The *msl-2* dosage compensation gene of *Drosophila* encodes a putative DNA-binding protein whose expression is sex specifically regulated by *Sex-lethal*

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**SUMMARY**

In *Drosophila* dosage compensation increases the rate of transcription of the male’s X chromosome and depends on four autosomal *male-specific lethal* genes. We have cloned the *msl-2* gene and shown that MSL-2 protein is co-localized with the other three MSL proteins at hundreds of sites along the male polytene X chromosome and that this binding requires the other three MSL proteins. *msl-2* encodes a protein with a putative DNA-binding domain: the RING finger. MSL-2 protein is not produced in females and sequences in both the 5’ and 3’ UTRs are important for this sex-specific regulation. Furthermore, *msl-2* pre-mRNA is alternatively spliced in a *Sex-lethal*-dependent fashion in its 5’ UTR.

Key words: dosage compensation, *Drosophila*, male-specific lethal, *msl-2*, DNA binding, sex specification, RNA

**INTRODUCTION**

In many species, sex is determined by differences in the number of copies of a single chromosome. In such cases, a process of dosage compensation may evolve to allow the different number of copies of genes in the two sexes to produce the same amount of functional product. In *D. melanogaster*, *C. elegans* and mammals, where dosage compensation regulates X chromosome activity, it appears that substantially different mechanisms of dosage compensation have evolved. In mammals dosage compensation is achieved by the inactivation of one of the two female X chromosomes (for reviews see Lyon, 1992; Borsani and Ballabio, 1993). In *C. elegans* dosage compensation is brought about by decreasing the rate of transcription of genes on both of the female’s X chromosomes (for review see Hsu and Meyer, 1993). In *D. melanogaster* in contrast, dosage compensation occurs by doubling the transcription rate of the genes on the male’s X chromosome (for review see Baker et al., 1994).

Despite the overt differences between dosage compensation in flies, worms and mammals, in all three cases, dosage compensation appears to be intimately associated with global changes in the chromatin structure of the X chromosome in the sex in which dosage compensation occurs. In mammals the inactive X chromosome is compacted heterochromatin, while in the interphase polytene salivary gland chromosomes of flies, the hypertranscribed X chromosome of the male can be seen to have a more open, “puffed” chromatin structure than the female’s X chromosomes or the autosomes of either sex (Dobzhansky, 1957). In *C. elegans* the recent finding (Chuang et al., 1994), that one of the genes controlling dosage compensation encodes a protein that associates specifically with the female’s X chromosomes and has sequence homology to proteins that are known to be involved in changes in chromatin condensation in other systems, strongly suggests that here too dosage compensation is linked to an alteration in the chromatin structure of the X chromosome. In addition, in both flies and mammals, dosage compensation is correlated with a change in the distribution of histone H4 acetylated at lysine 16 (H4Ac16) (Turner et al., 1992; Jeppesen and Turner, 1993): in mammals, H4Ac16 is underrepresented on the inactive X chromosome while, in flies, the male’s X chromosome is enriched in H4Ac16.

Dosage compensation in flies is brought about by the interaction of trans-acting regulatory factors with cis-acting dosage compensation sequences widely scattered along the X chromosome (for review see Baker et al., 1994). While nearly all genes on the X chromosome in flies are dosage compensated, dosage compensation is not a chromosomal process, but rather acts at the level of single genes, or small regions of the X chromosome. X chromosomal genes put into an autosomal site by either translocations or P-element-mediated transformation frequently remain dosage compensated.

Trans-acting regulatory factors necessary for hypertranscription of the male’s X chromosome throughout most developmental stages are encoded by four autosomal, male-specific lethal genes, *maleless* (*mle*), *male-specific lethal-1* (*msl-1*), *male-specific lethal-2* (*msl-2*) and *male-specific lethal-3* (*msl-3*). (There is some evidence that dosage compensation in early embryogenesis is not mediated by the known *msls*. For review see Baker et al., 1994.) These four genes are collectively referred to as the *msls*. Loss-of-function mutations of the *msls* have no phenotypic effects in females. Three of the four *msl* genes, *msl-1*, *msl-3* and *mle*, have been molecularly characterized (Kuroda et al., 1991; Gorman et al., 1993, 1995; Palmer et al., 1993, 1994; Hilfiker et al., 1994). All three genes encode...
proteins that are bound to hundreds of sites along the length of the male, but not the female, X chromosomes. The similarity of this distribution to that expected for the cis-acting dosage compensation sites along the X suggests that these MSL proteins may directly mediate dosage compensation. Studies with antibodies to these three MSL proteins showed that they are bound to the same sites along the X chromosome (Bone et al., 1994; Gorman et al., 1995). Moreover, the intensities of the anti-MSL-1, anti-MSL-3 and anti-MLE staining at any given site are strongly correlated suggesting that they are present at these sites in similar relative amounts (Gorman et al., 1995). Further evidence for a close association of these proteins comes from the findings that the X chromosome binding of any of these three MSL proteins requires the wild-type function of all four known msl genes (Gorman et al., 1993, 1995; Palmer et al., 1994). This co-dependence of MSL binding has led to the suggestion that the MSL proteins form a complex (Gorman et al., 1993).

How the MSL proteins function to bring about the hypertranscription of the male’s X chromosome is not currently known. The sequences of the MSL-1, MSL-3 and MLE proteins have not been particularly revealing with respect to the molecular functions of these proteins. The MSL-1 and MSL-3 proteins do not have significant homology to any proteins of known function (Palmer et al., 1993; Gorman et al., 1995). However, the MLE protein is a Drosophila homologue of a mammalian RNA helicase (Lee and Hurwitz, 1993) and is also closely related to three putative RNA-dependent ATPases from yeast (Kuroda et al., 1991). It is unclear how this relates to MLE’s role in dosage compensation. However, significant insight into the mechanism of dosage compensation has come from the finding that H4Ac16 is distributed in a pattern nearly identical to that of the MSLs (Bone et al., 1994). Moreover, the presence of H4Ac16 in this pattern on the male X chromosome is dependent on the functions of the four msl genes (Bone et al., 1994). These data suggest that the MSLs may alter the chromatin structure of the X chromosome.

The sex-specificity of dosage compensation is brought about by the female-specific SXL protein, which negatively regulates dosage compensation in females (Cline, 1978; Lucchesi and Skripsky, 1981). At the molecular level, the negative regulation of dosage compensation by SXL can be seen most directly in females that are mosaic with respect to SXL expression (Cline, 1978; Lucchesi and Skripsky, 1981). In these individuals, the MSL-1, MSL-2, MSL-3 proteins do not have significant homology to any proteins of known function (Palmer et al., 1993; Gorman et al., 1995). However, the MLE protein is a Drosophila homologue of a mammalian RNA helicase (Lee and Hurwitz, 1993) and is also closely related to three putative RNA-dependent ATPases from yeast (Kuroda et al., 1991). It is unclear how this relates to MLE’s role in dosage compensation. However, significant insight into the mechanism of dosage compensation has come from the finding that H4Ac16 is distributed in a pattern nearly identical to that of the MSLs (Bone et al., 1994). Moreover, the presence of H4Ac16 in this pattern on the male X chromosome is dependent on the functions of the four msl genes (Bone et al., 1994). These data suggest that the MSLs may alter the chromatin structure of the X chromosome.

In this paper, we describe the cloning and molecular characterization of the msl-2 gene. We have found that msl-2 encodes a protein that associates with hundreds of sites on the male, but not the female, X chromosomes. The sites of MSL-2 association are coincident with those of the other MSLs and largely coincident with the X chromosomal sites at which H4Ac16 is found. Furthermore, MSL-2 X chromosome binding depends on the wild-type function of all three of the other MSLs and also depends on the absence of SXL. We also report that MSL-2 protein expression is sex-specifically regulated and that sequences in both the 5' and 3' UTR are essential for this regulation. Moreover, msl-2 pre-mRNA is alternatively spliced and this splicing is dependent on Sex-lethal (Sxl). Finally, MSL-2 encodes a protein with a RING finger domain, a motif implicated in DNA binding, and thus may function to target the putative MSL complex to the male's X chromosome.

MATERIALS AND METHODS

Drosophila stocks

All mutants and rearrangements not specifically referenced are described in Lindsley and Zimm (1992). The msl-2 allele was used for all of the experiments described and is referred to throughout as msl-2.

Physical localization of the msl-2 gene

To physically map the msl-2 gene, we used T(Y;2)2s to generate terminal duplications (Lindsley et al., 1972) and determined whether these duplications covered the male lethality of a homozygous msl-2 mutation. T(Y;2)2s generated by Lindsley et al. (1972) [T(Y;2)G146, (23E1-2); T(Y;2)G120, (23F5-6)] were obtained from the Bloomington Stock Center. T(Y;2)188.9, (23F1-2) was obtained from J. Sekelsky. A T(Y;2) is a reciprocal translocation between a Y chromosome and a second chromosome and consists of the proximal (centromeric) portion of the Y capped by the distal portion of the second chromosome (2pYp) and the distal portion of the Y chromosome attached to the proximal (centromeric) portion of the second chromosome (2pYp). The short arm and long arm of the Y chromosome are marked with y' and B', respectively; these markers are used to follow the Yp2p and Yp2p parts of the translocation. To generate terminal duplications with T(Y;2)G146 and T(Y;2)188.9, where the Yp2p portion of the translocation had all Y chromosome fertility factors, attached-X/Y(Y;2)CyO males were crossed to msl-2 homozygous females and X/Yp2p; msl-2/CyO sons crossed to msl-2/CyO females and the viability of their X/Yp2p; msl-2/CyO sons examined. In the case of T(Y;2)G120, where the Yp2p portion of the translocation lacked some Y fertility factors, attached-X/Y(Y;2)G120/Cy, cn males were crossed to w1118; msl-2 cn females. Daughters that were attached-X/Y(Y;2)G120/Cy, cn were crossed to attached-X; Y(Y;2)G120/Cy, cn males to generate attached-X/Y(Y;2)G120/msl-2 msl-2 males. These males were crossed to msl-2 cn/CyO females and viability of X/Yp2p; msl-2 cn/msl-2 cn males determined.

An additional duplication in this region was provided by T(2;3)D275, a transposition of region 23E1-2-25A into the third chromosome at approximately salivary region 74. To test whether this duplication carried msl-2* males that were msl-2/CyO; Dp(2;3)D275/TM3 were crossed to msl-2 homozygous females and the viability of msl-2/msl-2; Dp(2;3)D275/+ sons determined.

Deficiencies in the region of msl-2 were generated by combining parts of T(2;3)s with one break in the region of the second chromosome containing msl-2 and a second break in the centric heterochromatin of the third chromosome. The T(2;3)s used were T(2;3)16 (23B; 3 het), T(2;3)188.16 (23E3; 3 het), T(2;3)H7 (23D1-2; 3 het), T(2;3)DD30 (24A1-2; 3 het), T(2;3)DD42, (23E1-2; 3 het) and T(2;3)DD62 (23F2-3; 3 het) and were obtained from J. Sekelsky. Interstitial deficiencies for the region where msl-2 is located were con-
structed from pairs of these translocations by combining the 2D3P portion of the translocation with the more distal 2L breakpoint with the 2P3P portion of the translocation with the more proximal second chromosome breakpoint. To generate such a deficiency between two translocations T(2;3)i and T(2;3)j, where T(2;3)i has the more distal second chromosome breakpoint, T(2;3)j/CyO and T(2;3)i/CyO flies were crossed to Sp/CyO; Sh/TM2 flies. Female progeny that were T(2;3)i/CyO; TM2 were crossed to male progeny that were T(2;3)j/Sp;Sh and single progeny that should be 2P3P/2D3P/Sh (assuming only alternate and adjacent 1 segregations occurred in the parents) were crossed to b Sco/SMI flies to establish a stock of the interstitial deficiency (2P3P/2D3P) balanced over SMI. Males from this stock were crossed to msl-2 homozygous females and the viability of 2P3P/2D3P/msl-2 males examined. In addition, J. Sekelsky provided the segmental deficiency Df(H7xDTD62), (23D1-2; 2F2-3) that he constructed from these translocations.

We confirmed the cytological breakpoints of all translocations and synthetic deficiencies used. In addition, all deficiencies were confirmed by in situ hybridization with genomic clones from this region (see below).

To isolate clones from the 23F1-2 region, where msl-2 mapped cytogenetically, DNA was isolated by pulse-field gel electrophoresis from a YAC clone (YAC E02-92; D. Hartl, personal communication) derived from 23F1-2. A probe was prepared from this DNA by hexamer labeling and used to screen a lambda library of Drosophila genomic DNA. As a YAC clone of Drosophila DNA will likely contain some middle repeat sequences, we also prepared a hexamer labeled probe from an equal amount of wild-type (Canton S) Drosophila genomic DNA and hybridized this probe to a set of replica filters of the library. Plaques that hybridized to the YAC probe, but not to the Canton S probe, should contain unique sequences from distal 23E and were picked. This initial screen yielded a lambda clone that was used in turn to screen a cosmid library of Drosophila genomic DNA (provided from J. Tomkun). Two cosmids, EC5 and EC8, homologous to that phage were isolated and their orientation relative to the chromosome was determined by in situ hybridization to salivary gland chromosomes. These experiments showed that EC5 and EC8 spanned the two breakpoints that delimited the region containing msl-2.

**Transformation experiments**

Genomic SpeI-NotI, KpnI-KpnI, SpeI-SpeI and XbaI-XbaI fragments of 11.2, 11.0, 11.5 and 5.0 kb, respectively, were subcloned into the CaSpeR4 transformation vector (Pirrotta, 1988). These constructs were injected at 0.3 mg/ml together with 0.1 mg/ml of Δ 2-3 helper (Laski et al., 1986) into w118 embryos under standard conditions (Spradling, 1986). Go adults were crossed to w118; Sp/CyO; Sh/TM2 flies of the opposite sex. w118; +/CyO; +/Sh or TM2 male progeny of that cross that carried the P element were crossed to w118; msl-2 (or msl-2Δ5kb)/CyO females. The segregation in the latter cross established the chromosome on which the P element was present. Inserts on either the X or third chromosome were established in balanced stocks which carried an msl-2 allele balanced on the second chromosome. Whether or not the DNA carried by the P element rescued msl-2 was assessed by determining the viability of homozygous msl-2 males that were heterozygous for the transformation construct.

**cDNA and genomic sequencing**

cDNAs were isolated from either a lambda ZAP head library (DiAntonio et al., 1993), a larval imaginal disc library (provided by A. Cowman) or male and female third instar larval libraries (provided by S. Elledge). cDNA inserts were subcloned into Bluescript vectors or rescued as plasmids from lambda ZAP and sequenced by the dideoxy method using standard procedures (Sambrook et al., 1989). The genomic region that contained msl-2 was subcloned into Bluescript vectors and sequenced by similar methods.

**Antibody production**

Anti-MSL-2 antibodies were raised against three different MSL-2 fusion proteins. MSL-2 fusion proteins were made by subcloning a 360 bp EcoRI Xhol restriction fragment (ab1 – amino acids 414-534), a 408 bp Xhol-PspI restriction fragment (ab2 – amino acids 534-669) and a 1.3 kb BamHI-PspI restriction fragment (ab3 – amino acids 50-464) in frame into the pWR 590 (Guo et al., 1984) vector to express β-GALACTOSIDASE-MSL-2 fusion proteins. To obtain fusion proteins for immunization, one liter cultures were induced with 1 mM IPTG. Cultures were centrifuged and pellets were resuspended in 50 mM Tris-HCl (pH 7.9), 200 mM NaCl, 2 mM EDTA, 2 mM β-mercaptoethanol, 100 μg/ml PMSF. Cells were lysed by treating with 0.2 mg/ml lysozyme on ice for 15 minutes, followed by the addition of 1% Triton X-100 and 10 minutes at room temperature prior to sonication for 3x15 seconds. The insoluble fraction was collected over a 40% sucrose cushion by centrifugation for 30 minutes at 14,000 revs/minute and resuspended in extraction buffer [8 M urea, 0.5 M NaCl, 0.5 M Tris-HCl (pH 7.9), 30 mM β-mercaptoethanol]. Following overnight dialysis against PBS, protein was mixed with MPL+TDM+CWS adjuvant (Ribi ImmunoChem Res.) and injected into rats at 100 μg/250 μl per boost. Rats were boosted at two week intervals. For affinity purification of antibodies 100-200 μl of serum was added to 10 ml of block solution [PBS with 0.1% NP40 and 5% baby milk] and incubated on a western of total protein from IPTG induced cultures of the pWR 590 vector without insert for 1 hour at room temperature. The supernatant was added to a western containing MSL-2 fusion protein and incubated overnight at 4°C. Antibodies were eluted with 2× 1 ml of 0.1 M glycine (pH 2.5), neutralized with 600 μl of 2 M Tris-HCl (pH 7.9) and concentrated using Amicon centricron 100 concentrators.

**Western analyses**

Westerns were done as described in Gorman et al. (1995) with the exception that antibodies 1 and 2 were preabsorbed on westerns of female larvae for 1 hour at room temperature.

**Polytene chromosome immunofluorescence**

Salivary glands were dissected and stained as described in Gorman et al. (1995) except that the secondary antibody used to detect MSL-2 was conjugated with Cy3, a fluorescent tag. All three MSL-2 antibodies were used for all chromosome stains at a dilution of 1:50. Anti-MSL-1 antibody (Gorman et al., 1995) was used at a dilution of 1:50 for co-localization of MSL-2 and MSL-1.

**Primer extensions**

Primer extensions were performed as described in Sambrook et al. (1989) with the exceptions that poly(A)-selected instead of total RNA was used as the template and annealing was 2-3 hours at 45°C rather than overnight.

**Reverse transcription PCR**

First-strand cDNAs were synthesized by reverse transcription using eight primers staggered at 500 bp intervals across the msl-2 gene. Approximately 500 ng of poly(A)-selected Canton S male, Canton S female or Sxl(f1)/Sxl(fh1) RNA was used as a template in the reverse transcription reactions. PCR reactions were performed using standard conditions with staggered primer pairs that covered the entire msl-2 transcription unit.

**Northern analyses**

Northern analyses were performed using 4-6 μg per lane of the following poly(A)-selected RNAs: Canton S female and male, female and male progeny of tudor mothers, and msl-2 female. Progeny of tudor mothers lack a germline and therefore allow somatic and germline transcripts to be distinguished by northern blot. RNAs were electrophoresed, transferred and probed using standard procedures.
RESULTS

Molecular cloning of msl-2

To localize msl-2, we made use of a set of terminal duplications and interstitial duplications and deficiencies generated from T(Y;2)s and T(2;3)s (see Experimental Procedures for details). Complementation tests between these deficiencies and duplications and msl-2 were consistent and localized msl-2 between the breakpoints of T(Y;2)188.9 and T(2;3)62 both of which are broken at 23F1-2 (Fig. 1). The T(Y;2)188.9 and T(2;3)62 breakpoints are spanned by two overlapping cosmids, EC8 and EC5, respectively (see Experimental Procedures for details of the cloning of this region). Southern blot analysis showed that the genomic interval delimited by the two breakpoints is approximately 30 kb, while the genomic region spanned by the two cosmids is approximately 55 kb (Fig. 2A).

In order to get some idea of the number and precise locations of the transcription units present in this region cDNAs were isolated. A probe comprising a mixture of three genomic fragments that together covered the region spanned by the T(Y;2)188.9 and T(2;3)62 breakpoints, as well as a small amount of flanking DNA, was used to screen cDNA libraries. cDNA clones isolated from this screen were put into groups by cross hybridization. To determine whether the cDNAs came from the region between the T(Y;2)188.9 and T(2;3)62 breakpoints, they were checked by in situ hybridization and/or using hybridization with genomic DNA clones.

We identified the msl-2 transcription unit in this region by P-element-mediated germline transformation (Spradling, 1986). Genomic SpeI-NorI (11.2 kb), KpnI-KpnI (11.0 kb) and SpeI-SpeI (11.5 kb) fragments, each of which covered two or three cDNA classes (Fig. 2B), were subcloned into the Casper-4 transformation vector and used to establish germline transformants in the w1118 stock (see Experimental Procedures for details). Three third chromosome inserts of both the SpeI-NorI and SpeI-SpeI constructs and one third chromosome insert of the KpnI-KpnI construct were obtained and tested for rescue of the male lethality caused by msl-2. Only the KpnI-KpnI transformant rescued the male lethality, indicating that one of the three transcription units in this 11 kb fragment was msl-2 (Fig. 2B).

Fig. 1. Summary of the results of complementation tests between msl-2 and duplications and deficiencies. Open boxes designate the extent of deficiencies and filled boxes designate the extent of duplications. The relative order of close breakpoints (i.e. less than one major band) is given only for breakpoints ordered by in situ hybridization with genomic DNA clones.

Fig. 2. (A) The two cosmids EC5 and EC8 that span the region of genetic complementation are shown. The locations of the T(Y;2) 188.9 and the T(2;3)DTD62 breakpoints are indicated above the EC5 and EC8 cosmids respectively. R, EcoRI, N, NorI, K, KpnI, S, SpeI. (B) A schematic diagram of the three initial genomic rescue constructs. The number of cDNA classes contained within each construct is indicated. Enzyme sites as in A. (C) Expanded diagram of the KpnI genomic rescue fragment. The three transcription units are shown underneath the genomic clone. Their orientations are indicated by arrows at their 3′ ends. The 5 kb Xbal rescue construct that contains only the proximalmost transcription unit is indicated underneath the KpnI construct. Note that the Xbal construct removes part of the 3′ end of msl-2. X, XbaI.
To determine which of these three transcription units corresponded to \textit{msl-2}, restriction fragments of cosmid EC8 that contained only one of these transcription units were subcloned into the Casper-4 transformation vector and used to transform the \textit{w^{118}} stock. Three third chromosomal inserts of a 5.0 kb \textit{XbaI}-\textit{XbaI} fragment that overlapped the proximal end of the \textit{KpnI} rescue fragment and contained only the most proximal transcription unit present in that fragment (Fig. 2C) were obtained and shown to also rescue \textit{msl-2} male lethality, indicating that this transcription unit corresponds to \textit{msl-2}.

**Transcript analysis**

Probing northern blots of poly(A)+ RNA from males and females with \textit{msl-2} probes revealed male- and female-specific transcripts of approximately 3.7 kb and 3.8 kb (Fig. 3A). (We also observe a broad band at 1.8 kb on this northern. Unlike the 3.7/3.8 kb transcripts, this broad band is not reproducibly detected with other double-stranded \textit{msl-2} probes and is likely to represent an artifact of the riboprobe preparation.) That the 3.8 kb transcript is a bona fide \textit{msl-2} transcript is strongly suggested by the finding that this transcript is greatly reduced, if not absent, in \textit{msl-2} mutant females. The size difference between the 3.8/3.7 kb sex-specific transcripts is seen reproducibly on multiple northerns. This finding raises the possibility that \textit{msl-2} may be the direct dosage compensation target of \textit{Sxl} regulation and thus the gene whose regulation results in the sex specificity of dosage compensation.

**\textit{msl-2} gene structure and sequence analysis**

14 \textit{msl-2} cDNAs were isolated from male-specific, female-specific and mixed sex libraries. cDNAs were initially characterized by restriction mapping, in situ hybridization to polytene chromosomes and Southern blot analysis. One 3.8 kb cDNA (5.2-10), that was nearly full length based on northern analysis and primer extensions, was isolated from a sex non-specific library. This cDNA, which turned out to represent an unspliced \textit{msl-2} RNA (see below), was completely sequenced as were the ends of several other cDNA clones. None of the cDNAs obtained from the sex-specific libraries extended far enough 5' to be informative with regard to potential sex-specific differences in the 5' UTR of \textit{msl-2} (see below). Comparison of the structures of cDNAs from sex-specific libraries revealed no differences in the coding or 3' UTR regions of \textit{msl-2}. In addition, we sequenced a four kb genomic interval that encompasses the \textit{msl-2} transcription unit. The \textit{msl-2} cDNA sequenced contained a 450 nucleotide 5' UTR, a 773 amino acid open reading frame and a 1 kb 3' UTR (Fig. 4B). Comparison of cDNA and genomic sequences revealed a single intron of 52 nt within the \textit{msl-2} ORF (Fig. 4A).

In order to understand the basis for the size difference between the male and female \textit{msl-2} mRNAs, we used RT-PCR to compare male and female \textit{msl-2} RNA (see Experimental Procedures for details). These experiments identified a sex-specific size difference in the 5' UTR of \textit{msl-2} (Fig. 3B Lanes 1 and 3). No differences were detected between males and females in the ORF or 3' UTR. Cloning and sequencing of the sex-specific 5' UTR PCR products revealed that the male and female \textit{msl-2} mRNAs differ by the presence of a single 133 nt intron that is spliced out of the mRNA in the male, but retained in the female. Since this male-specific intron is in the 5' UTR of \textit{msl-2} RNA, the male- and female-specific \textit{msl-2} mRNAs contain the same ORF. While there is a significant amount of the larger, unspliced form detected in wild-type males, the crucial point is that there is a form specific to males, where \textit{msl-2} is functional. There is no reason to believe that the male-specific splice need occur with total efficiency to have sex-specificity of dosage compensation.

![Fig. 3.](image-url)

Fig. 3. (A) Northern analysis of 4-6 \textmu g of poly(A)+ RNA prepared from: lane 1, Canton S females; lane 2, Canton S males; lane 3, \textit{tudor} females; lane 4, \textit{tudor} males; lane 5, \textit{msl-2} females. The blot was probed with a 560 bp single-stranded riboprobe synthesized from the 5' end of \textit{msl-2}. Probing this blot with RP49 showed that the lanes were equivalently loaded. (B) Reverse Transcription PCR with primers flanking the sex-specific splice junction in the 5' UTR of \textit{msl-2}. Lane 1, Canton S male; lane 2, \textit{Sxl}^{F1}/\textit{Sxl}^{hiv1} female; lane 3, Canton S female. As seen on the gel, we detect a significant amount of the larger unspliced species in the male (see text). Southern analysis of the pictured gel with a \textit{msl-2} 5' UTR probe reveals strong hybridization only to the 162 and 295 nucleotide species. A small amount of the 162 nt species in Canton S female (that can not be seen on the ethidium stained gel) is also observed on the Southern blot. We do not know if this represents the bona fide male splice form and, if it does, whether it is present in biologically significant amounts. PCR reactions were also performed on the starting male and female RNAs to control for genomic DNA contamination. No amplification was observed (data not shown).
cluster known as the RING finger domain (Fig. 4B; Freemont et al., 1991; Lovering et al., 1993). RING fingers have been identified in over 27 proteins from a wide range of species. The RING finger is a zinc-binding domain and has been suggested to be a DNA-binding domain because many RING finger proteins are implicated in processes that are mediated through DNA or chromatin interactions (Lovering et al., 1993; von Arnim and Deng, 1993). Although no RING finger protein has yet been shown to be a sequence-specific DNA-binding protein, the RING finger has been shown to have a non-specific DNA-binding capability (Lovering et al., 1993). Multiple alignments with a subset of other RING finger proteins created using thePILEUP program are shown in Fig. 4C. The finding of a potential DNA-binding domain in MSL-2 is particularly exciting since this may provide the mechanism by which the MSL proteins are targeted to the X chromosome. This idea is considered more fully in the Discussion.

MSL-2 has another cysteine-rich cluster between positions 527 and 573 (Fig. 4B). Multiple cysteine clusters are a common feature of other RING finger proteins (von Arnim and Deng, 1993). The second cysteine cluster appears to be related to the PHD finger, a cysteine-rich element thought to be involved in chromatin-mediated transcriptional regulation (Aasland et al., 1995). MSL-2 possesses 5 of the 8 conserved histidines and cysteines (it does not contain the three cysteines found after the second loop in other PHD members) and is therefore an imperfect or truncated PHD finger. The imperfect alignment of MSL-2 with other PHD members coupled with the fact that the PHD finger has yet to be established as a bona fide domain, hampers the assessment of the significance of this relationship. However, the known chromatin-mediated functions of many of the PHD proteins are intriguing in light of the proposed role of regulated chromatin structure in dosage compensation.

Two other features of the MSL-2 protein sequence of potential interest are the presence of a proline-rich region near the carboxy terminus and the presence of a repetitive motif between positions 313 and 475 (Fig. 4B). There are eight regularly spaced copies of a 15 amino acid motif that are related to each other to varying degrees. Two copies are identical at 13 of 15 positions, while others on average have 6 out of 15 identities. The significance of these repeats is not known.

The 3’ and 5’ UTRs of msl-2
Both the 5’ and 3’ UTRs of msl-2 contain poly(U) tracts that resemble the SXL consensus binding site as defined by Samuels et al. (1994). There are two long U tracts in the 5’ UTR and six shorter tracts in the 3’ UTR. Recently a more complex binding site consisting of U stretches interspersed with G residues has been identified by in vitro selection experiments (Singh et al., 1995; Sakashita and Sakamoto, 1994). The potential SXL sites in the UTRs of msl-2 are more like the earlier identified simple U stretches. Three small upstream open reading frames (uORFs) are also observed in the 5’ UTR (Fig. 4).

Nucleotide searches also identified a 450 base pair region of the 3’ UTR with significant similarity to flanking sequences from a number of Drosophila genes. This similarity region can be divided into two subregions. The 3’ 200 nucleotides of the region are 60-80% identical with the flanking regions of many Drosophila genes (including suppressor of forked, retinal degeneration gene c and the beta subunit of integrin) and contains a small repetitive element called Lefka (Fig. 4B; Madueño et al., 1995). The flanking sequences of two Drosophila U1 snRNA genes designated 82.1 and 82.3 share a larger region of similarity with the msl-2 3’ UTR. The nucleotide sequence of this region of the msl-2 3’ UTR is 83 and 82% identical across its 450 nucleotide length to flanking sequences of U1 82.1 and U1 82.3, respectively. (Nucleotide sequences were compared using the BESTFIT program GCG, version 8.) No such similarity is found for the two other U1-related sequences present in this chromosomal region, U1 82.2 and a U1 pseudogene. Whether this region of extended similarity represents a more full-length copy of the Lefka element or has some other significance is not known.

The MSL-2 protein associates with the male X
To investigate the role of MSL-2 in dosage compensation and to examine the regulation of msl-2, we produced polyclonal rat antibodies to three different parts of the MSL-2 protein. The first antibodies (ab1) were raised against a β-galactosidase-MSL-2 (aa 414-534) fusion protein, the second antibodies (ab2) were raised against a β-galactosidase-MSL-2 (aa 534-669) fusion protein and the third antibodies (ab3) were raised against a β-galactosidase-MSL-2 (aa 50-464) fusion protein. All three antibodies were affinity purified before use.

To examine the intracellular location of the MSL-2 protein, anti-MSL-2 antibodies (ab1) were used to stain polytene salivary gland chromosomes. No anti-MSL-2 staining is detected in females (Fig. 5A-B) consistent with the apparent absence of MSL-2 protein on western blots of proteins from females (see below). In males, the MSL-2 protein is associated with hundreds of sites along the length of the X chromosome (Fig. 5C-D) suggesting that MSL-2 like the other MSLs may directly mediate dosage compensation. In males, there are also about 20-30 sites of MSL-2 association with the autosomes, similar to what is seen using MSL-1 and MSL-3 antibodies (Palmer et al., 1993; Gorman et al., 1995). These autosomal sites are detected by both of our anti-MSL-2 antibodies.

It has been previously shown that the MSL-1, MSL-3 and
MLE and H4Ac16 are all present at the same sites along the male’s X chromosome (Bone et al., 1994; Gorman et al., 1995). We asked whether MSL-2 co-localized with these proteins. Salivary gland polytene chromosomes simultaneously stained with MSL-2 and MSL-1 antibodies showed that they were localized at identical sets of X chromosome sites. (Fig. 5E-H). In addition, the relative intensities of staining along the X for MSL-2 and MSL-1 are correlated, as had been previously seen for MSL-1, MSL-3 and MLE (Gorman et al., 1995), suggesting that all the MSL proteins are present at these sites in similar relative amounts.

The MSL-1, MSL-3 and MLE proteins are also co-localized at 20-30 autosomal sites while MLE is present at an additional 20-30 autosomal sites. Examination of chromosomes doubly stained for MSL-2 and MSL-1 showed that they are present at a common set of autosomal sites.

**MSL-2 protein binding requires the other MSLs**

Previous studies have shown that the MSL-1, MSL-3 and MLE proteins co-localize along the length of the X chromosome and are able to associate with the X chromosome only if a full complement of the four known MSL proteins is present; in the presence of mutations in any one of the msl genes, these three proteins fail to bind to the X chromosome (Gorman et al., 1993, 1995; Palmer et al., 1994). These findings have led to the suggestion that the MSL proteins form a complex. Our finding that the MSL-2 protein contains a potential DNA-binding domain (RING finger) raised the possibility that MSL-2 protein might be capable of binding the X chromosome in the absence of the other MSL proteins. We therefore examined whether the MSL-2 protein was able to associate with the X chromosome in the presence of mutations in the other msl genes.

For mle and msl-3 we took advantage of the fact that while males homozygous for these mutations die during the late larval/early pupal period, they are sometimes healthy enough to yield usable salivary chromo-

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**Fig. 5.** Anti-MSL-2 and anti-MSL-1 staining of wild-type male and female chromosomes. (A) DNA stain of wild-type female. (B) Anti-MSL-2 stain of wild-type female. (C) DNA stain of wild-type male. (D) Anti-MSL-2 stain of wild-type male. (E-G) A higher magnification of a wild-type male X chromosome stained for DNA (E), MSL-1(F) and MSL-2(G). (H) A double exposure of the same X chromosome seen in E-G.
Regulation of dosage compensation

We therefore made salivary squashes of males homozygous for mle\textsuperscript{eml18} or msl-3\textsuperscript{mak} and stained them with anti-MSL-2 antibodies. In both cases no staining was seen at most X chromosome sites, although we could detect around 20-30 weakly staining bands. MSL-1 is reported to bind to about 40 sites on the X in an msl-3 mutant background (Palmer et al., 1994). To compare the residual sites bound by MSL-1 and MSL-2, we stained homozygous msl-3\textsuperscript{mak} males simultaneously with anti-MSL-2 and anti-MSL-1 antibodies. This experiment showed that they co-localized (data not shown).

To examine the dependence of MSL-2 binding on msl-1, we made use of the previous findings that Sxl\textsuperscript{f1}/Sxl\textsuperscript{fhv1} females are mosaic for SXL expression and the MSL-1, MSL-3 and MLE proteins associate in a male-like pattern with the X chromosomes in those salivary gland cells of Sxl\textsuperscript{f1}/Sxl\textsuperscript{fhv1} females that do not express SXL (Gorman et al., 1993, 1995; Palmer et al., 1994). In double staining experiments with MSL-2 and SXL antibodies, we found that MSL-2 is present in those cells of Sxl\textsuperscript{f1}/Sxl\textsuperscript{fhv1} females that do not express SXL and is bound to the X chromosomes in a male-like pattern. We then used MSL-2 and SXL antibodies to stain chromosomes of Sxl\textsuperscript{f1}/Sxl\textsuperscript{fhv1}; msl-1/msl-1 females. No MSL-2 X chromosome banding was detected in those cells that lacked anti-SXL staining. These experiments thus show that MSL-2, like the other MSLs, requires the presence of all known MSL proteins in order to bind to the X chromosome.

**MSL-2 protein is expressed only in males**

When used to probe western blots of proteins from wild-type males ab2 (Fig. 6A), ab3 (Fig. 6B) and ab1 (data not shown)
detect a prominent band of approximately $115 \times 10^3 M_r$ which is close to the molecular weight inferred from the cDNA sequence. There are no other common bands that are detected by all three antibodies. That the $115 \times 10^3 M_r$ band corresponds to MSL-2 is shown by the fact that this band is absent in extracts of msl-2 homozygous male larvae (Fig. 6A). The reappearance of the $115 \times 10^3 M_r$ band in extracts of msl-2 homozygous male larvae who carry two copies of the Xba genomic rescue fragment further substantiates that the $115 \times 10^3 M_r$ band is in fact MSL-2 (Fig. 6B). We also note the presence of a prominent band at approximately $68 \times 10^3 M_r$ in msl-2 mutant males which is detected by ab1 and ab2, but not by ab3 (Fig. 6 and data not shown). The molecular nature of the msl-2 mutation is not known, therefore whether this prominent band represents mutant protein is unclear. When ab2 (Fig. 6A), ab3 (Fig. 6B) or ab1 (data not shown) are used to probe western blots of proteins from wild-type female larvae no MSL-2 protein is detected, indicating that MSL-2 is expressed in a sex-specific manner.

Sex-specific regulation of msl-2 pre mRNA

The finding that the male and female msl-2 mRNAs contain the same ORF, when coupled with the finding that MSL-2 protein is found only in males, strongly suggests that msl-2 expression is regulated post-transcriptionally in a sex-specific manner. We have made two kinds of observations that delimit the mechanisms by which the male-specific synthesis of MSL-2 protein is achieved.

First, in the process of mapping msl-2 cDNAs back to the region of genomic rescue, we realized that the smaller of the two genomic rescue fragments lacked almost the entire 3' UTR. The truncation was confirmed by genomic sequencing. Because of the interesting sequence elements present in the 3' UTR (i.e. regions of homology with U1 snRNA, the Lefka repetitive element and, in particular, the potential SXL-binding sites), we investigated the potential regulatory role of the 3' UTR on MSL-2 sex-specific protein expression.

In females carrying two copies of the 3' UTR truncated transgene, a significant level of MSL-2 X chromosome-specific staining is detected (Fig. 7A,C). In these females MSL-2 is present at approximately 50-60 sites scattered along the length of the female X-chromosomes. In females who carry only one dose of the transgene, MSL-2 is observed at approximately half the sites of the two dose females (data not shown). The staining seen in these two dose females is greatly reduced both in number of bands stained and the intensity of these bands relative to sibling males carrying the same transgene (data not shown). Similar levels of staining in females are observed for three independent insertion lines for this construct. No such staining is ever seen in wild-type females, nor is staining detected in females carrying the larger genomic construct which contains the complete msl-2 3' UTR. One possibility is that the X-chromosome binding observed in these 3' UTR truncated transgenic females could result from inappropriately high levels of msl-2 transcription due to position effects on the transgenes. The fact that similar levels of female staining are seen for three independent insertions and no such staining is seen for a full-length transgene argue against this possibility. Moreover, total protein from larvae transgenic for either the full-length or truncated rescue constructs were compared with wild-type male and female larvae by western analyses (Fig. 6B and data not shown). MSL-2 expression in msl-2 mutant males carrying two copies of the truncated transgene was similar to wild-type males (Fig. 6B). Notably, we do not detect a significant amount of MSL-2 in females transgenic for the 3' UTR truncated construct (Fig. 6B). This is not so surprising in view of the limited number and reduced intensity of sites stained in these females.

All three of the other MSLs were found to be associated with these same 50-60 sites in females carrying the truncated msl-2 transgene (Fig. 7B,D and data not shown). The presence of the other MSLs would suggest that the expression of MSL-2 in females leads to the assembly of male-type dosage compensation complexes. It is worth noting that, although there is a low level of MSL binding in these transgenic females, there is no significant reduction in viability. The above observations suggest two conclusions. First they clearly implicate the 3' UTR in the negative regulation of MSL-2 expression in females. However, since only low levels of MSL protein binding are seen in females carrying the truncated transgene, and since MSL-2 protein is not detected at significant levels on western blots of these females, these results also suggest that there is probably an additional way in which MSL-2 protein expression is prevented in females.

The 5' UTR is a good candidate for the site of additional regulation both because it also contains potential SXL-binding sites and because it undergoes a sex-specific splice. As the potential SXL-binding sites are near the male-specific splice junctions in msl-2 pre-mRNA, it seemed likely that SXL directly regulated msl-2 by blocking splicing of the 133 nucleotide intron in females. In order to demonstrate that the sex-specific splice in the 5' UTR of msl-2 is dependent on wild-type Sxl function, we analyzed msl-2 RNA from heteroallelic Sxl$^{B1}$/Sxl$^{Bw1}$ mutant females by RT-PCR. We detect both male and female forms of msl-2 RNA in these females indicating that the absence of the male product is dependent on Sxl function (Fig. 3B, lane 2).

It may be significant that the two SXL consensus binding sites are found within the male-specific intron and thus are not removed from female msl-2 transcripts. In this regard, it is also worth noting the presence of a two amino acid upstream open reading frame (uORF) within the retained female intron. The potential significance of this female-specific uORF, as well as other possible mechanisms of 5' UTR mediated sex-specific protein expression, are considered below.

DISCUSSION

MSL-2 association with chromosomes

We have found that the MSL-2 protein specifically binds hundreds of sites along the length of the male, but not the female, X chromosomes suggesting that MSL-2 may be a direct mediator of dosage compensation. We have also shown that MSL-2 co-localizes with MSL-1 along the X chromosome. Since previous studies showed that MSL-1, MSL-3 and MLE are co-localized, our finding indicates that all these proteins are found at the same X-chromosomal sites. Moreover, the band intensities of MSL-1 and MSL-2 are correlated suggesting that these proteins are present in similar relative amounts at each of the sites at which they are present.
MSL-2 also reproducibly associates with 20-30 autosomal sites in the male and it is co-localized with MSL-1 at these sites. MSL-3, MLE and MSL-1 are also localized at the same 20-30 autosomal sites (Bone et al., 1994; Gorman et al., 1995). Taken together these observations suggest that the MSLs may function to transcriptionally regulate a small group of autosomal genes in males.

In females, the absence of MSL-2 from the X chromosome is dependent on the expression of Sxl. In Sxl(+/+)trans heterozygous females, which are mosaic for SXL expression, there is, at the cellular level, a perfect inverse correlation between the presence of SXL and the presence of MSL-2. Thus like the other three MSLs, MSL-2 chromosome association is strictly dependent on the absence of SXL. These experiments established a functional link between SXL expression and the absence of MSL-2 X chromosome staining in the female. Evidence for a direct regulatory link between Sxl and msl-2 is discussed below.

In males MSL-2’s binding to the X chromosome is dependent on the presence of functional products from the other msl genes. Thus MSL-2 staining is completely absent in SXL non-expressing cells of Sxl(+/+)trans: msl-1 mutant females and greatly reduced (to 20-30 sites) in both mle and msl-3 mutant males. The sites of MSL-2 and MSL-1 association in the msl-2 background are coincident. It is not known whether the residual binding of MSL-1 and MSL-2 in mle and msl-3 mutants is functionally significant.

The observations that MSL-2 associates specifically with the male X, that the sites of its association are coincident with those of the other MSLs and that the association of each of the MSLs is dependent on the wild-type function of all of the MSLs strongly support the idea that the MSLs mediate dosage compensation through the formation of an oligomeric complex (Gorman et al., 1993).

**On the sex-specificity of dosage compensation**

We have found that the MSL-2 protein is undetectable in wild-type females. The sex-specificity of MSL-2 protein expression suggests a mechanism for how the sex-specificity of dosage compensation is conferred. Because it has been shown that the X-chromosome association of each of the four known MSL proteins requires the presence of functional products from all four of the msl genes, the absence of MSL-2 in the female would prevent X chromosome binding of the other three MSLs and in so doing would presumably prevent hypertranscription of the female’s X chromosomes.

**Regulation of MSL-2 expression**

SXL is an RNA-binding protein that controls the expression of its two target genes in the somatic sex-determination hierarchy by regulating the splicing of their pre-mRNAs within their ORFs (for review see Mattox et al., 1992): in males the default splicing pathway includes stop codons in the mature mRNAs of these genes whereas the alternative splices directed by SXL in females removes these stop codons. This has led to the idea that SXL would likely control dosage compensation in an analogous fashion – by negatively regulating one or more of the msls at the level of splicing. It has been further suggested that msl-2 might be the dosage compensation target of SXL because of the genetic observation that, unlike the other msls, there is no maternal effect of msl-2 on the lethal phase of homozygous sons (Baker et al., 1994). Thus msl-2 does not appear to make a functional product in the female germline, where SXL is expressed, whereas the other msls do produce functional products in the female germline.

We have demonstrated here that there is a difference in the splicing of msl-2 pre-mRNA between the two sexes. A 133 nucleotide intron in the 5' UTR of msl-2 is retained in females, but removed in males. The presence of putative SXL-binding sites near the sex-specific 5' UTR splice junctions, coupled with the demonstration that the inhibition of the male-specific splicing is dependent on the presence of the wild-type function of SXL strongly supports the idea that msl-2 may be the direct target of SXL through which dosage compensation is mediated.

The alternative splice in the 5' UTR of msl-2 does not result in a difference between the ORF in males and females. Furthermore, the northern analysis demonstrates that msl-2 pre-mRNA is present at roughly equal levels in males and females indicating that there is no major difference in message stability between the sexes. Taken together with our finding that MSL-2 protein is absent in females, these observations indicate that the regulatory mechanism that controls msl-2 must, at some level, prevent the translation of msl-2 mRNA in the female. In this regard, we note that there are potential SXL-binding sites in the 3' UTR as well as the two potential SXL-binding sites present in the male-specific intron in the 5' UTR.

That sequences in the 3' UTR play a role in the regulation of MSL-2 expression is demonstrated by our finding that removing the 3' UTR leads to detectable binding of MSL-2 to the X chromosome in females. Females that carry two copies of the genomic 3' UTR truncated rescue construct show MSL-2 associated with the X-chromosome at approximately 50-60 sites.

In addition to detecting MSL-2 associated with 50-60 sites on the X chromosomes of these transgenic females, all three of the other MSLs are also associated with these same sites. The presence of the other MSLs suggests that the expression of MSL-2 due to truncation of the 3' UTR results in the assembly of a male-type MSL complex. If it is the absence of the SXL-binding sites that causes MSL-2 expression in these 3' UTR truncated transgenic females, then it seems likely that SXL is acting through these sites at some level other than splicing to control MSL-2 expression.

While the above observations clearly establish a role for the 3' UTR in the regulation of MSL-2 expression, the fact that the 3' UTR truncation does not result in male levels of MSL-2 binding implies that there are additional msl-2 sequences that regulate msl-2 expression in females. The presence of potential SXL-binding sites and the alternative splicing event make the 5' UTR a strong candidate for a region that may contain other sequences critical to the regulation of MSL-2.

Our results regarding the regulatory role of the 3' UTR are consistent with the observations of Kelly et al. (1995) who have also demonstrated that female X chromosome binding is seen when the 3' UTR is removed, but that female viability is not affected. In addition, Kelly et al. (1995) have found that the simultaneous removal of the 5' and 3' UTRs greatly reduces the viability of transgenic females and MSL-2 X chromosome binding increases dramatically to a level comparable to that seen in wild-type males. It is difficult to ascertain from these experiments the relative contributions of the 5' and 3' UTRs in...
MSL-2 regulation because the effects of removing only the 5’ UTR were not determined. One must conclude, however, that both the 5’ and 3’ UTRs are essential for proper MSL-2 regulation.

**msl-2: putative target of SXL**

The demonstration that there is a sex-specific alternative splice in the 5’ UTR of msl-2 pre-mRNA and that there are potential SXL-binding sites in the proximity of the splice junctions strongly suggests that SXL may directly control MSL-2. The observation that in Sxlfl/Sxlfhv1 females both male and female splice forms are detected further supports the idea that MSL-2 is a direct target of SXL. Proof of such direct regulation will await the biochemical demonstration that SXL and msl-2 RNA physically interact and that the sex-specific splice of msl-2 directly depends on SXL.

In view of the fact that the alternative splice does not affect the coding sequence of msl-2, but that the splice and perhaps some other feature of the 5’ UTR are necessary for proper regulation of MSL-2 expression, it is necessary to consider other potential levels of regulation that may be mediated via the 5’ UTR. Two possibilities include translational masking and the regulation of translation initiation (for review see Curtis et al., 1995).

Translational masking, also referred to as message sequestration, could explain the absence of MSL-2 expression in females. One simple model is that SXL binds to the putative SXL-binding sites in the 5’ UTR and, because SXL is a nuclear protein, msl-2 pre-mRNA can not be transported out of the nucleus and hence is not available for translation. This idea is also compatible with the role of the 3’ UTR in regulation. There is precedent for this kind of translational control (Ahringer and Kimble, 1991; Goodwin et al., 1993; Ostareck-Lederer et al., 1994). However, our finding that there is a male-specific intron in the proximity of the SXL-binding sites in the 5’ UTR suggests that the actual mechanism of regulation through the 5’ UTR is probably more complex.

Another possibility is that msl-2 is regulated at the level of translation initiation. In this regard, it is worth noting that there is a two amino acid open reading frame in the male-specific intron that if used would result in the termination of translation before the bona fide initiation site of msl-2 could be reached. Precedent for the suppression of translational initiation by upstream open reading frames (uORFs) is provided by studies on the translational control of GCN4 in Saccharomyces cerevisiae (for reviews see Hinnenbusch 1990, 1994). GCN4 is translated at high levels in response to amino acid starvation and its translation is controlled by two three amino acid uORFs: uORF1 and uORF4. uORF4 acts to repress translation of GCN4 in non-starved state, while uORF1 can overcome the uORF4-mediated translational repression of GCN4 under starvation conditions. The two amino acid uORF4 in GCN4 has been shown to be a strong translational repressor of GCN4. GCN4 constructs that contain only uORF4 in their 5’ UTR are translated at only 10% of the levels of GCN4 constructs that have no uORFs regardless of whether or not cells are starved for amino acids. It will be of interest to determine if the female-specific msl-2 uORF functions to repress MSL-2 translation initiation in wild-type females. Regulation of translational initiation is just one possible mechanism of translational control. To explore these and other possibilities systematic analysis and mutagenesis of potential regulatory elements in the 5’ UTR need to be undertaken.

**Targeting the MSLs to the male X chromosome**

msl-2 has been shown to encode a protein with a putative DNA-binding domain – the RING finger (Lovering et al., 1993; von Arnim and Deng, 1993). Specific DNA binding of this domain has yet to be demonstrated for any members of this family; however, it has been shown that this domain can bind DNA non-specifically.

In other RING finger proteins where their functions are known, these functions can be mediated through DNA binding or chromatin association. Site-specific recombination, transcriptional regulation and DNA repair are examples of the diverse functions that RING finger proteins are known to mediate. Some examples of proteins with RING fingers include:
Rad16 – a helicase domain containing DNA repair gene in yeast with homology to proteins known to be involved in chromatin structure (Bang et al., 1992; Schild et al., 1992), RAG-1 – a human gene involved in the activation of recombination (Schatz et al., 1989), SS-A/Ro – a component of a ribonucleoprotein particle (Pruin et al., 1991), and BMI-1 and Mel18 – two highly homologous mammalian nuclear localized oncoproteins with potential roles in B cell lymphomagenesis (Ishida et al., 1993). Two polycyto group genes that also have RING fingers, Posterior sex combs (Psc) and supressor(2) of zeste (su(2)Z) encode the Drosophila homologues of BMI-1 and Mel-18. Interestingly, PSC and SU(2)Z have been shown to bind at 70-80 sites on polytene chromosomes and are implicated in the regulation of chromatin structure (Patel et al., 1993).

As was pointed out in the Introduction, none of the other MSLs have any sequence elements that can be implicated in DNA binding. It is therefore attractive to postulate that MSL-2 interacts directly with male X-chromosomal DNA and in so doing targets the putative MSL complex to the male X. It should be pointed out that if MSL-2 does bind DNA it can not do so stably alone in vivo as evidenced by the observation that MSL-2 does not associate with the male’s X chromosome in the absence of any of the other MSLs. If MSL-2 does directly bind DNA, it may provide a valuable tool in the identification of the uncharacterized cis-acting sequence elements that are required for dosage compensation.

Conclusion – a model

Our findings with regard to MSL-2’s role in the process of dosage compensation are summarized in Fig. 8. We have shown that msl-2 encodes a protein with a potential DNA-binding motif. Moreover msl-2 pre-mRNA is alternatively spliced in a SXL-dependent fashion. In males, the male-specific splice form of msl-2 mRNA is efficiently translated and MSL-2 may mediate dosage compensation by targeting a complex of MSL proteins to the male’s X chromosome. In contrast, in females, SXL functions to block removal of the male-specific msl-2 intron as well as to repress the translation of female msl-2 pre-mRNA. Our data support both splicing dependent and splicing independent mechanisms of SXL-mediated regulation. Sequences in both the 5’ and 3’ UTRs are important for the observed translational regulation of msl-2. This results in the absence of MSL-2 in females, which inhibits the formation of functional MSL complexes, and hence X chromosome transcription proceeds at the basal female level.

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