Copyright © 1997 by the Genetics Society of America

Drosophila Male-Specific Lethal-2 Protein: Structure/Function Analysis and Dependence on MSL-1 for Chromosome Association

Laura M. Lyman,* Kyle Copps,† Luca Rastelli,‡ Richard L. Kelley* and Mitzi I. Kuroda*,†,‡

**Department of Cell Biology,* †*Program in Cell and Molecular Biology and* ‡*Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas 77030*

Manuscript received March 25, 1997
Accepted for publication August 18, 1997

ABSTRACT

MSL-2 is required for the male-specific assembly of a dosage compensation regulatory complex on the *X* chromosome of *Drosophila melanogaster.* We found that MSL-2 binds in a reproducible, partial pattern to the male *X* chromosome in the absence of MLE or MSL-3, or when ectopically expressed at a low level in females. Moreover, the pattern of MSL-2 binding corresponds precisely in each case to that of MSL-1, suggesting that the two proteins function together to associate with the *X.* Consistent with this hypothesis, we isolated EMS-induced loss of function *msl-1* and *msl-2* alleles in a screen for suppressors of the toxic effects of MSL-2 expression in females. We also used site-directed mutagenesis to determine the importance of the MSL-2 RING finger domain and second cysteine-rich motif. The mutations, including those in conserved zinc coordinating cysteines, confirm that the RING finger is essential for MSL-2 function, while suggesting a less stringent requirement for an intact second motif.

MANY organisms determine sex on the basis of differential chromosome inheritance, with *XX* individuals developing as females, and *XY* individuals developing as males. Dosage compensation is the mechanism through which *X*-linked gene expression is made equivalent in the two sexes. Dosage compensation in *Drosophila melanogaster* occurs primarily through the transcriptional upregulation of the single male *X* chromosome (reviewed in Lucchesi and Manning 1987; Baker *et al.* 1994; Kelley and Kuroda 1995; Lucchesi 1996). Four factors required for this process are encoded by the *maleless* (*mle*), *male-specific lethal-1* (*msl-1*), *male-specific lethal-2* (*msl-2*), and *male-specific lethal-3* (*msl-3*) genes. The four loci are collectively referred to as the *msls*. Males homozygous mutant for these genes die during late larval to early pupal stages, presumably due to the cumulative shortage of *X*-encoded products.

Genetic data demonstrate no increase in the severity of phenotype when multiple *msls* are disrupted, suggesting that the individual loci function in a common pathway (Sanchez and Nothiger 1983). Consistent with their role as critical factors for the dosage compensation process, the gene products for all four *msl* loci localize specifically to the male *X* chromosome (Kuroda *et al.* 1991; Palmer *et al.* 1993; Bashaw and Baker 1995; Gorman *et al.* 1995; Kelley *et al.* 1995; Zhou *et al.* 1995). Furthermore, each of the MSL proteins binds to the *X* chromosome in a precisely coincident pattern, and this pattern is dependent on all four msl^+ functions (Gorman *et al.* 1993, 1995; Bone *et al.* 1994; Hilfiker *et al.* 1994; Palmer *et al.* 1994; Bashaw and Baker 1995). These data have been interpreted as evidence that the MSL proteins form a multi-subunit complex. Consistent with this view, the MSL-1 and MSL-2 proteins co-immunoprecipitate from male nuclear extracts (Kelley *et al.* 1995). There is also indirect evidence for one or more RNA components of the complex (Richter *et al.* 1996; Amrein and Axel 1997; Meller *et al.* 1997).

Insight into the function of the MSL complex was provided by the discovery of the male *X* chromosomal localization of histone H4 monoacetylated at lysine 16 (H4Ac16, Turner *et al.* 1992). The significance of specific histone acetylation to the transcriptional upregulation of the male *X* chromosome is supported by recent work in yeast and humans, showing a direct link between histone acetylation and gene activation (Brownell *et al.* 1996; Mizzen *et al.* 1996; reviewed in Pennisi 1997). The enrichment of H4Ac16 on the Drosophila male *X* chromosome is dependent upon the wild-type activity of all four *msls* (Bone *et al.* 1994), and the recently discovered *males absent on the first* (*mof*) locus (Hilfiker *et al.* 1997). The MOF protein is a strong candidate to encode a male-specific histone acetyltransferase that functions in dosage compensation. Thus, a primary function of the MSL protein complex may be to target *mof* activity to the *X* chromosome (Hilfiker *et al.* 1997).

Assembly of the MSL complex on the *X* chromosome occurs only in males. In females, complex assembly is prevented by the negative regulation of MSL-2 protein expression by the product of the *Sex lethal* locus. Ectopic expression of MSL-2 is sufficient to assemble all four MSL proteins on both female *X* chromosomes, indicating that *msl-2* is the primary target of sex-specific regula-

Corresponding author: Mitzi I. Kuroda, Howard Hughes Medical Institute, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. E-mail: mkuroda@bcm.tmc.edu

tion (KELLEY *et al.* 1995; BASHAW and BAKER 1997; KELLEY *et al.* 1997).

It is not known how the MSL complex recognizes the *X* chromosome, as none of the characterized MSL proteins contain a classical DNA binding domain. MSL-1 contains two highly acidic stretches, reminiscent of proteins that can interact with nucleosomes (PALMER *et al.* 1993). MSL-2 contains a putative zinc binding domain known as the RING finger (BASHAW and BAKER 1995; KELLEY *et al.* 1995; ZHOU *et al.* 1995). Two *msl-2* mutant alleles have lesions in this segment of the protein, including a single amino acid deletion between the first and second conserved cysteines of the RING finger (msl-$2^{\gamma136}$; ZHOU *et al.* 1995). The RING finger has been implicated in protein-protein interactions and/or nucleic acid affinity in other family members (REDDY *et al.* 1991; LOVERING *et al.* 1993; MU *et al.* 1994; BORDEN *et al.* 1995; ELENBAAS *et al.* 1996). A second cysteine-rich region resides in the central portion of MSL-2 (BASHAW and BAKER 1995; ZHOU *et al.* 1995). This domain was aligned with the PHD finger, a motif suggested to function in protein-protein interaction (BASHAW and BAKER 1995; reviewed in AASLAND *et al.* 1995), or with a metallothionein-like domain, suggested to regulate zinc availability to the RING finger (ZHOU *et al.* 1995; LUCCHESI 1996 and references therein). However, both alignments show only partial conservation of the individual motifs.

To further define the role of MSL-2 in complex formation and chromosome association, we examined the genetic requirements for MSL-2 association with the *X* chromosome. We found that MSL-2 and MSL-1 colocalize to a reproducible subset of their wild-type *X* chromosome sites in the absence of either *mle* or *msl-3*. MSL-2 chromosome association requires MSL-1, and therefore the two proteins are co-dependent on one another for their interaction with the *X* chromosome. To understand the relevance of structural motifs within MSL-2, a site-directed mutagenesis strategy was employed. Our results suggest that the incomplete PHD/metallothionein domain is not essential and confirm that the amino-terminal RING finger is indispensible for MSL-2 *in vivo* activity.

MATERIALS AND METHODS

Polytene chromosome spreads and immunofluorescent staining: Preparation and incubation with primary antibodies for chromosomes from Sxl^{f1}/Sxl^{fhv1}; *msl* heteroallelic animals was performed as follows. Salivary glands from third-instar larvae were dissected and fixed in phosphate-buffered saline (PBS) at pH 7.2 containing 3.7% formaldehyde and 0.1% Triton X-100 for 1 min. The solution was then replaced with 50% acetic acid and 3.7% formaldehyde for 2 min. Glands were transferred in 3 μl of fixative to siliconized cover slips and squashed onto slides. Slides were immediately frozen in liquid nitrogen, and the cover slips removed with a razor blade. Preparations were dehydrated in 95% ethanol for a minimum of 15 min before washing in PBS for 30 min, and PBT (PBS plus 0.2% Triton X-100) for 30 min. Individual slides were blocked in PBT with 2% BSA for a minimum of 30 min before incubation with primary antibody overnight at 4° in a humidified chamber. Goat anti-MSL-2 was used at a dilution of 1:50, and rabbit anti-MSL-1 at 1:30. Both primary antibodies were incubated simultaneously. After primary antibody incubation, slides were blocked in PBT with 2% BSA for 1 hr and reacted with biotin conjugated anti-goat antibodies preabsorbed against Drosophila embryos at a concentration of 1:500 for 2 hr at room temperature. Slides were again washed in PBT and blocked in PBT with 2% BSA. Tertiary incubation with Cy-3 conjugated anti-biotin (Jackson Laboratories) and anti-rabbit FITC-conjugated secondary antibodies occurred for 2 hr at room temperature. FITC-conjugated secondary antibodies (Jackson Laboratories) were preabsorbed against fixed Drosophila embryos at a concentration of 1:10, and sample incubation was done at a final dilution of 1:200 at room temperature for 2 hr, following a 30-min incubation with PBT and 2% BSA. Cy-3-conjugated antibodies were used at a final dilution of 1:2000. DNA was counterstained for 7–10 sec with 2 μg/ml bis-benzimide (Hoechst 33258, Sigma). Samples were mounted under a cover slip with 80% glycerol and 2% n-propyl gallate. Chromosomes were viewed using epifluorescent optics with a Zeiss Axioscope and photographed using Kodak Ektachrome 400. Data in Table 1 were compiled through the comparison of at least three chromosome preparations of each genotype.

Western blots: Crude extracts were prepared from third instar larvae through homogenization in 10 μl/animal of Laemmli loading buffer containing the following proteinase inhibitors: 1 μg/ml of pepstatin, 1 μg/ml of leupeptin, 1 μM benzamidine, 10 μM aprotinin, 1 μg/ml antipain, 1 μg/ml of soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride. The extracts were immediately boiled for a minimum of 5 min. Preparations were spun at maximum speed in a microfuge for 30 sec and frozen at −80° until analyzed. Extracts were not stored for >30 days. Loaded samples were balanced against one another for equivalent protein concentrations by Coomassie staining. Approximately 0.5 to one fly (or larva) was loaded. Samples were electrophoresed through 4% stacking-6% polyacrylamide-SDS Laemmli gels overnight for 1000 volt-hr. Proteins were transferred in CAPS (MATSUDAIRA 1987) buffer to nitrocellulose using an EC electroblot apparatus for 2.5–3.0 hr at 500 mA. Western blots were incubated with primary antibody overnight at room temperature at dilutions determined for individual antibody purifications and visualized using an alkaline phosphatase-conjugated goat anti-rabbit detection system (Promega).

Drosophila stocks: Flies were raised on standard cornmeal-yeast-agar-molasses medium containing propionic acid. All stocks not specifically mentioned are described in LINDSLEY and ZIMM (1992) or in PALMER *et al.* (1994).

Crosses to generate *Sxl; msl* double mutants for Western analysis and/or immunostaining were performed at 18°. *Sxl; msl-2* double mutants were as follows: *cm* Sxl^{fhv1} *ct; msl-2 b pr cn wxt bw* females × Sxl^{f1} *oc ptg v/Y; msl-2 cn bw/In (2LR) Gla Bc Elp* males. *Sxl; msl-1* double mutants were as follows: *cm* Sxl^{fhv1} *ct*; $msl\text{-}1^{\gamma216}$ *cn bw* females × Sxl^{f1} *oc ptg v/Y*; $msl\text{-}1^{b}$ *cn bw/In (2LR) Gla Bc Elp* males or *cm* Sxl^{fhv1} *ct*; $msl\text{-}1^{\gamma269}$ *cn bw* females × Sxl^{f1} *oc ptg v/Y*; $msl\text{-}1^{\gamma269}$ *cn bw/In (2LR) Gla Bc Elp* males or *cm* Sxl^{fhv1} *ct*; $msl\text{-}1^{b}$ females × Sxl^{f1} *oc ptg v/Y*; $msl\text{-}1^{b}$ *cn bw/In (2LR) Gla Bc Elp* males. *Sxl; msl-3* double mutants were as follows: *cm* Sxl^{fhv1} *ct*; $msl\text{-}3^{P}$ *red* females × Sxl^{f1} *oc ptg v/Y*; $msl\text{-}3^{P}$ *red/TM6B Tb* males. *Sxl; mle* double mutants were as follows: *cm* Sxl^{fhv1} *ct; pr* mle^{1} females × Sxl^{f1} *oc ptg v/Y; pr* mle^{1}*/In (2LR) Gla Bc Elp*. Homozygous *msl-2, msl-1,* and *mle* larvae were identified by the absence of the *Bc* marker. Homozygous *msl-3* larvae were identified by the absence of the *Tb* marker.

Crosses to generate *msl-3* and *mle* mutant males for Western analysis of MSL-2 were performed at 18°. *mle* mutants were *y; mle*^{pml8} *cn bw/Cyo y*$^{+}$ females × *y/Y; mle*^{pml8} *cn bw/CyO y*$^{+}$ males. *msl-3* mutants were *msl-3*^{P} *red/TM3* females × *msl-3*^{mak} *red e/TM3* males. Homozygous *mle* males were identified by the absence of the *y*$^{+}$ mouth-hook marker carried on the *CyO* balancer. *msl-3*^{P}/*msl-3*^{mak} larvae were identified by their red Malpighian tubules. Larvae for all crosses were sexed by gonad size as viewed through the cuticle.

Dominant suppressor screens: Chemical mutagenesis was performed on *yw; H83M2-6I/+* adult males that had aged 5 days at room temperature. Animals were separated into sets of 100/bottle and starved for 6–10 hr. A 1% sucrose solution containing 0.26% EMS (Sigma) was added to Whatman paper lining the fly containers, and flies were left to drink the solution overnight (15–18 hr). At that time, the flies were transferred to fresh bottles with food and left to recover for 24 hr before mating. Approximately 200–300 virgin females were mated to the EMS-treated males. After 4 days the males were removed and the females transferred to fresh food to complete laying their fertilized eggs. Male progeny eclosing from these bottles were mass-mated to their nontransgenic sisters. The progeny from this second cross were sorted beginning on day 1 of eclosion through day 4. All transgenic females recovered on days 1–3 were mated to test for fertility. Based on the number of transgenic brothers produced, we examined a maximum of 19,000 chromosomes in this screen.

Site-directed mutagenesis: Oligonucleotide primers were designed in the sense and anti-sense direction to incorporate specific mismatches into the MSL-2 coding sequence such that the desired amino acid substitution occurred upon translation (HIGUCHI *et al.* 1988). Two individual PCR amplifications of 15 cycles each were performed on the starting DNA substrate (15 ng) with each set of mutant primers and the appropriate 5′ and 3′ external primers. Full length product from these reactions was purified on 0.8% low-melt agarose and melted for 30 min at 65° in a 1:1 dilution with autoclaved water. Two microliters of each product were combined into a third PCR amplification reaction with the two external primers for 15 cycles. The PCR fragment encompassing the targeted region was used to replace wild-type sequences in the pM2NGN transformation vector. pM2NGN contains a complete *msl-2* gene (6 kb) cloned into pCaSpeR (PIRROTTA 1988) in which the *Cla*I and *Xma*I sites in the 5′ untranslated region have been converted to *Not*I and *Bgl*II sites, respectively (KELLEY *et al.* 1997), and an *Nco*I site has been introduced at the start codon of *msl-2*.

To confirm the targeted mutations were present and that no random PCR-induced mutations had occurred, each site-directed mutant was sequenced across the region between the external primers. Standard dideoxy sequencing reactions were performed utilizing the Sequenase DNA sequencing kit (USB) and resolved through electrophoresis on a 6% polyacrylamide/7 M urea gel. One positive construct for each mutant was purified over a Qiagen DNA column and prepared for microinjection.

Germline transformation: Purified DNA was microinjected into *y w;* P[Δ2-3] Ki/+ embryos that contain a stable source of transposase (ROBERTSON *et al.* 1988). Transgenic flies were recovered and tested for *msl-2* complementation. Multiple lines carrying insertions on the *X* or third chromosome were assayed.

RESULTS

Co-localization of MSL-1 and MSL-2 in partial MSL complexes: We tested whether or not MSL-2 might possess *X*-chromatin binding activity in the absence of any of the other MSLs by examining its localization pattern on polytene chromosomes. Since perturbation of *msl* function in males results in poor chromosomal morphology, we performed our analysis in female cells that had adopted the male sexual fate. Females of the genotype *Sxl*^{f1}/*Sxl*^{fhv1} fail to activate the *Sxl* locus in a subset of their cells and thus are mosaic for cells that follow either the male or female pathway for dosage compensation (GORMAN *et al.* 1993). In *XX* cells that have adopted a male fate, MSL-2 interacts in a full male-like pattern on the two female *X* chromosomes (KELLEY *et al.* 1995 and inset, Figure 1e). However, mosaic animals lacking *mle* or *msl-3* show partial immunostaining patterns for MSL-2 (Figure 1a–d), as previously seen for MSL-1 (PALMER *et al.* 1994). We mapped the cytological locations of MSL-2 immunostaining and found that the sites occur in a reproducible, overlapping pattern in the two mutants (Table 1). Furthermore, MSL-1 and MSL-2 are precisely co-localized at the *msl-3*- and *mle*-independent sites (Table 1 and data not shown). In the *msl-3* mutant these sites number ~30–40. The lack of MLE appears to have a more severe effect, as chromosome binding is detectable at a smaller subset of sites, all common to the *msl-3* mutant pattern. In contrast, MSL-2 localization to the *X* chromosomes is completely abolished in the absence of MSL-1 (Figure 1, e and f). Thus, MSL-2 and MSL-1 are co-dependent for chromosome binding, even at the *mle*- and *msl-3*-independent subset of sites.

We found that a partial MSL-2 pattern is observed in *msl-1*^{b} mutants (*Sxl*^{f1}/*Sxl*^{fhv1}; *msl-1*$^{\gamma216}$/*msl-1*^{b} and *Sxl*^{f1}/*Sxl*^{fhv1}; *msl-1*^{b}, Figure 2a and data not shown). *msl-1*^{b} is a putative point mutant (BELOTE and LUCCHESI 1980; PALMER *et al.* 1993). When MSL-1 function is compromised, MSL-2 is only localized to sites where the mutant form of MSL-1 is bound (Figure 2b). When a reverse approach was used in which *msl-2* expression was derepressed in females through the removal of SXL binding sites in the 3′ untranslated region of *msl-2* transcripts (MM2SV; KELLEY *et al.* 1997), a similar partial pattern of MSL-2:MSL-1 *X* chromosome interaction was observed (Figure 2, d–f). In this case, MSL-2 was present in limiting quantities, and MSL-1 was precisely restricted to the sites of MSL-2 localization. We mapped the cytological locations of the MSL complexes in each genotype (Table 1). In all cases the patterns overlapped, suggesting the presence of specific high affinity binding sites spaced along the length of the *X* chromosome.

We performed Western blots of *msl* mutants to determine whether the failure of MSL-2 to bind the *X* chromosome in a wild-type pattern was due to lack of MSL-2 protein accumulation (Figure 3). No dramatic difference in MSL-2 levels was detected between homozygous *msl-3* or *mle* males and their heterozygous brothers. Because *msl-1* homozygous male larvae produce poor quality protein extracts, we again used *Sxl*^{f1}/*Sxl*^{fhv1} females

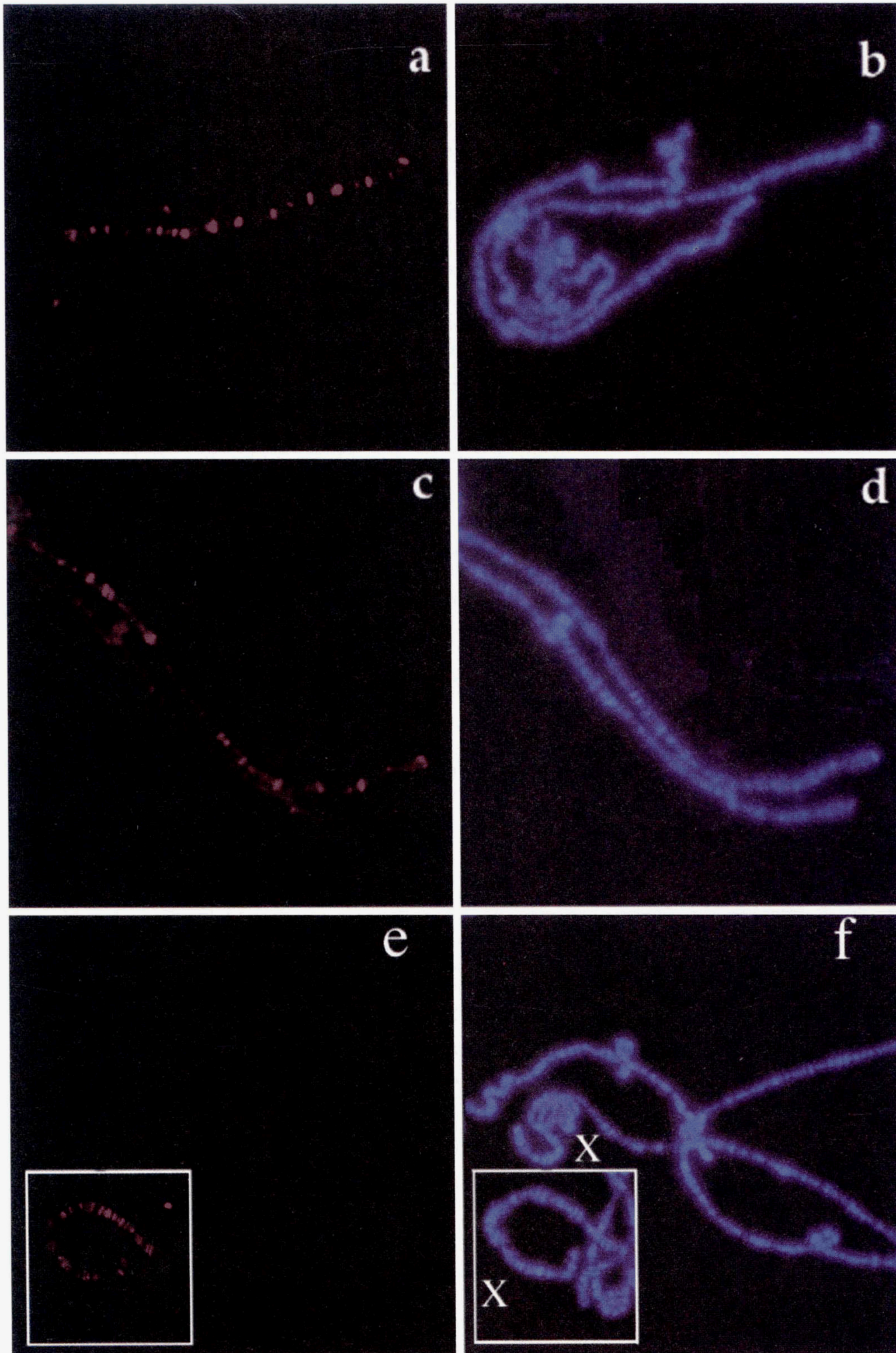

FIGURE 1.—Indirect immunofluorescence to detect MSL-2 in *msl*$^-$ genetic backgrounds. (Left) Anti-MSL-2 detection in *Sxl*^{f1}/*Sxl*^{fhv1}; *msl*$^-$ salivary gland polytene nuclei. (Right) The same nuclei counterstained with Hoechst 33258 to visualize all chromosome arms. MSL-2 maintains interaction at ~30 sites in the absence of *msl-3* (a), and slightly fewer in *mle* mutant nuclei (c). No MSL-2 protein is observed on the *X* chromosome in an *msl-1*$^{\gamma 269}$ homozygote (e). The *X* chromosome is indicated in f. The insets in e and f show the full anti-MSL-2 staining pattern in a *Sxl*^{f1}/*Sxl*^{fhv1}; *msl-1*$^{\gamma 269}$/+ heterozygous sister.

that were either homozygous mutant or heterozygous for *msl-1*. By this assay, MSL-2 levels were not dramatically decreased by lack of *msl-1* function. Individual larvae in this experiment are mosaic and may contain a different percentage of cells taking the male or female fate, therefore a quantitative comparison of MSL-2 levels (expressed only in "male" cells) was not possible. However, these data suggest that the observed reduction in MSL-2 chromosome binding in the *msl* mutants examined is due primarily to the functional absence of any one of the putative complex components, rather than to effects on MSL-2 expression or stability.

Dominant suppressor screen of MSL-2-dependent female lethality: Ectopic MSL-2 expression in females re-

TABLE 1

Cytological location of MSL-2:MSL-1 binding sites in the absence of *msl-3* or *mle*

Locus	msl-3/MM2SV	mle	msl-1$^{\gamma 216}$/msl-1^{b}
1C	++	++	++
2D	++	—	—
3F	++	++	++
4C	++	++	—
5C	++	++	++
5D	++	++	++
7A	++	++	++
7E	++	++	—
8A	+	+	—
8EF—double band	++	++	++
9D	+	—	—
10C	++	—	++
11A	+	—	—
11B-D cluster	++	++	+
12B	+	+	—
12F	++	++	++
13A	++	++	++
13D	++	++	++
13E	++	++	++
14B	++	++	+
14F	++	+	+
15E	++	++	++
16D	++	++	+
17B	++	++	—
17F	++	++	++
18D	++	++	++
19B	+	—	—
19F	++	++	++
20A	+	ND	ND

++ indicates that the individual locus was consistently detectable, while + notes a position that was not detected in every nucleus. ND refers to sites not determined for particular genotypes.

sults in a dominant female phenotype. Females expressing MSL-2 from the hsp83 promoter (H83M2) either die or are developmentally delayed and sterile (KELLEY *et al.* 1995). A powerful use of this constitutive MSL-2 transgene is for the isolation of genetic suppressors of the dominant phenotype. The strongest H83M2 line available for this purpose contains two tightly linked transgene insertions on the third chromosome (line 6I, data not shown). The female phenotypes of H83M2-6I can be suppressed by removing one wild-type copy of *msl-1*, or both functional copies of *msl-3* or *mle* (KELLEY *et al.* 1995). Therefore, we performed an EMS screen for dominant suppressors of the lethality and late eclosion phenotypes of H83M2 females, expecting to recover mutations in *msl-1* and perhaps novel, limiting components for dosage compensation (Figure 4). The results of this screen yielded one *msl-1* mutant and three putative mutations mapping to the chromosome containing the two H83M2 transgenes (third chromosome). Further characterization of these three suppressor lines revealed that they still expressed full length MSL-2 protein and could still complement *msl-2* mutants (Figure 5 and data not shown), suggesting that at least one of the H83M2 transgenes was intact for *msl-2* function. We analyzed one of these mutants (M63) in greater detail due to the presence of both wild-type and apparently truncated MSL-2 protein on Western blots (Figure 5). PCR amplification of the transgenic DNA from this stock revealed an intact copy of H83M2 and an additional smaller product. DNA sequence analysis of this PCR product revealed an in frame deletion of 121 amino acids, resulting in an MSL-2 protein lacking residues 19–140, including the RING finger (residues 39–82). Subsequent separation of this RING finger deletion transgene from its wild-type counterpart by *P*-element transposition (Figure 5) showed that this mutant does not appear to exert a dominant negative effect on males carrying at least one intact copy of *msl-2*. However, the RING finger deletion mutant protein fails to bind the *X* chromosome in transgenic females or complement *msl-2* mutant males (data not shown), suggesting that the RING finger domain is essential to MSL-2 function.

Mutagenesis of the MSL-2 RING finger and additional cysteine-rich motifs: In addition to the RING finger homology previously noted in the amino terminus of MSL-2, we distinguished overlapping similarity to the zinc finger of a nuclear receptor subfamily including LXRα (Figure 6A, WILLY *et al.* 1995). This alternative configuration of cysteines within the nuclear receptor zinc finger structure is mutually exclusive with that of the RING finger. The region of similarity between LXR and MSL-2 is limited to the first zinc finger of LXR (Figure 6A). The striking feature of this alignment is the strict conservation of the alpha helical sequence (CEGCxxF) that is required for DNA binding activity of the nuclear receptor (for review, see FREEDMAN and LUISI 1993; TSAI and O'MALLEY 1994). This region, termed the P-box, consists of five absolutely conserved residues split by two residues required for sequence specific DNA contact. Since MSL-2 has all five of the conserved residues, it was conceivable that MSL-2 might utilize this alpha helical structure to bind target sequences along the *X* chromosome.

We used site-directed mutagenesis to test whether MSL-2 was likely to fold into a RING finger or a nuclear receptor zinc finger structure. Six mutant MSL-2 constructs were generated through PCR-mediated mutagenesis (Figure 6, B and C). In each case one or more residues expected to play a vital role in the structure or function of a single motif were altered and then tested *in vivo* using transgenic animals. Two cysteine to alanine mutants, CA-1 and CA-3, are designed to destroy any zinc binding ability at position C_3 or C_7 of the RING finger (RING finger cysteines form a C_3HC_4 metal binding structure) (Figure 6B). In the appropriately folded structure, these residues are predicted to coordinate a common zinc atom with two other amino

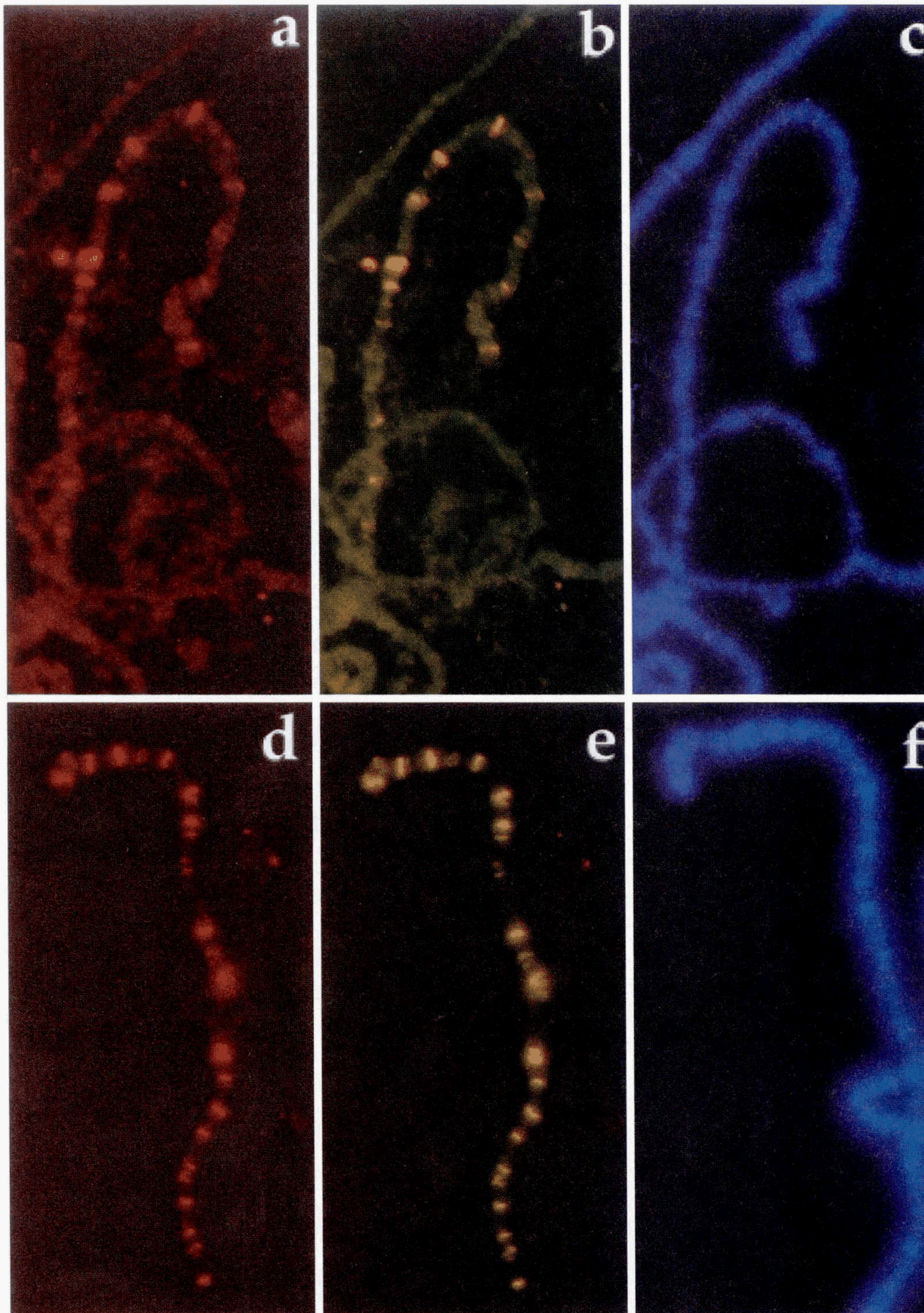

FIGURE 2.—MSL-2 and MSL-1 colocalize with one another as the wild-type pattern is altered. Polytene spreads from Sxl^{f1}/Sxl^{fhv1}; $msl\text{-}1^{\gamma 216}$/$msl\text{-}1^{b}$ females (top) and MM2SV females (bottom). MSL-2 protein (red) is detected at multiple sites spaced along the *X* chromosomes (a and d). Double exposure micrographs (b and e) illustrate the colocalization of MSL-2 and MSL-1 at these sites (MSL-1 alone not shown). (c and f) The identical chromosomes stained with Hoechst 33258.

acid partners (H_4 and C_8). Three other mutations target the putative P-box sequence. Adjacent positions known to be essential for DNA contact were modified in two cases (EA and EP), and the third involved replacement of the putative sequence-specific recognition residues in MSL-2 to those found in LXR (SK).

Transgenic *msl-2* rescue data collected for the individual mutants reveal that only the transgenes lacking two of the cysteines that form part of the RING finger failed to support male development (Table 2). Strains expressing mutations within the P-box of the nuclear receptor homology were viable in the absence of endogenous *msl-2*. Thus, our data eliminate the possibility for a significant role for the P-box motif and support an essential function for the MSL-2 RING finger.

The substitution of the serine-aspartic acid (SD) residues with the LXR sequence lysine-glycine (KG) lowered MSL-2 activity. Of the 11 independent insertions recovered, only one gave complete male rescue (SK 8) (APPENDIX A). Eight of the other 10 lines produced

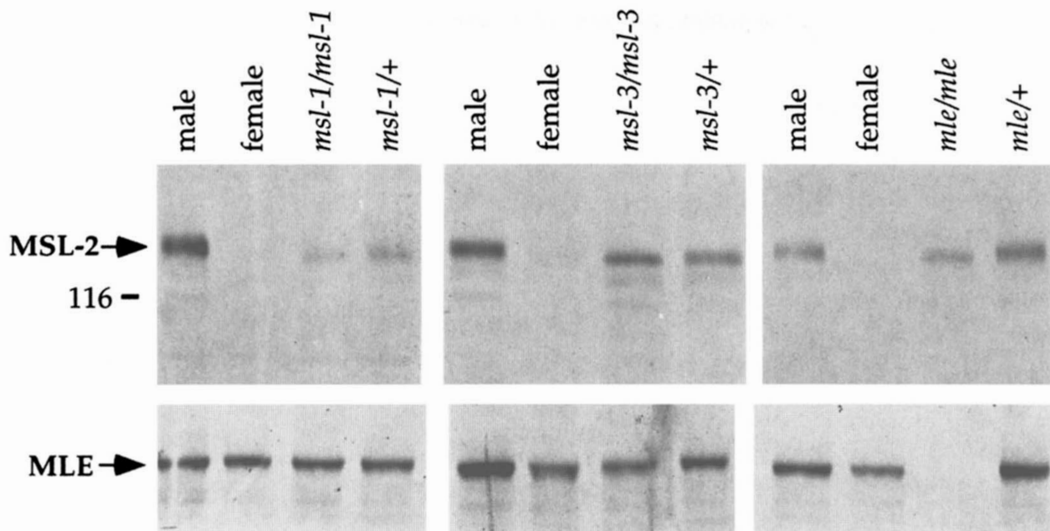

FIGURE 3.—MSL-2 protein expression in the absence of *msl-1, msl-3,* and *mle.* (Top) Western analysis of MSL-2 protein levels in wild-type males and females, as well as animals deficient for *msl-1* (left), *msl-3* (center), and *mle* (right). Sxl^{f1}/Sxl^{fhv1} heteroallelic females were utilized to generate the $msl\text{-}1^{\gamma 216}/msl\text{-}1^{b}$ background, while both the *msl-3* and the *mle* experiments were performed in mutant males. MSL-2 protein levels cannot be compared between Sxl^{f1}/Sxl^{fhv1}; $msl\text{-}1^{\gamma 216}/msl\text{-}1^{b}$ females and their Sxl^{f1}/Sxl^{fhv1}; $msl\text{-}1^{\gamma 216}/+$ sisters as the number of male-like cells expressing the MSL-2 protein will vary for each individual larva. (Bottom) MLE protein levels are shown as a control for total protein loaded.

males, but their development was delayed, and they were recovered at a lower percentage than expected. Furthermore, two transgenic strains occasionally produced males with the "wings held-out" phenotype previously characterized for partial loss of function mle^{-} males (RICHTER *et al.* 1996). These males were not fertile and most died soon after eclosion. These data indicate that mutations in this segment of the protein can result in diminished MSL-2 function, but do not indicate the nature of the defect.

We also mutagenized a second cysteine-rich domain in the central portion of MSL-2. This region has been suggested to contain a metallothionein-like cysteine cluster or a PHD finger. However, both alignments show only partial conservation of the individual motifs. In the case of the metallothionein domain, only eight of 20 cysteines are found (ZHOU *et al.* 1995). Similarly, MSL-2 possesses only the first five of eight hydrophobic

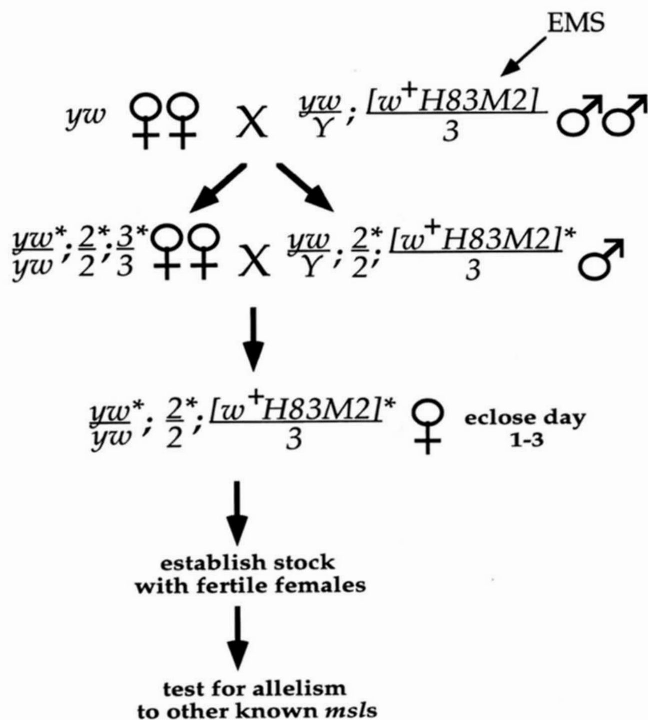

FIGURE 4.—Screen for dominant suppressors of ectopic MSL-2. The strategy was designed to recover healthy females expressing ectopic MSL-2 (H83M2). Stable stocks were established from fertile females. Chromosomes that were confirmed to rescue females were tested for complementation of *msl-1, msl-3,* and *mle.*

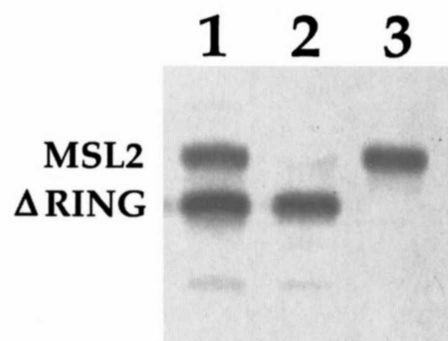

FIGURE 5.—Western analysis of the M63 RING finger deletion mutant. MSL-2 protein from adult females was detected with anti-MSL-2 antibodies. Lane 1, two protein bands are detected in the parental M63 mutant stock. Lane 2, an excision derivative stock that produces only the shorter MSL-2 protein and fails to rescue *msl-2* mutant males. Lane 3, an excision derivative of M63 that makes only the full length, functional MSL-2 protein.

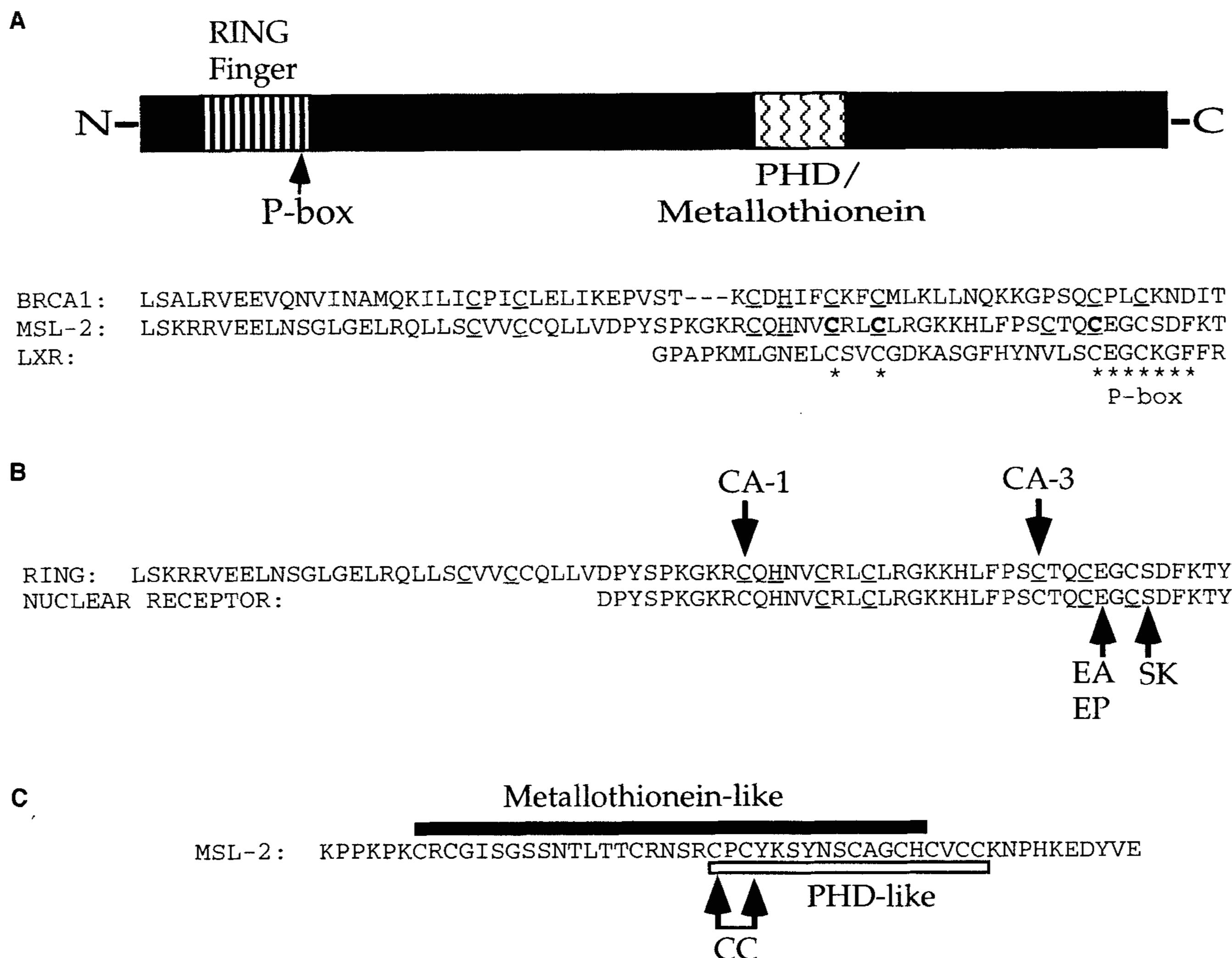

FIGURE 6.—Mutagenesis of the MSL-2 cysteine-rich domains. (A) The protein structure of MSL-2 is outlined to highlight the two cysteine-rich regions within the coding sequence. The amino-terminal domain has homology to the RING finger zinc-binding structure. The carboxy-terminal cluster has been related to either the PHD finger or a metallothionein-like sequence. Alternative alignment of the amino-terminal cysteines displays homology to a second form of zinc finger found in the nuclear receptor family. Similarity to this structure is conserved within the region of the P-box known to confer specific DNA binding activity to the nuclear receptors. As shown, the residues involved in the RING finger (underlined) and those within the nuclear receptor structure (*) are mutually exclusive. Those common to both are in bold. (B) To distinguish between the alternative alignments for the amino-terminal cysteine cluster, specific residues critical to single domains were modified (arrows) through PCR-directed mutagenesis. (C) Residues predicted to form the incomplete PHD finger and the metallothionein-like domain are overlapping. Therefore to determine the significance of the region as a whole, cysteines common to the two motifs were replaced with alanines (arrows) to prevent any potential zinc interaction through these positions.

residues characteristic of the PHD finger (AASLAND *et al.* 1995; BASHAW and BAKER 1995), and the spacing between these residues is not conserved with other family members. To determine the significance of either configuration, two cysteines contained in both proposed motifs were changed to alanines (Figure 6c). The *msl-2* mutant male progeny carrying the CC mutant transgene exhibited delayed development and reduced viability in all but two transgenic lines, but were fertile despite the late eclosion (data not shown). Again, this suggests that protein function has been diminished by the sequence changes. Any such decrease, however, is not sufficient to cause complete lethality, suggesting that conformation of the MSL-2 protein in this region may not correspond to either the metallothionein or PHD models.

DISCUSSION

The RING finger domain is essential for function of MSL-2: The RING finger is a zinc binding domain found in a growing number of proteins with diverse biological and biochemical functions (SAURIN *et al.* 1996). The loss of function phenotypes of (1) site-directed mutations in conserved RING finger cysteines and an EMS-generated RING finger deletion (this

TABLE 2

The RING finger is essential for MSL-2 function *in vivo*

Transgene	Region disrupted	Percent male viability (%)
Wild type	None	55–100
CA-1	RING finger	0
CA-3	RING finger	0
EA	P-box	54–100
EP	P-box	53–100
SK	P-box	0–92
CC	PHD/metallothionein	16–64

The range of male rescue is shown as compared to homozygous mutant sisters (see APPENDIX A).

work) and (2) two preexisting *msl-2* alleles with lesions in the RING finger region (ZHOU *et al.* 1995) are each indicative of an essential role for the RING finger in the function of MSL-2. In several cases, the RING finger has been implicated in protein-protein interactions, and our results are consistent with such a function in MSL complex formation or stability (BORDEN *et al.* 1995; EVERETT *et al.* 1995; RODGERS *et al.* 1996). Our results do not exclude additional or alternative roles for the MSL-2 RING finger domain in DNA or RNA binding. However, from our site-directed mutagenesis, we can rule out a requirement for the overlapping P-box motif in DNA recognition by MSL-2.

MSL-2 requires MSL-1 for *X* chromosome contact: Although each of the MSL proteins is dependent upon the others for binding to the *X* in the wild-type pattern, some important differences exist in the binding of the remaining MSLs in the absence of any single protein. In the absence of either MLE or MSL-3 we find that MSL-2 and MSL-1 remain bound at a subset of the wild-type sites. In addition, we show that when MSL-1 function is abolished, MSL-2 is not present on the *X*, but that if MSL-1 function is only compromised, MSL-2 is present at the reduced set of MSL-1 binding sites. Two alternative mechanisms could explain this obligate requirement of MSL-2:MSL-1 colocalization in *X* chromosome interaction. First, it is possible that MSL-1 is the sole subunit responsible for chromosome binding. In this model, it is the failure of MSL-1 to accumulate in the absence of MSL-2 that prohibits association. Alternatively, the two proteins may form the appropriate recognition motif only when bound together. A similar form of facilitation is seen with the yeast mating type-specific protein $\alpha 1$ and PRTF, a general protein found in all mating types (BENDER and SPRAGUE 1987). Such an interaction could explain the absence of a classical DNA binding domain within either protein. In both models the regulation of dosage compensation is critically sensitive to the relative concentrations of MSL-1 and MSL-2.

Consistent with an interdependent relationship between MSL-1 and MSL-2, we found that loss-of-function *msl-1* alleles can be isolated as dominant suppressors of MSL-2-dependent female lethality or developmental delay. We failed to identify any novel suppressors in the small EMS screen presented in this report, or in a much larger gamma-ray mutagenesis performed subsequently, in which 38 new *msl-1* mutants were isolated (R. L. KELLEY, unpublished results). Therefore, our results suggest that in the presence of excess MSL-2, MSL-1 is the predominant limiting component for dosage compensation in females. We conclude that any additional dosage compensation components must not be limiting in amount in females under these conditions.

Incomplete complex formation on the *X* chromosome: No MSL protein can bind in the absence of MSL-1 and MSL-2, suggesting that these subunits are the most central to complex assembly. The existence of a precise set of sites that are independent of MSL-3 and MLE argues that there are at least two classes of binding sites along the *X* chromosome. These "high affinity" sites could be nucleation centers from which the MSL complex might spread, but this is inconsistent with previous data demonstrating that *X*-linked loci, which do not map to the observed high affinity sites, appear dosage compensated when moved to autosomes (*e.g.*, KRUMM *et al.* 1985). An alternative possibility is that the high affinity sites, which are the only locations at which putative partial complexes are detected, might be sites of assembly of the MSL complex. In this model, only fully assembled, functional complexes would be competent to associate with all additional sites on the *X* chromosome.

We thank R. RICHMAN for assistance and advice regarding antibodies and Western blots, K. CHANG for advice on cloning and mutagenesis and for critical reading of the manuscript, and J. RAMPERSAD-AMMONS for excellent technical assistance. This work was supported by the National Institutes of Health grant GM-45744 and the Texas Higher Education Advanced Technology Program. L.R. is a Postdoctoral Associate and M.I.K. is an Associate Investigator of the Howard Hughes Medical Institute.

LITERATURE CITED

AASLAND, R., T. J. GIBSON and A. F. STEWART, 1995 The PHD finger: implications for chromatin-mediated transcriptional regulation. Trends Biochem. Sci. **20:** 56–59.

AMREIN, H., and R. AXEL, 1997 Genes expressed in neurons of adult male *Drosophila*. Cell **88:** 459–469.

BAKER, B. S., M. GORMAN and I. MARIN, 1994 Dosage compensation in *Drosophila*. Annu. Rev. Genet. **28:** 491–521.

BASHAW, G. J., and B. S. BAKER, 1995 The *msl-2* dosage compensation gene of *Drosophila* encodes a putative DNA-binding protein whose expression is sex-specifically regulated by *Sex-lethal*. Development **121:** 3245–3258.

BASHAW, G. J., and B. S. BAKER, 1997 The regulation of the Drosophila *msl-2* gene reveals a function for Sex-lethal in translational control. Cell **89:** 789–798.

BELOTE, J. M., and J. C. LUCCHESI, 1980 Male-specific lethal mutations of *Drosophila melanogaster*. Genetics **96:** 165–186.

BENDER, A., and G. F. SPRAGUE, 1987 MATα1 protein, a yeast transcription activator, binds synergistically with a second protein to a set of cell-type-specific genes. Cell **50:** 681–691.

BONE, J. R., J. LAVENDER, R. RICHMAN, M. J. PALMER, B. M. TURNER *et al.*, 1994 Acetylated histone H4 on the male X chromosome is

associated with dosage compensation in *Drosophila*. Genes Dev. **8:** 96–104.

Borden, K. L., M. N. Boddy, J. Lally, N. J. O'Reilly, S. Martin *et al.*, 1995 The solution structure of the RING finger domain from the acute promyelocytic leukaemia proto-oncoprotein PML. EMBO J. **14:** 1532–1541.

Brownell, J. E., J. Zhou, T. Ranalli, R. Kobayashi, D. G. Edmondson *et al.*, 1996 Tetrahymena histone acetyltransferase A: a transcriptional co-activator linking gene expression to histone acetylation. Cell **84:** 843–851.

Elenbaas, B., M. Dobbelstein, J. Roth, T. Shenk and A. Levine, 1996 The MDM2 oncoprotein binds specifically to RNA through its RING finger domain. Mol. Med. **2:** 439–451.

Everett, R., P. O'Hare, D. O'Rourke, P. Barlow and A. Orr, 1995 Point mutations in the herpes simplex virus type 1 Vmw110 RING finger affect activation of gene expression, viral growth, and interaction with PML-containing nuclear structures. J. Virol. **69:** 7339–7344.

Freedman, L. P., and B. F. Luisi, 1993 On the mechanism of DNA binding by nuclear hormone receptors: a structural and functional perspective. J. Cell. Biochem. **51:** 140–150.

Gorman, M., M. I. Kuroda and B. S. Baker, 1993 Regulation of the sex-specific binding of the *maleless* dosage compensation protein to the male X chromosome in *Drosophila*. Cell **72:** 39–50.

Gorman, M., A. Franke and B. Baker, 1995 Molecular characterization of the *male-specific lethal-3* gene and investigations of the regulation of dosage compensation in *Drosophila*. Development **121:** 463–475.

Higuchi, R., B. Krummel and R. K. Saiki, 1988 A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res. **16:** 7351–7367.

Hilfiker, A., D. Hilfiker-Kleiner, A. Pannuti and J. C. Lucchesi, 1997 *mof*, a putative acetyl transferase gene related to the Tip60 and MOZ human genes and to the SAS genes of yeast, is required for dosage compensation in *Drosophila*. EMBO J. **16:** 2054–2060.

Hilfiker, A., Y. Yang, D. H. Hayes, C. A. Beard, J. E. Manning *et al.*, 1994 Dosage compensation in *Drosophila*: the X-chromosomal binding of MSL-1 and MLE is dependent on *Sxl* activity. EMBO J. **13:** 3542–3550.

Kelley, R. K., I. Solovyeva, L. M. Lyman, R. Richman, V. Solovyev *et al.*, 1995 Expression of Msl-2 causes assembly of dosage compensation regulators on the X chromosomes and female lethality in Drosophila. Cell **81:** 861–877.

Kelley, R. L., and M. I. Kuroda, 1995 Equality for X chromosomes. Science **270:** 1607–1610.

Kelley, R. L., J. Wang, L. Bell and M. I. Kuroda, 1997 *Sex lethal* controls dosage compensation in *Drosophila* by a nonsplicing mechanism. Nature **387:** 195–199.

Krumm, A., G. E. Roth and G. Korge, 1985 Transformation of salivary gland secretion protein gene *Sgs-4* in *Drosophila*: stage- and tissue-specific regulation, dosage compensation, and position effect. Proc. Natl. Acad. Sci. USA **82:** 5055–5059.

Kuroda, M. I., M. J. Kernan, R. Kreber, B. Ganetzky and B. S. Baker, 1991 The *maleless* protein associates with the X chromosome to regulate dosage compensation in *Drosophila*. Cell **66:** 935–947.

Lindsley, D. L., and G. G. Zimm, 1992 *The genome of Drosophila melanogaster*. Academic Press, New York.

Lovering, R., I. M. Hanson, K. L. B. Borden, S. Martin, N. J. O'Reilly *et al.*, 1993 Identification and preliminary characterization of a protein motif related to the zinc finger. Proc. Natl. Acad. Sci. USA **90:** 2112–2116.

Lucchesi, J. C., 1996 Dosage compensation in *Drosophila* and the 'complex' world of transcriptional regulation. BioEssays **18:** 541–547.

Lucchesi, J. C., and J. E. Manning, 1987 Gene dosage compensation in *Drosophila melanogaster*. Adv. Genet. **24:** 371–429.

Matsudaira, P., 1987 Sequence from picomole quantitites of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262:** 10035–10038.

Meller, V. H., K. H. Wu, G. Roman, M. I. Kuroda and R. L. Davis, 1997 roX1 RNA paints the X chromosome of male Drosophila and is regulated by the dosage compensation system. Cell **88:** 445–457.

Mizzen, C. A., X.-J. Yang, T. Kokubo, J. E. Brownell, A. J. Bannister *et al.*, 1996 The TAFII250 subunit of TFIID has histone acetyltransferase activity. Cell **87:** 1261–1270.

Mu, Z., K. Chin, J. Liu, G. Lozano and K. Chang, 1994 PML, a growth suppressor disrupted in acute promyelocytic leukemia. Mol. Cell. Biol. **14:** 6858–6867.

Palmer, M. J., V. A. Mergner, R. Richman, J. E. Manning, M. I. Kuroda *et al.*, 1993 The *male-specific lethal-one* (*msl-1*) gene of *Drosophila melanogaster* encodes a novel protein that associates with the X chromosome. Genetics **134:** 545–557.

Palmer, M. J., R. Richman, L. Richter and M. I. Kuroda, 1994 Sex-specific regulation of the *male-specific lethal-1* dosage compensation gene in *Drosophila*. Genes Dev. **8:** 698–706.

Pennisi, E., 1997 Opening the way to gene activity. Science **275:** 155–157.

Pirrotta, V., 1988 Vectors for P-element transformation in Drosophila, pp. 437–456 in *Vectors: A Survey of Molecular Cloning Vectors and Their Uses*, edited by R. L. Rodriguez and D. T. Denhardt. Butterworths, Boston.

Reddy, B., M. Kloc and L. D. Etkin, 1991 The cloning and characterization of a maternally expressed novel zinc finger nuclear phosphoprotein (xnf7) in *Xenopus laevis*. Dev. Biol. **148:** 107–116.

Richter, L., J. R. Bone and M. I. Kuroda, 1996 RNA-dependent association of the *Drosophila* maleless protein with the male X chromosome. Genes Cells **1:** 325–336.

Robertson, A. M., C. R. Preston, R. W. Phillis, D. Johnson-Schlitz, W. K. Benz *et al.*, 1988 A stable genomic source of P-element transposase in *Drosophila melanogster*. Genetics **118:** 461–463.

Rodgers, K. K., Z. Bu, K. G. Fleming, D. G. Schatz, D. M. Engelman *et al.*, 1996 A zinc-binding domain involved in the dimerization of RAG1. J. Mol. Biol. **260:** 70–84.

Sanchez, L., and R. Nothiger, 1983 Sex determination and dosage compensation in *Drosophila melanogaster*: production of male clones in XX females. EMBO J. **2:** 485–491.

Saurin, A. J., K. L. B. Borden, M. N. Boddy and P. S. Freemont, 1996 Does this have a familiar RING? Trends Biochem. Sci. **21:** 208–214.

Tsai, M.-J., and B. W. O'Malley, 1994 Molecular mechanisms of action of steriod/thyroid receptor superfamily members. Annu. Rev. Biochem. **63:** 451–486.

Turner, B. M., A. J. Birley and J. Lavender, 1992 Histone H4 isoforms acetylated at specific lysine residues define individual chromosomes and chromatin domains in *Drosophila* polytene nuclei. Cell **69:** 376–384.

Willy, P., K. Umesono, E. Ong, R. Evans, R. Heyman *et al.*, 1995 LXR, a nuclear receptor that defines a distinct retinoid response pathway. Genes Dev. **9:** 1033–1045.

Zhou, S., Y. Yang, M. S. Scott, A. Pannuti, K. C. Fehr *et al.*, 1995 Male-specific lethal 2, a dosage compensation gene of *Drosophila*, undergoes sex-specific regulation and encodes a protein with a RING finger and a metallothionein-like cysteine cluster. EMBO J. **14:** 2884–2895.

Communicating editor: V. G. Finnerty

APPENDIX A

Percentage of *msl-2* male rescue as compared to homozygous transgenic sisters

	RING finger		P-box			PHD/metall.
Wild type	CA-1	CA-3	EA	EP	SK	CC
96 (37)	0 (23)	0 (19)	64 (28)	79 (19)	33 (12)	58 (38)
100 (28)	0 (20)	0 (14)	104 (23)	70 (20)	18 (28)	64 (11)
55 (29)	0 (17)	0 (14)	109 (11)	0 (7)	92 (12)	16 (25)
86 (7)	0 (3)	0 (9)	54 (11)	107 (15)	25 (12)	49 (33)
100 (8)	0 (27)	0 (5)	87 (23)	62 (8)	50 (24)	29 (7)
167 (6)	0 (32)	0 (13)	54 (11)	78 (9)	0 (13)	
	0 (20)	0 (15)	94 (16)	0 (13)	0 (7)	
	0 (33)	0 (15)	123 (13)	66 (12)	33 (27)	
	0 (21)			71 (17)	0 (6)	
				53 (17)	58 (26)	
				150 (6)	8 (38)	

Values are percentages; numbers in parentheses indicate the number homozygous transgenic sisters recovered.