative control without DNA; RT–, control reactions in the absence of reverse transcription; RT+, reactions performed on reverse transcribed samples; S+, PCR reaction

the two ends interacted synergistically to repress MSL-2 protein expression, one would expect that the double-mutant transgenes would not cause a substantial increase in MSL-2 expression above that observed for the single mutants, while if the two ends were additive, one would expect a large increase in MSL-2 expression in the double mutant. In females carrying double-mutant transgenes, there was only a modest increase in MSL-2 staining relative to the single mutants (Figures 3E and 3F). Moreover, the single and double mutants give similar ranges of female expression if all inserts are considered (Figure 1A). This suggests that the two ends confer repression synergistically, but owing to the difficulty in rigorously quantitating the effects of the single and double mutants, this data cannot exclude additivity. Experiments in SL-2 cells were better able to distinguish between these possibilities (see below).

RNAse protection was performed with a probe specific to the *Actin* leader plus the linker region of the constructs to compare directly the mRNA produced by the transgenes. This probe protects different-sized fragments from the transgenes and the endogenous *Actin* gene, allowing us to use the latter as an internal control. This probe does not recognize endogenous *msl-2*. For all transgenes, females produce roughly 60% as much RNA as do males (Figure 4A). Most importantly, similar levels of RNA were observed in females carrying a wild-type transgene and females carrying transgenes with mutations in the SXL-binding sites of the UTRs. Thus, the dramatic differences in protein detected between males and females carrying wild-type *Actin-msl-2* transgenes and between females carrying wild-type and females carrying mutant *Actin-msl-2* transgenes cannot be attributed to differences in mRNA level.

The Male-Specific Intron Functions Primarily to Allow SXL Binding

To address the role of the male-specific intron in the 5' UTR, two constructs were assayed. The first carried a point mutation that eliminates the start codon of the uORF in the intron. The second carried mutations in the 5' and 3' splice sites of the intron. Point mutation of the start codon of the uORF (Δ uORF) does not result in significant production of MSL-2 in females. No binding above the low level observed for the wild-type cDNA construct is detected on X chromosomes of females carrying the Δ uORF construct (data not shown). RNase protection indicates that the amount of *msl-2* RNA produced from the Δ uORF construct is comparable to the amount produced by the other transgenes (data not shown). Mutation of the splice junctions (Δ spl) does not prevent rescue of *msl-2* mutant males. Moreover, the MSL staining patterns of *msl-2* males carrying either the wild-type or the Δ spl transgenes are indistinguishable

performed on a plasmid with the male spliced form; U+, PCR reaction performed on a plasmid with the female unspliced form.

(C) Western analysis of *msl-2* males rescued by the *FC* and *FC* Δ spl transgenes were performed using anti-MSL-2 antibodies. Lanes are as indicated above the gel. Equivalent levels of the large background band common to each sample shows that the lanes were equally loaded.

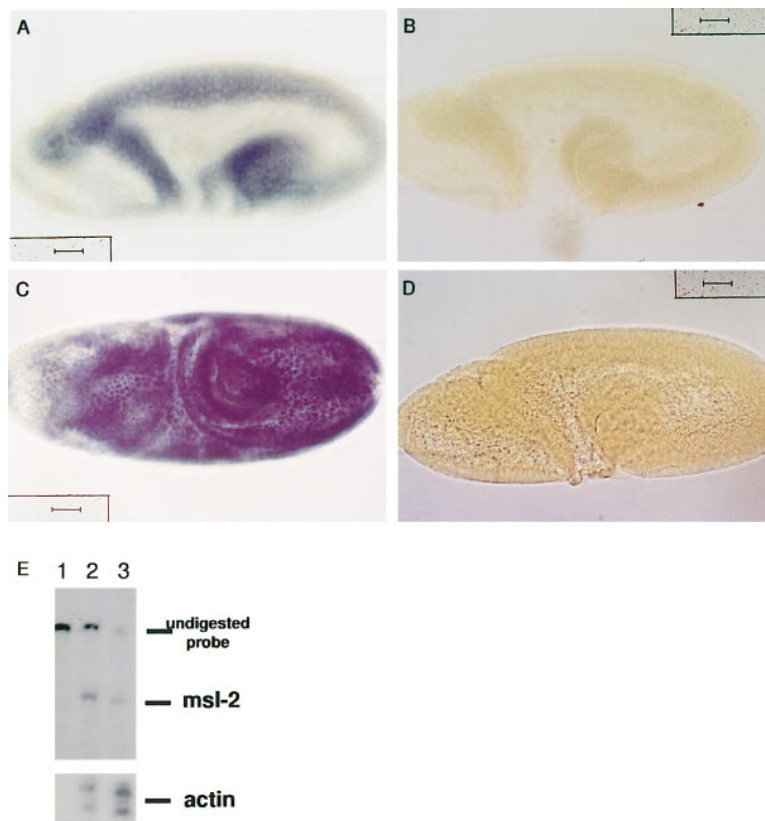


Figure 5. In Situ Localization of Endogenous *msl-2* mRNA

(A) Early gastrula-stage embryos were stained with antisense digoxigenin-labeled *msl-2* RNA probes. Staining was detected with alkaline phosphatase (note predominant staining surrounding the nuclei, seen as white spots).

(B) Negative control hybridized with sense-strand digoxigenin-labeled *msl-2* RNA probe gives no signal. Longer exposures with sense-stranded *msl-2* probes show some staining. The staining observed is consistently less than that seen with antisense probes exposed for the same time and does not show as discrete a localization.

(C) Anti-SXL staining of a female embryo provides a visual contrast for nuclear localization (contrast [A] and [C]).

(D) Anti-SXL staining of a male embryo provides a negative control for SXL staining.

(E) Quantitative comparison of endogenous *msl-2* RNA levels in males and females. Lane 1, tRNA negative control; lane 2, *w¹¹⁸* male RNA; lane 3, *w¹¹⁸* female RNA. RNAs were probed simultaneously with an *Actin* probe as a loading control. Signals were quantified using a Bio-Rad Phosphor Imager and the Molecular Analyst software. Two independent determinations gave similar female/male ratios when corrected for differences in actin signal (female/male = 0.32 ± 0.03). Signal derived from undigested probe is indicated.

(data not shown). Thus, removal of the intron is not strictly required for MSL-2 translation. To determine if there were subtle quantitative effects of retaining the intron in males, Western analysis was performed on *msl-2* mutant males rescued by either the wild-type or Δspl transgenes. Both transgenes produced equivalent amounts of protein in males, and the Δspl transgene actually produces lower levels of mRNA (Figures 4A and 4C). RNase protection analysis and RT-PCR were used to confirm that the majority of RNA from the Δspl males and females is in the expected unspliced configuration (Figure 4B). Thus, it appears that there is no substantial inhibition of translation associated solely with the presence of the male-specific intron. Taken together, the results from the $\Delta uORF$ and Δspl transgenes suggest that the primary regulatory function of the male-specific intron in females is exerted by its SXL-binding sites.

Endogenous *msl-2* RNA Is Not Retained in the Nucleus of Wild-Type Females

To determine whether *msl-2* translation is indirectly prevented in females by retention of *msl-2* mRNA in the nucleus, or *msl-2* translation is more directly blocked, we examined the subcellular distribution of *msl-2* RNA in both sexes. RNA in situ hybridization to wild-type embryos was performed with three different digoxigenin-labeled antisense *msl-2* RNA probes. While there was some variability in intensity of staining, all embryos examined ($n > 200$) showed predominant cytoplasmic localization (Figure 5A). No signal was detected with probes made from the sense strand of *msl-2*, nor was

staining observed with other unrelated sense-strand RNA probes (Figure 5B; data not shown). Thus, there is no qualitative difference in the subcellular distribution of *msl-2* transcripts in males and females, and the data are not consistent with nuclear retention as a mechanism for repression. To provide a visual contrast between nuclear and cytoplasmic localization, embryos were also stained with anti-SXL antibodies (Figures 5C and 5D; also, compare Figures 5A and 5C). As previously reported (Bopp et al., 1991), we observed predominant nuclear staining with a low level of cytoplasmic signal in females (Figure 5C). No staining is detected in males (Figure 5D).

Owing to differences in previous reports, we have reexamined the expression levels of *msl-2* RNA in males and females (Bashaw and Baker, 1995; Kelley et al., 1995; Zhou et al., 1995). Quantitatively comparing male and female levels by RNase protection, we find a 3.2-fold lower level of *msl-2* RNA in females (Figure 5E). Although this difference in RNA level may contribute to *msl-2* regulation, we do not believe that it is sufficient to explain the observed results, for two primary reasons. First, a 3.2-fold decrease in RNA level cannot by itself explain a complete absence of MSL-2 protein in females (Figure 4C). Second, this difference between male and female RNA levels is only half as great in males and females that carry the wild-type *Actin-*msl-2** transgene, but this transgene is still subject to strong regulation. These results indicate that the sex-specific differences in endogenous *msl-2* RNA levels depend in part on the sequence differences between these transgenes and

the endogenous gene. Our experiments cannot distinguish between the possibilities of decreased transcription or decreased stability as a basis for reduced RNA levels in females.

The UTRs of *msl-2* Confer SXL-Dependent Regulation on a Heterologous ORF

To investigate more quantitatively the mechanism of *msl-2* regulation, we established a transient transfection assay in *Drosophila* SL-2 tissue-culture cells. These cells are known to be male with respect to *Sxl* expression (Ryner and Baker, 1991). Constructs were designed in which the β -*gal* coding sequence was inserted in between the 5' and 3' UTRs of *msl-2*, under the control of the *hsp70* promoter (Figure 1B).

Cells transfected with the *msl-2*- β -*gal* plasmid produce significant amounts of β -*gal* activity in the absence of heat shock, and this activity increases linearly with increasing amount of reporter plasmid (data not shown). To determine if the *msl-2*- β -*gal* reporter could be regulated by *Sxl*, cells were cotransfected with either (i) *msl-2*- β -*gal* reporter, a plasmid carrying the *chloramphenicol acetyl transferase* (*cat*) gene, and a plasmid carrying a female-specific *Sxl* cDNA (Wang and Bell, 1994) under the control of the *hsp70* promoter, or (ii) *msl-2*- β -*gal* reporter, *cat* plasmid, and a plasmid containing only the *hsp70* promoter (Figure 1B). In cells that receive *Sxl* plasmid, there is a 7-fold reduction in β -*gal* activity relative to cells that did not receive *Sxl* plasmid ($p < 0.01$ by t test, hereafter), while the CAT activity produced by the two cell types is not statistically different (by anova analysis) (Figures 6A and 6B). Thus, the UTRs of *msl-2* confer a significant level of *Sxl*-dependent regulation on the β -*gal* reporter. Because the *Sxl* construct and our reporter constructs are under the control of the same promoter, there is a window of time in which *msl-2*- β -*gal* RNA is present, and there has not been time for a significant accumulation of SXL protein. Thus, the true repressive effect of *Sxl* may be greater than the 7-fold difference we observe.

The Regulation of *msl-2*- β -*gal* Requires the Synergistic Function of the SXL-Binding Sites

To investigate how SXL acts to repress *msl-2*- β -*gal* expression in SL-2 cells, the mutations assayed in transgenic flies were assayed in tissue culture. Mutating the SXL-binding sites in the 5' UTR of the *msl-2*- β -*gal* mRNA results in only 2-fold repression in the presence of SXL ($p < 0.01$). Mutating the SXL-binding sites in the 3' UTR results in only 1.7-fold repression ($p < 0.01$). These results indicate that the 7-fold repression observed for the wild-type *FC* construct in the presence of SXL cannot be explained by additive effects of the two UTRs. Simultaneous mutation of both UTRs resulted in complete loss of repression (Figure 6A). CAT activities were statistically similar for all transfections (by anova analysis) (Figure 6B). These results corroborate the results from transgenic flies and add weight to the suggestion that the two UTRs function synergistically to confer SXL-mediated repression. They also indicate that regulation in tissue culture is likely to reflect accurately regulation in flies in a qualitative sense.

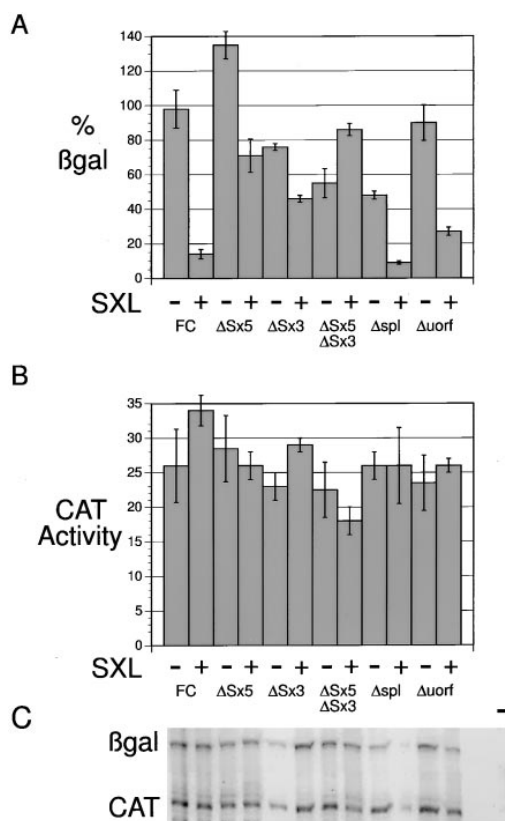


Figure 6. *msl-2*- β -*gal* Regulation in SL-2 Cells

(A) Relative β -*gal* activities of reporter constructs in the presence and absence of SXL. The constructs are indicated along the X axis, and the β -*gal* activity is given in percent on the Y axis. Each data point was generated from analysis of activities from triplicate samples with the exception of *FC* Δ *Sx3*, which was done in duplicate. In the case of the double-mutant construct, there is an apparent increase in β -*gal* activity in the presence of SXL (55% versus 86%). Although the difference is significant, it is small (1.5-fold), and of all the comparisons it gives the lowest score by t test ($p < 0.05$; for all other comparisons, $p < 0.01$ – 0.001). Therefore, we feel that the difference may have occurred by chance and is not biologically relevant.

(B) Relative *cat* activities corresponding to the β -*gal* activities in (A). Error was calculated as standard error from the mean (SEM) in both (A) and (B). β -*gal* activities were normalized to *cat* activities to correct for differences in transfection efficiency.

(C) RNase protection analysis of cytoplasmic RNAs prepared from transfections presented in (A) and (B). Lanes are in register with the histograms in (A) and (B) and correspond to the same samples as in (A) and (B). The far right lane labeled (–) is a negative tRNA control.

The results for the mutations in the splice sites and uORF of the male-specific intron are also similar to what was observed in transgenic flies. The only exception is that there is a 2-fold reduction in the expression of the Δ *spl* mutant in the absence of SXL, relative to the level produced by the wild-type *FC* in the absence of SXL ($p < 0.001$); the almost 2-fold increase in the β -*gal* activity produced by the Δ uORF construct in the presence of SXL relative to wild-type is not statistically significant. These observations suggest that the intron itself may have some subtle function in inhibiting translation that is revealed by the more quantitative SL-2 cell assays.

msl-2-β-gal Is Regulated at the Level of Translation

Two possible mechanisms of SXL-mediated posttranscriptional regulation that have been proposed are that SXL binds *msl-2* mRNA and prevents its export from the nucleus, and that SXL binds *msl-2* mRNA and directly blocks its translation. The observation that *msl-2* RNA is predominantly localized in the cytoplasm of both males and females argues in favor of a direct role for SXL in translational regulation.

To distinguish further between these two possibilities, cytoplasmic RNA was purified from SL-2 cells transiently transfected with wild-type or mutant *msl-2-β-gal* reporters, both in the presence and in the absence of SXL. RNase protection assays with a probe specific to *β-gal* indicate that the amount of *msl-2-β-gal* RNA produced was equivalent in cells that received wild-type *msl-2-β-gal* with or without SXL (Figure 6C). Furthermore, mutations in the SXL-binding sites of the UTRs, which result in significant increases in *β-gal* activity in the presence of SXL, do not significantly affect the amount of mRNA produced. RNase protection with a probe specific to *cat* was performed simultaneously on each sample to serve as an internal control for RNA level. The ratio of *β-gal/cat* RNA is equivalent in all cases, indicating that observed differences in *β-gal* activity are not due to differences in RNA level (Figure 6C). Since the levels of cytoplasmic reporter RNAs are equivalent, these data argue that the regulation of *msl-2-β-gal* occurs at the level of translation.

SXL Can Directly Bind both UTRs of *msl-2*

To determine if SXL protein directly interacts with *msl-2* RNA, as the above results strongly predict, we performed *in vitro* binding assays using fragments derived from the UTRs of *msl-2* as probes. Previous work has established that SXL purified after overexpression in bacteria binds with very high affinity to stretches of poly(U). A stretch of 8 Us is sufficient to allow high affinity binding, while shorter U stretches can be bound if presented in a favorable context (Samuels et al., 1994; Wang and Bell, 1994). In the case of the *msl-2* 5' UTR, there are 2 poly(U) stretches of 11 and 16 nt, respectively, while in the 3' UTR there are 4 poly(U) stretches of 7, 8, 8, and 7 nt, all within a region of 115 nt. Based on these observations, it seemed likely that RNA gel-shift assays that use purified SXL would give positive results. In light of this, we attempted to isolate SXL-binding activity from an *in vivo* source where it is functioning in regulation. Extracts were prepared from SL-2 cells transfected with the *Sxl* female cDNA plasmid or mock transfected with the *hsp70* promoter plasmid. These extracts were then used in UV cross-linking assays with probes from the *msl-2* UTRs (Figure 7). In the case of both UTRs, a strong binding activity is detected in the predicted size range for female SXL protein (Figure 7).

Several lines of evidence support the contention that this activity corresponds to SXL protein: (i) the activity is dependent on transfection with the SXL plasmid (Figures 7A, lanes 3 and 4, and 7B, lanes 3 and 4); (ii) the activity is not detected with probes in the antisense

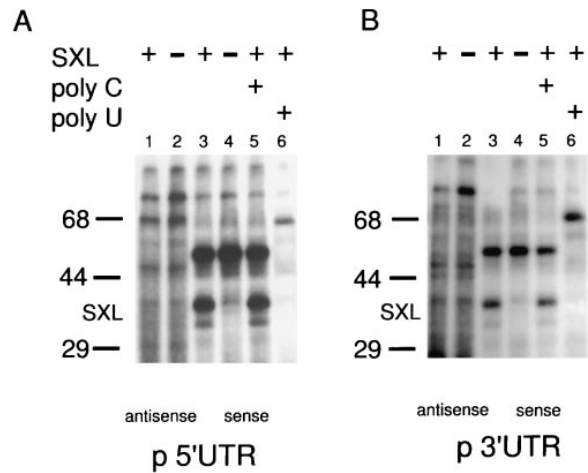


Figure 7. UV Cross-Linking of SXL Protein to the UTRs of *msl-2*. The 5' UTR data are presented in (A), and the 3' UTR are presented in (B). Results with antisense negative-control probes are shown in lanes 1 and 2, and results with sense probes are shown in lanes 3–6. Presence and absence of SXL are indicated with (+) and (–) above the gel. Equal amounts of poly(C) and poly(U) competitor were included in lanes 5 and 6, respectively. The expected size range of SXL is indicated to the left of each gel.

orientation (Figures 7A, lanes 1 and 2, and 7B, lanes 1 and 2); (iii) the activity is strongly competed by excess cold poly(U) oligonucleotide competitor but not by an equivalent amount of excess cold poly(C) competitor (Figures 7A, lanes 5 and 6, and 7B, lanes 5 and 6); and (iv) the activity is not detected with a 5' UTR probe with multiple substitutions in the poly(U) stretch (data not shown), and it is not detected with a 3' UTR probe missing the four poly(U) stretches (data not shown). Taken together, these data argue that SXL directly binds to both the 5' and 3' UTRs of *msl-2* and that this binding is essential for appropriate translational regulation.

Discussion

We have used two parallel approaches, analyses of transgenic flies, and transient transfection of SL-2 cells to characterize the *cis*-acting requirements for the male-specific synthesis of MSL-2 protein and to examine how the SXL protein functions to confer this regulation. The major conclusions from both sets of experiments are both complementary and consistent. They indicate that *msl-2* is regulated at the level of translation and that SXL directly interacts with *msl-2* RNA to confer repression.

Cis-Acting Sequences Required for the Sex-Specific Translation of *msl-2*

We have examined the role of the SXL-binding sites in the 5' and 3' UTRs, as well as two other potential regulatory elements in the 5' UTR of *msl-2*. Mutation of the SXL-binding sites at either end of *msl-2* results in similar high levels of ectopic expression of MSL-2 protein in transgenic females, indicating that sites at both ends are required for appropriate translational regulation. Mutation in both ends simultaneously does not dramatically affect the level of expression above what is seen in the

single mutants, suggesting that the two ends of *msl-2* RNA function synergistically to confer repression.

Similar results are obtained in SL-2 cells: mutation of either the 5' or 3' SXL-binding sites results in a significant loss of SXL-dependent repression, while simultaneous mutation of both ends only modestly diminishes SXL-dependent repression relative to what is seen in the single mutants. This argues that the roles of the two UTRs are not additive in conferring SXL-mediated repression, and these more quantitative data make a stronger argument for synergy. The results showing that repression is dependent on transfection with SXL, that repression is dependent on SXL-binding sites, and that SXL can interact with *msl-2* RNA in vitro argue strongly that SXL inhibits translation by directly associating with *msl-2* RNA.

There are numerous examples of 3' UTR sequences mediating translational repression (for review, see Decker and Parker, 1995) and other examples where sequences in the 5' UTR confer translational repression (for review, see Curtis et al., 1995). However, we know of no other case of an mRNA that has target sequences for the same translational regulatory factor in both the 5' and 3' UTRs. The requirement for binding sites at both ends to achieve appropriate regulation would suggest that SXL-mediated repression of *msl-2* represents a novel form of translational control. The ability of SXL protein to bind cooperatively (Wang and Bell, 1994), and the synergy observed for the two UTRs of *msl-2* in conferring SXL-mediated repression, raise the possibility that SXL binding at the two ends of *msl-2* RNA alters the structure of the mRNA and closes up or circularizes it, thereby blocking access of the translational machinery.

Why Bother with Regulated Alternative Splicing?

We also examined the potential role of the male-specific intron of *msl-2*. Our results from both tissue culture and transgenic flies argue that the primary role for the retention of this intron in females is to provide SXL-binding sites. Thus, the intron has little or no intrinsic role in blocking translation—neither through the presence of a small uORF nor through sequence-specific effects of the intron. Although in tissue culture, the intron appears to result in a small reduction of β -*gal* activity in the absence of SXL (2-fold), the lack of an effect in flies and the rather modest regulation in SL-2 cells suggest that it is not of major importance. Indeed, it is unclear if the 2-fold reduction in SL-2 cells is biologically significant, as preliminary results indicate that *Drosophila virilis*, a distant relative of *D. melanogaster* where it is known that the MSLs mediate dosage compensation (Bone and Kuroda, 1996; Marin et al., 1996), does not have this intron. In *D. virilis*, the 5' UTR of *msl-2* has three closely spaced SXL-binding sites, but there are no sex-specific splicing differences in the UTR (G. J. B. and B. S. B., unpublished data).

A Splicing Regulator Involved in Translation

The discovery that SXL has a role in regulating translation is surprising, in light of the well-characterized role

of SXL as a splicing regulator. The fact that SXL is predominantly localized to the nucleus of cells is consistent with its role in regulating splicing. While nuclear localization may appear inconsistent with a role in translation, it is not possible to conclude, based on predominant nuclear localization, that there is not a cytoplasmic pool of SXL. Indeed, the heterogeneous nuclear RNP protein A1, which is involved in nuclear pre-mRNA processing, provides an example of a protein originally thought to be strictly nuclear that has since been shown to shuttle between the nucleus and the cytoplasm (Siomi and Dreyfuss, 1995). Finally, the finding that the *Drosophila* BICOID protein, which is a homeobox transcription factor, also has a direct role in the translational control of *caudal* provides another case of a protein thought to function solely in the nucleus that has recently been shown to act in the cytoplasm as well (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996). Thus, SXL joins a growing number of proteins that have functional roles in both the nucleus and the cytoplasm.

Experimental Procedures

Actin P Element Constructs

To make the *Actin-5c* promoter-driven *msl-2* female cDNA (*FC*) constructs, the full-length *msl-2* cDNA designated 5.2-10 (which contains the male-specific intron) was removed from SKII Bluescript as a 3.8 kb NotI-SalI fragment. Overhangs were filled with Klenow, and BglII linkers were attached, followed by BglII digestion and insertion into the BamHI site of pCaSpeR-*Actin* (Thummel et al., 1988). The resulting construct contains *msl-2* in between the *Actin-5c* promoter and the *Actin-5c* polyadenylation signals. To make the male-specific cDNA, an RT-PCR fragment containing the male 5' UTR was subcloned into the full-length female cDNA 5.2-10. The male-specific cDNA, here designated *MC*, was inserted into the *Actin* promoter vector as described above.

Tissue-Culture Constructs

To generate constructs that contain the β -*galactosidase* gene in between the 5' and 3' UTRs of *msl-2* driven by the *hsp70* promoter, the 5' UTR and the sequences encoding the first 48 amino acids of *msl-2* were first cloned as a 560 nt BamHI fragment into the BamHI site of PC4- β -*gal* (Thummel et al., 1988). This construct is a translational fusion of MSL-2 position 48 to position 8 of β -*gal* and contains SV40 polyadenylation signals, but does not have a promoter. The *msl-2* 5' UTR- β -*gal*-SV40 was removed by EcoRI digestion and cloned downstream of the *hsp70* promoter into the EcoRI site of *phsp70*, which contains the *hsp70* promoter as a 400 nt 5' SalI 3' EcoRI fragment in pKSII. This clone was modified to create a unique XbaI site in between the β -*gal* and SV40 sequences for rapid insertion of different 3' UTRs, and the BamHI site in the pKSII polylinker was removed, so that the only BamHI sites were those that would release the 560 nt *msl-2* fragment. The wild-type 3' UTR of *msl-2* was cloned into the unique XbaI site to give *msl-2*- β -*gal*.

Site-Directed Mutagenesis

5' UTR mutants were made in pBam5'U (a 560 nt BamHI pSKII clone derived from cDNA 5.2-10 [Bashaw and Baker, 1995]) by unique site-elimination mutagenesis for the Δ uORF and Δ spl mutants, and by PCR-based mutagenesis for the SXL-binding site mutants. 3' UTR deletions were generated as follows: the large deletion by digestion of cDNA 5.2-10 with XbaI, and the smaller deletion removing the four SXL-binding sites was generated by PCR. The 5' UTR mutants, Δ uORF and Δ spl, were cloned into the *msl-2*- β -*gal* construct, replacing the wild-type UTR. They were cloned into the *Actin* construct stepwise. Mutant UTRs were first added to *MC* as Apal-SmaI fragments replacing the male 5' end. Full-length mutants were inserted into the *Actin* vector as described above. Cloning of the SXL-binding-site mutant required an additional step to generate a

Δ SXL 5' UTR BamHI fragment. A 260 nt ClaI-PmlI fragment that replaced the poly(U) runs with other nucleotides was generated by PCR and cloned into the endogenous *msl-2* PmlI site and the polylinker ClaI site of pBam5'U. All mutations were confirmed by sequencing, both upon generation and in the final clones. 3' UTR deletions were inserted as XbaI fragments into the *Actin-5c* constructs and the *msl-2*- β -*gal* constructs, respectively. 3' UTR deletions were confirmed by restriction digestion.

Oligonucleotides

For the mutagenic oligos, the substitutions are in bold, and the wild-type sequences they replace are indicated after each oligo and are underlined in parentheses. A list of the mutagenic oligos follows: Δ uORF, CA TTA ACA AGT ACT TGA GAC C (A); Δ spl-5', GCT TGG ACA ATT TTT TTT AGTTGC (GT); Δ spl-3', CGT GAA ACA TTC TGA TAA CG (AG); Δ SXL-5'-1, CCG AAC TGC AG (TTT TTT TTT TTT); Δ SXL-5'-2, CTG CAG TGA TCC GAA G (TTT TTT TTT TTT TTT T). A list of *msl-2* PCR oligos follows: Δ SXL 3' PCR (3'-5' nt 3642-3622—XbaI linker underlined), CGTCTAGA CTT TTT AGG CTC CAC AGC ATC C; m2-3.2 (3'-5' nt 500-480), AGA TTC GAA GCG GAG CGC AT; m2-3.1 (3'-5' nt 300-280), GGG CTA GTT ACC TGC AAT TC; m2-5.2 (5'-3' nt +1-+20), GTT CGC TCA GCA AAA TAT TGC; The sequence of the *Actin* PCR oligo actm2 is as follows. CCG AAT TCT CAT ATC ACT ACC GTT TGA G.

Transgenic Flies

w¹¹¹⁸ embryos were injected with the constructs and Δ 2-3 helper plasmid as described (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Multiple independent inserts (from 2-20) for each construct were obtained. Representative inserts were crossed into an *msl-2* mutant background to assay for rescue of *msl-2* males. Experiments in females carrying the transgenes were typically performed in a wild-type background, since wild-type females do not make any detectable MSL-2.

It was reported that high levels of MSL-2 in females result in developmental delay and a significant reduction in viability (Kelley et al., 1995). None of the inserts that were used for comparison in this study have significant effects on female viability when carried in single copy. Many of the single- and double-mutant UTR lines do cause some female-specific developmental and viability defects when two doses of the transgene are present, as do single copies of a few particularly strong mutant lines. The inserts used for comparison were selected to avoid strong effects on female viability, in order to eliminate the caveat of looking at a selected population of females. For example, if some of the inserts had strong effects on female viability, the individuals chosen for analysis might be biased for lower levels of MSL-2 expression.

Polytene Chromosome Immunofluorescence

Chromosome stainings were performed as described (Gorman et al., 1995). All experiments were done with anti-MSL-2 B-Pst antibody (Bashaw and Baker, 1995) or an anti-MSL-1 antibody (Gorman et al., 1995). Anti-MSL-1 was used for most experiments. MSL-1 staining is a valid indicator of MSL-2 protein presence both in wild-type males and females who ectopically express MSL-2 (Bashaw and Baker, 1995; Kelley et al., 1995).

Western Blots

Western analyses were performed as described (Bashaw and Baker, 1995). The anti-MSL-2 B-Pst antibody was used for all experiments. Signal was detected using an ECL Western blot kit and autoradiography.

RNase Protection

Flies

Poly(A)⁺ RNA was prepared from adults carrying single copies of the various transgenes and also from *w¹¹¹⁸* adults that carried no transgenes using standard procedures (Sambrook et al., 1989). Five micrograms of poly(A) RNA was used in each assay. RNAs were probed with a 150 nt riboprobe derived from the *Actin-5c* leader sequence and common cloning region of the *msl-2* transgenes. *w¹¹¹⁸* RNAs were also probed with a gene-specific *msl-2* probe that was generated by subcloning a 600 nt BamHI-SacI fragment from the

N-terminal coding region of MSL-2 into pSKII. RNase protection assays were performed as described (Ryner and Baker, 1991) with the exception that probes were not gel purified.

Tissue Culture

Cytoplasmic RNA was prepared as described (Berk and Sharp, 1978) and treated with RNase-free DNase in order to remove any contaminating DNA from the transfections. Twenty micrograms of cytoplasmic RNA was used per assay. RNase protection assays were as described above. The *cat* probe was made by subcloning a 300 nt BamHI-EcoRI fragment into pSKII, and the β -*gal* probe was made by subcloning a 400 nt HpaI-ClaI fragment from the middle of the β -*gal* coding sequence.

³²P-labeled riboprobes were synthesized by linearizing the various plasmids with appropriate enzymes and transcribing in vitro from either the T3 or T7 promoters.

Whole-Mount In Situ and Antibody Staining of Embryos

In situ hybridization to whole-mount embryos was performed with antisense digoxigenin-labeled *msl-2* RNA probes as described (Tautz and Pfeifle, 1989). The antisense *msl-2* probes used are derived from subclones from cDNA 5.2-10 and detect the following regions of *msl-2* transcripts: probe 1, nt 1-585; probe 2, nt 585-1159; probe 3, nt 1826-2444. The results in Figure 5 were obtained with probe 2. The *msl-2* sense-strand negative control is derived from nt 585-1826. The non-*msl-2* sense-strand negative control probe was provided by Y. Lie. Anti-SXL staining of embryos was performed as described (Franke et al., 1996).

Tissue Culture

SL-2 cells were transfected as described (Ryner and Baker, 1991) with the following modifications. Cells (4×10^6) were seeded on 60 mm tissue-culture plates. Each transfection contained 20 μ g of DNA: 1.5 μ g of *Sxl* plasmid or *hsp-70* plasmid, 0.25 μ g of *msl-2*- β -*gal* reporter plasmid, 0.25 μ g of *cat* reporter plasmid, and 18 μ g of pGEM plasmid as carrier. Transfections were harvested and one-fourth of the cells were used for β -*gal* and *cat* assays; the rest of the cells were used to prepare RNA (see above).

β -gal Assays

β -*gal* assays were performed as described (Jones et al., 1995).

cat Assays

cat assays were performed as described (Neumann et al., 1987), with the exception that the SL-2 extracts used were the same as for β -*gal* assays.

UV Cross-Linking Assays

For extract preparation, cells were harvested by centrifugation, washed in ice-cold PBS, and then lysed in 150 μ l of a buffer containing 150 mM NaCl, 50 mM Tris (pH 7.8), 1% Triton X-100, 1 mM PMSF, 2 μ g/ml leupeptin, 2 μ g/ml pepstatin, 2 mM benzamide, 0.5 mM EDTA. Cells were incubated 5 min on ice in the above lysis buffer and then centrifuged for 15 min at 15,000 rpm at 4°C to remove nuclei. The supernatant was removed, and 1-5 μ l was used in UV cross-linking assays. UV cross-linking was performed as described (Smibert et al., 1996) with sense and antisense probes from the 5' and 3' UTRs, respectively. For the 5' UTR, a 260 nt SacI subclone was used to make probes. For the 3' UTR, a 275 nt PstI-EcoRI subclone was used to make probes.

Statistical Analysis

β -*gal* activities were compared by t test for comparison between means (Sokal and Rohlf, 1981). *cat* activities were compared by single-classification analysis of variance (anova) with unequal sample sizes (Sokal and Rohlf, 1981).

Acknowledgments

We would like to thank members of the Baker and Macdonald labs for valuable discussions throughout the course of this work. We thank Carl Thummel for the gift of plasmids and Guennet Bohm for the preparation of media and fly food. We are especially grateful to

Ignacio Marin and Craig Smibert for valuable comments on the manuscript and for enthusiastically sharing their technical expertise. G. J. B. also thanks Fernanda and Alexander for long-term support. This research was supported by a grant from the NIGMS.

Received March 11, 1997; revised May 7, 1997.

References

- Baker, B.S., Gorman, M., and Marin, I. (1994). Dosage compensation in *Drosophila*. *Annu. Rev. Genet.* **28**, 491–521.
- Bashaw, G.J., and Baker, B.S. (1995). The *msl-2* dosage compensation gene of *Drosophila* encodes a putative DNA-binding protein whose expression is sex specifically regulated by *Sex-lethal*. *Development* **121**, 3245–3258.
- Bell, L.R., Horabin, J.I., Schedl, P., and Cline, T.W. (1991). Positive autoregulation of *Sex-lethal* by alternative splicing maintains the female determined state in *Drosophila*. *Cell* **65**, 229–239.
- Berk, A.J., and Sharp, P.A. (1978). Structure of the adenovirus 2 early mRNAs. *Cell* **14**, 695–711.
- Boggs, R.T., Gregor, P., Idriss, S., Belote, J.M., and McKeown, M. (1987). Regulation of sexual differentiation in *D. melanogaster* via alternative splicing of RNA from the *transformer* gene. *Cell* **50**, 739–747.
- Bone, J.R., and Kuroda, M.I. (1996). Dosage compensation regulatory proteins and the evolution of sex chromosomes in *Drosophila*. *Genetics* **144**, 705–713.
- Bopp, D., Bell, L.R., Cline, T.W., and Schedl, P. (1991). Developmental distribution of female-specific *Sex-lethal* proteins in *Drosophila melanogaster*. *Genes Dev.* **5**, 403–415.
- Cline, T.W. (1993). The *Drosophila* sex determination signal: how do flies count to two? *Trends Genet.* **9**, 385–390.
- Cline, T.W., and Meyer, B.J. (1996). Vive la différence: males vs. females in flies vs. worms. *Annu. Rev. Genet.* **30**, 637–702.
- Curtis, D., Lehmann, R., and Zamore, P.D. (1995). Translational regulation in development. *Cell* **81**, 171–178.
- Decker, C.J., and Parker, R. (1995). Diversity of cytoplasmic functions for the 3' untranslated region of eukaryotic transcripts. *Curr. Opin. Cell Biol.* **7**, 386–392.
- Dubnau, J., and Struhl, G. (1996). RNA recognition and translational regulation by a homeodomain protein. *Nature* **379**, 694–699.
- Franke, A., Dernburg, A., Bashaw, G.J., and Baker, B.S. (1996). Evidence that MSL-mediated dosage compensation in *Drosophila* begins at blastoderm. *Development* **122**, 2751–2760.
- Gorman, M., Kuroda, M.I., and Baker, B.S. (1993). Regulation of the sex-specific binding of the maleless dosage compensation protein to the male X chromosome in *Drosophila*. *Cell* **72**, 39–49.
- Gorman, M., Franke, A., and Baker, B.S. (1995). Molecular characterization of the male-specific lethal-3 gene and investigations of the regulation of dosage compensation in *Drosophila*. *Development* **121**, 463–475.
- Hilfiker, A., Yang, Y., Hayes, D.H., Beard, C.A., Manning, J.E., and Lucchesi, J.C. (1994). Dosage compensation in *Drosophila*: the X-chromosomal binding of MSL-1 and MLE is dependent on Sxl activity. *EMBO J.* **13**, 3542–3550.
- Hinnebusch, A.G. (1994). Translational control of GCN4: an in vivo barometer of initiation-factor activity. *Trends Biochem. Sci.* **19**, 409–414.
- Jones, F.E., Smibert, C.A., and Smiley, J.R. (1995). Mutational analysis of the herpes simplex virus virion host shutoff protein: evidence that vhs functions in the absence of other viral proteins. *J. Virol.* **69**, 4863–4871.
- Kelley, R.L., and Kuroda, M.I. (1995). Equality for X chromosomes. *Science* **270**, 1607–1610.
- Kelley, R.L., Solovyeva, I., Lyman, L.M., Richman, R., Solovyev, V., and Kuroda, M.I. (1995). Expression of *Msl-2* causes assembly of dosage compensation regulators on the X chromosomes and female lethality in *Drosophila*. *Cell* **81**, 867–877.
- Lucchesi, J.C., and Manning, J.E. (1987). Gene dosage compensation in *Drosophila melanogaster*. *Adv. Genet.* **24**, 371–429.
- Marin, I., Franke, A., Bashaw, G.J., and Baker, B.S. (1996). The dosage compensation system of *Drosophila* is co-opted by newly evolved X chromosomes. *Nature* **383**, 160–163.
- Nagoshi, R.N., McKeown, M., Burtis, K.C., Belote, J.M., and Baker, B.S. (1988). The control of alternative splicing at genes regulating sexual differentiation in *D. melanogaster*. *Cell* **53**, 229–236.
- Neumann, J.R., Morency, C.A., and Russian, K.O. (1987). A novel rapid assay for chloramphenicol acetyltransferase gene expression. *Biotechniques* **5**, 444–448.
- Rivera-Pomar, R., Niessing, D., Schmidt-Ott, U., Gehring, W.J., and Jackle, H. (1996). RNA binding and translational suppression by bicoid. *Nature* **379**, 746–749.
- Rubin, G., and Spradling, A. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348–353.
- Ryner, L.C., and Baker, B.S. (1991). Regulation of *doublesex* pre-mRNA processing occurs by 3'-splice site activation. *Genes Dev.* **5**, 2071–2085.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).
- Samuels, M.E., Bopp, D., Colvin, R.A., Roscigno, R.F., Garcia-Blanco, M.A., and Schedl, P. (1994). RNA binding by Sxl proteins in vitro and in vivo. *Mol. Cell. Biol.* **14**, 4975–4990.
- Siomi, H., and Dreyfuss, G. (1995). A nuclear localization domain in the hnRNP A1 protein. *J. Cell Biol.* **129**, 551–560.
- Smibert, C.A., Wilson, J.E., Kerr, K., and Macdonald, P.M. (1996). *smaug* protein represses translation of unlocalized nanos mRNA in the *Drosophila* embryo. *Genes Dev.* **10**, 2600–2609.
- Sokal, R.R., and Rohlf, F.J. (1981). *Biometry* (New York: W.H. Freeman and Co.).
- Sosnowski, B.A., Belote, J.M., and McKeown, M. (1989). Sex-specific alternative splicing of RNA from the *transformer* gene results from sequence-dependent splice site blockage. *Cell* **58**, 449–459.
- Spradling, A., and Rubin, G. (1982). Transposition of cloned P elements into *Drosophila* germ line chromosomes. *Science* **218**, 341–347.
- Tautz, D., and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98**, 81–85.
- Thummel, C.S., Boulet, A.M., and Lipshitz, H.D. (1988). Vectors for *Drosophila* P-element-mediated transformation and tissue culture transfection. *Gene* **74**, 445–456.
- Wang, J., and Bell, L.R. (1994). The *Sex-lethal* amino terminus mediates cooperative interactions in RNA binding and is essential for splicing regulation. *Genes Dev.* **8**, 2072–2085.
- Zhou, S., Yang, Y., Scott, M.J., Pannuti, A., Fehr, K.C., Eisen, A., Koonin, E.V., Fouts, D.L., Wrightsman, R., Manning, J.E., and Lucchesi, J.C. (1995). Male-specific lethal 2, a dosage compensation gene of *Drosophila*, undergoes sex-specific regulation and encodes a protein with a RING finger and a metallothionein-like cysteine cluster. *EMBO J.* **14**, 2884–2895.