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

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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; HiUffaker et al., 1994). Sexl also controls sex determination (reviewed by Cline, 1993; Cline and Meyer, 1996). Sexl encodes an RNA-binding protein that regulates its own expression by regulating splicing (Bell et al., 1991). In females, Sexl protein-directed splicing of Sexl pre-mRNA generates an open reading frame (ORF). In males, default splicing results in an mRNA with premature stop codons. Sexl also regulates transformer (tra) (Boggs et al., 1987; Nagoshi et al., 1988; Sosnowski et al., 1989); in females, the Sexl-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 Sexl would regulate dosage compensation similarly, by controlling the splicing of the pre-mRNA of one or more of the msls, 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 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 Sexl, there is a 7-fold decrease in β-gal activity relative to cells that do not receive Sexl; 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 mRNA. 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

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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 females at 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±gal and cat reporter constructs. Features of the msl-2±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).
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 FCΔSXS transgene (A and B), the FCΔSXSΔ splice junction mutant (C and D), or the FCΔSXSΔ transgene (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%; FCΔ3 male, 59%; female, 40%; FCΔSX3 male, 120%; female, 69%; FCΔSX5 male, 119%; female, 69%; FCΔSX5/ΔSX3 male, 120%; female, 65%; FCΔsp 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 RNA; RT--, control reactions in the absence of reverse transcription; RT+, reactions performed on reverse transcribed samples; S+, PCR reaction 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 FCΔsp 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.

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 (ΔuORF) 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 ΔuORF construct (data not shown). RNase protection indicates that the amount of msl-2 RNA produced from the ΔuORF construct is comparable to the amount produced by the other transgenes (data not shown). Mutation of the splice junctions (Δsp) 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 Δsp transgenes are indistinguishable from those of wild-type msl-2 males. 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.
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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, w1118 male RNA; lane 3, w1118 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 $= 0.32 \pm 0.03$). 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 Δsp1 transgenes. Both transgenes produced equivalent amounts of protein in males, and the Δsp1 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 Δsp1 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 ΔuORF and Δsp1 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 if 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 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.

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 Δspl 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 ΔuORF 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 decreases 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 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 translation repression (for review, see Curtis et al., 1995). However, we know of no other case of an mRNA that has target sequences (for review, see Curtis et al., 1995). However, we know 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.

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.

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 a 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 Dro sophila 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).

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 pcA5pER-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 80 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 phs70, which contains the hsp70 promoter as a 400 nt 5' Sal placic fragment. 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 ΔuORF and ΔSpI 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, ΔuORF and ΔSpI, 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-Smal 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
Sex-lethal and Translational Control

±93XL 5’ UTR BamHI fragment. A 260 nt ClaI-Pmi1 fragment that replaced the poly(U) runs with other nucleotides was generated by PCR and cloned into the endogenous msl-2 Pmi1 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 mutagenetic oligos follows: ±93uORF, CA TTA ACA GTG ACT TGA GAC C (Δ); ±93s1-5’, GCC TTG ACA ATT TTT TTT TTT AGTTGC (GT); ±93s1-3’, CGT GAA ACA TTC TGA TAA CG (Δ); ±93s1-5’-1, CCG AAC TGC AG (TTT TTT TTT TTT TTT); ±93s1-5’-2, CGT CAG TGA TCC GAA (TTT TTT TTT TTT TT). A list of msl-2 PCR oligos follows: ±93sXL 3’PCR (3’-5’ nt 3642–3622—XbaI linker underlined), CTGTCTAGA CCT TTT AGG CTC CAC AGC ATC C; m2-3.2 (3’-5’ nt 500–480), AGA TTC GAA GCG GAG GCG AT; m2-5.1 (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 TTG CTC CAT ACT ACC ACC GTT GAA G.

Transgenic Flies

±93W embryos were injected with the constructs and ±93-3 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 described 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 Assays

Files

Poly(A)± RNA was prepared from adults carrying single copies of the various transgenes and also from ±93W 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. ±93W 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 Hpal-Clai fragment from the middle of the β-gal coding sequence.

±93P-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 Situs 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-S-5XL 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 × 10⁶) were seeded on 60 mm tissue-culture plates. Each transfection contained 20 μg of DNA: 2 μg of the reporter plasmid, 0.25 μg of cat reporter plasmid, and 18 μg 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 μl of a buffer containing 150 mM NaCl, 50 mM Tris (pH 7.8), 1% Triton X-100, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 mM benzamidine, 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 μl 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 S-5’ and S-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).

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References


