The EMBO Journal Vol.17 No.18 pp.5409–5417, 1998

Complex formation by the *Drosophila* MSL proteins: role of the MSL2 RING finger in protein complex assembly

Kyle Copps[1], Ron Richman[2], Laura M.Lyman[3], Kimberly A.Chang[2,3], Joanne Rampersad-Ammons[2] and Mitzi I.Kuroda[1,2,3,4]

[1]Program in Cell and Molecular Biology, [2]Department of Cell Biology and [3]Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX 77030, USA

[4]Corresponding author
e-mail: mkuroda@bcm.tmc.edu

Drosophila **MSL proteins are thought to act within a complex to elevate transcription from the male X chromosome. We found that the MSL1, MSL2 and MSL3 proteins are associated in immunoprecipitations, chromatographic steps and in the yeast two-hybrid system, but that the MLE protein is not tightly complexed in these assays. We focused our analysis on the MSL2–MSL1 interaction, which is postulated to play a critical role in MSL complex association with the X chromosome. Using a modified two-hybrid assay, we isolated missense mutations in MSL2 that disrupt its interaction with MSL1. Eleven out of 12 mutated residues clustered around the first zinc-binding site of the RING finger domain were conserved in a *Drosophila virilis* MSL2 homolog. Two pre-existing *msl2* alleles, which fail to support male viability *in vivo*, have lesions in the same region of the RING finger. We tested these in the two-hybrid system and found that they are also defective in interaction with MSL1. Mutation of the second zinc-binding site had little effect on MSL1 binding, suggesting that this portion of the RING finger may have a distinct function. Our data support a model in which MSL2–MSL1 interaction nucleates assembly of an MSL complex, with which MLE is weakly or transiently associated.**

Keywords: dosage compensation/*male-specific lethal*/MSL complex/reverse two-hybrid/RING finger

Introduction

In many species, females develop with two X chromosomes, while males have only one. This imbalance has a potentially lethal effect that is countered by the evolution of various forms of X-linked gene dosage compensation. In the fruit fly *Drosophila*, dosage compensation is accomplished by ~2-fold hypertranscription of most genes on the male X chromosome (reviewed in Lucchesi and Manning, 1987; Baker *et al*., 1994). Several genes required for this phenomenon were identified in screens for mutations with recessive, male-specific lethal (*msl*) phenotypes (Fukunaga *et al*., 1975; Belote and Lucchesi, 1980; Uchida *et al*., 1981; Lucchesi *et al*., 1982; Hilfiker *et al*., 1997). The '*msl*s' include *msl1*, *msl2*, *msl3*, *maleless* (*mle*) and *males absent on the first* (*mof*). Each gene has been cloned and the encoded proteins have been characterized to varying degrees. MSL1 is a novel acidic protein (Palmer *et al*., 1993); MSL2, a putative zinc-binding protein (Bashaw and Baker, 1995; Kelley *et al*., 1995; Zhou *et al*., 1995); MSL3, a chromodomain protein (Gorman *et al*., 1995; Koonin *et al*., 1995); MLE, an RNA or DNA helicase of the DExH subfamily of ATPase/helicases (Kuroda *et al*., 1991; Lee *et al*., 1997); and MOF, a probable acetyltransferase (Hilfiker *et al*., 1997).

Abundant evidence suggests that the MSL proteins act within a multi-subunit complex (reviewed in Baker *et al*., 1994; Kelley and Kuroda, 1995; Cline and Meyer, 1996; Lucchesi, 1996). Immunofluorescence studies have shown that the MSLs co-localize at hundreds of discrete sites on the X chromosome in male somatic cells (Bone *et al*., 1994; Gorman *et al*., 1995; Kelley *et al*., 1995; Gu *et al*., 1998). Localization of each protein in the wild-type pattern requires all five msl^+ functions, suggesting that complex formation is a prerequisite for their association with most X chromosome sites. In support of this idea, both MSL1 and MSL3 appear to depend on interaction with the other proteins for stability (Palmer *et al*., 1994; Gorman *et al*., 1995; Kelley *et al*., 1995), and MSL1 and MSL2 have been co-immunoprecipitated (Kelley *et al*., 1995).

There is indirect evidence for the existence of one or more RNA components of the MSL complex. Two non-coding RNAs termed roX1 and roX2 were shown to require the MSLs for their male-specific accumulation, and roX1 was shown specifically to coat the male X chromosome (Amrein and Axel, 1997; Meller *et al*., 1997). Although a requirement for the roX RNAs in male viability has not been demonstrated, it is possible that their role in dosage compensation is masked by functional redundancy. In addition, it is noteworthy that MLE, the *Drosophila* homolog of human RNA helicase A, is removed specifically from the X chromosome by treatment of polytene chromosomes with RNase A (Richter *et al*., 1996).

The MSLs appear to function by a mechanism involving the modification of chromatin structure. In the presence of the wild-type MSLs, histone H4 that is mono-acetylated on Lys16 (H4Ac16) is associated preferentially with the X chromosome (Turner *et al*., 1992; Bone *et al*., 1994; Hilfiker *et al*., 1997). A link between histone acetylation and gene transcription was demonstrated recently by the finding that several proteins defined as transcriptional coactivators possess histone acetyltransferase activity (Brownell *et al*., 1996; Kuo *et al*., 1996; Ogryzko *et al*., 1996). Thus, a major function of the MSL complex may be to target the putative acetylase MOF to X chromatin (Hilfiker *et al*., 1997; Gu *et al*., 1998).

Interaction between MSL2 and MSL1 may serve an initiating role in the association of the MSLs with the

© Oxford University Press

X chromosome. First, ectopic expression of the male-specific MSL2 protein in females appears to stabilize MSL1, and causes the assembly of functional MSL complexes on their X chromosomes (Kelley *et al*., 1995). Secondly, the MSL2 and MSL1 proteins remain co-dependently co-localized at ~30 sites on the X chromosome in the absence of either MSL3, MLE or MOF (Lyman *et al*., 1997; Gu *et al*., 1998). Thus, MSL2 and MSL1 may comprise the chromatin-binding activity of the MSL complex, or may be the first of the known subunits to assemble at pre-defined sites.

The MSL2 protein contains a RING finger (C_3HC_4) domain. The RING finger is present in >60 proteins from plants, bacteria, animals and viruses, many of which are present in macromolecular complexes (reviewed in Freemont, 1993; Saurin *et al*., 1996). In some proteins, such as the breast cancer protein BRCA1, mutations in the RING finger are correlated with human cancers (Shattuck-Eidens *et al*., 1995). Solution ^{1}H-NMR structures have been obtained for the equine herpesvirus gene 63 protein (EHV-63) and the human acute promyelocytic leukemia proto-oncoprotein (PML) (Barlow *et al*., 1994; Borden *et al*., 1995). In each case, two zinc atoms are coordinated by interleaved pairs of cysteines, or cysteine plus histidine, in a 1–3, 2–4 pattern. A comparison of the EHV-63 and PML domains revealed significant structural differences, suggesting that the RING finger may serve as a scaffold for the evolution of different functions (Borden *et al*., 1995). A requirement for the MSL2 RING finger in male viability was demonstrated by site-directed mutation of conserved RING finger cysteines (Lyman *et al*., 1997); however, its function, whether in binding to DNA or to other proteins, has not been established.

In this report, we provide direct evidence of complex formation by the MSLs and explore the role of the MSL2 RING finger in this process. We find that the RING finger domain of MSL2 binds the MSL1 protein, and that residues around the first zinc-binding site of the RING finger are critical for this interaction. In addition, we find that MSL3 is tightly complexed with MSL2–MSL1 through interaction with MSL1, but that MLE may be only weakly, or transiently, associated with these proteins. These findings are discussed in relation to current models of dosage compensation and RING finger function.

Results

MSL1, MSL2 and MSL3 are tightly complexed in vivo

The MSL1 and MSL2 proteins previously were reported to co-immunoprecipitate (co-IP) (Kelley *et al*., 1995). We have extended this analysis to include MSL3 and MLE using extracts from *Drosophila* Schneider line 2 (SL2) cells. The male character of SL2 cells was documented previously by the presence of male-specific transcripts of the alternatively spliced sex-determination genes *Sxl* and *tra* (Ryner and Baker, 1991). We found that these cells also express the MSLs, including the male-specific protein MSL2 (Figure 1A–C). As in male larvae, the MSL-1 and MSL-2 proteins are restricted to a portion of the chromatin in nuclei (presumably the X), while MLE exhibits some general, possibly autosomal, staining in addition to its X-specific association (Kuroda *et al*., 1991). Assembly of the MSLs into apparently functional complexes in SL2 cells is demonstrated by the subnuclear co-localization of MSL1 and H4Ac16, a marker of dosage-compensated chromatin (Figure 1D–F).

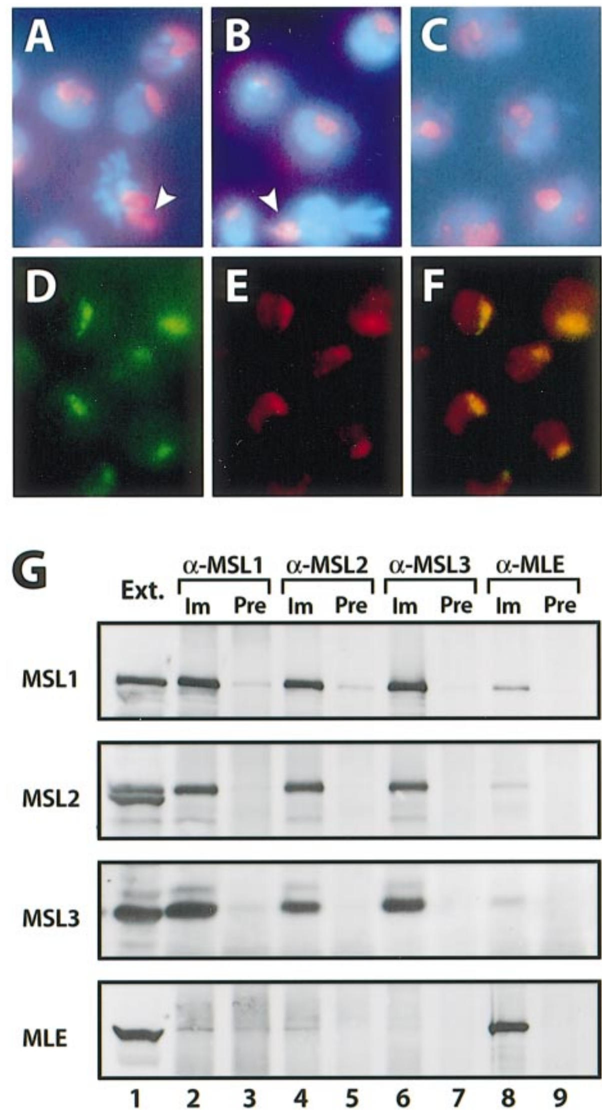

Fig. 1. Immunostaining and co-immunoprecipitation of the MSLs from *Drosophila* SL2 cells. (A–C) Separate fields showing cells stained for (**A**) MSL1, (**B**) MSL2 or (**C**) MLE (red) and counterstained with Hoechst 33258 to reveal DNA (blue). In each case, the MSL proteins are localized predominantly to single sites within nuclei, compatible with X chromosome binding. MLE also exhibits some general, possibly autosomal, staining. White arrows indicate association of MSL-1 and MSL-2 with mitotic chromosomes. (D–F) A single field stained for (**D**) MSL-1 or (**E**) H4Ac16, a marker of the dosage-compensated X chromosome in male flies. A double exposure micrograph of the same field (**F**) shows the co-localization of the two signals. (**G**) Co-IP Western blots. SL2 cell nuclear extract was incubated with immune (Im) and pre-immune (Pre) sera against each of the MSLs. Immunoprecipitates from 200 μg of extract (lanes 2–9) were probed for the presence of individual proteins on separate blots; lane 1 contains 40 μg of the extract. Only MLE was not co-precipitated significantly by antibodies against MSL1, MSL2 and MSL3; similar results were seen with extracts prepared from third instar male larvae by the same method (not shown).

For immunoprecipitation reactions, we incubated SL2 cell nuclear extract with immune and pre-immune sera against MSL1, MSL2, MSL3 and MLE (Figure 1G);

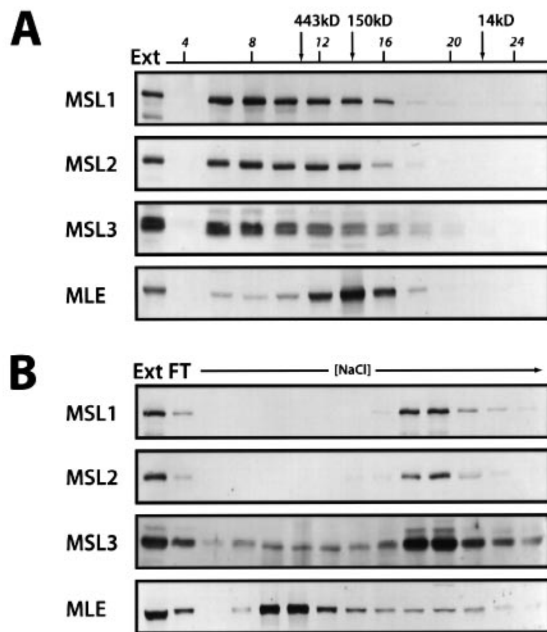

Fig. 2. MSL1, MSL2 and MSL3 co-fractionate on gel filtration and anion exchange chromatography. (**A**) Superose 12 chromatography. Increasing time (in fraction number) is indicated by the scale; the elution times of calibrating marker proteins (apoferritin, 443 kDa; alcohol dehydrogenase, 150 kDa; cytrochrome *c*, 14 kDa) are denoted by arrows. MSL1, MSL2 and MSL3 elute as a complex with an M_r >1 MDa. In contrast, elution of MLE (mol. wt 143 kDa) peaked in fraction 14 with the 150 kDa alcohol dehydrogenase marker. (**B**) Mono Q chromatography. MSL1 and MSL2 are predicted to have an acidic pI (~5.5) and are strongly retained by Mono Q resin at pH 8.0. Elution of the more neutral MSL3 protein (predicted pI ~7) peaks in the same fractions as MSL1 and MSL2, while the bulk of MLE (predicted pI ~7) is eluted in lower salt (Ext., Schneider extract; FT, unbound, or flowthrough, fraction).

extracts from third instar male larvae gave qualitatively equivalent results (data not shown). A significant portion of the total MSL1, MSL2 and MSL3 in SL2 extract was precipitated by antisera against any one of these proteins, demonstrating their tight association *in vivo* (Figure 1G, lanes 2, 4 and 6). In contrast, the fraction of MSL1, MSL2 and MSL3 precipitated by anti-MLE serum was small relative to the amount of MLE precipitated (Figure 1G, lane 8), suggesting that only a fraction of the total MLE was complexed with the other proteins. The simplest explanation for this finding is that there is a substantial pool of free MLE in nuclei *in vivo*; however, we cannot exclude the possibility that MLE is removed preferentially from the MSL complex during its extraction from chromatin.

We further characterized the soluble MSL complex from Schneider cells using gel filtration (Superose 12) and anion exchange (Mono Q) chromatography (Figure 2A and B). Consistent with our IP data, the MSL1, MSL2 and MSL3 proteins co-eluted from both columns, while the bulk of MLE appeared to run as a monomer. The elution of MSL1, MSL2 and MSL3 from Superose 12 chromatography was compatible with their presence in a complex with an M_r of >1 MDa, while the bulk of MLE (143 kDa) co-eluted with a 150 kDa marker protein. In subsequent analyses using Superose 6 chromatography, we found that the MSL complex co-elutes with the *Drosophila* brahma (BRM) complex (M_r ~2 MDa; data not shown). We also found that MSL1, MSL2 and MSL3 could be co-precipitated from their peak Mono Q fractions, suggesting that the chromatography was not generally disruptive to protein interaction (data not shown). Thus, the relative abundance of uncomplexed MLE in nuclear extracts may indicate that the association of MLE with the MSL complex is weaker than that of the other MSLs.

MSL2 and MSL3 interact with MSL1 in the two-hybrid system

To begin molecular dissection of the MSL complex, we assayed for interaction of MSL1, MSL2, MSL3 and MLE in the yeast two-hybrid system. Initially, we made fusions of the full-length proteins to the Gal4 DNA-binding domain (DB) and activation domain (AD) and tested for their ability to activate *GAL–lacZ* transcription in yeast. The DB–MSL1 and DB–MSL2 fusions strongly activated transcription of the *GAL–lacZ* reporter in the absence of AD-tagged partners. Therefore, these fusions were not informative for interactions with other proteins. The DB–MSL3 fusion also weakly activated transcription of the reporter; however, interactions with this protein could still be scored by the enhancement of *lacZ* activity in the presence of strongly interacting partners. Finally, DB–MLE did not activate the reporter in the absence of a partner. To permit two-hybrid assay of MSL1–MSL2 interaction in at least one of the two possible configurations, we examined the effect of C-terminal deletions in these proteins on their ability to potentiate *GAL–lacZ* transcription. As a series of truncated DB–MSL1 fusions retained the ability to activate the reporter (not shown), we constructed and used a non-activating MSL2 fusion [DB–MSL2 (1–190)], which contains only the N-terminal 190 MSL2 residues, including the RING finger domain (amino acids 37–87).

In the complete set of pairwise assays (Figure 3A), both DB–MSL2 (1–190) and DB–MSL3 interacted with AD–MSL1. In contrast, the DB–MLE fusion did not interact with MSL1, MSL2 or MSL3 fusions, but did interact with AD–MLE. It is unclear if the interaction of MLE with itself is relevant to its function *in vivo*; however, the interaction of MSL2 and MSL3 with MSL1 is consistent with our co-IP data and with the co-purification of these three proteins. In addition, this result is compatible with a network of presumed stability effects in which MSL2 stabilizes MSL1, and both MSL2 and MSL1 are required for the stability of MSL3 (Baker *et al.*, 1994; Gorman *et al.*, 1995; Kelley *et al.*, 1995). Therefore, the two-hybrid interactions between these proteins probably represent their actual interactions at sites on the X chromosome *in vivo*.

We screened a library composed of restriction fragments from the *msl1* cDNA to identify a region of MSL1 sufficient for interaction with DB–MSL2 (1–190). Several strongly interacting clones isolated from the screen were found to contain an identical *Sau*3A1 fragment encoding amino acids 48–321 of the MSL1 open reading frame (Figure 3B). Subsequent assay of overlapping subclones within this ~1 kb fragment showed that amino acids 85–186 of MSL1 were sufficient for interaction with MSL2 in the two-hybrid system; the same region was not sufficient for interaction with MSL3 or with other

A

DNA binding domain hybrid / lacZ activity	activation domain hybrid: MLE	MSL1	MSL2	MSL3	pACT2 vector
MLE	++	–	–	–	–
MSL1	++	++	++	++	++
MSL2 (aa 1-190)	–	+++	–	–	–
MSL3	+	+++	+	+	+

B

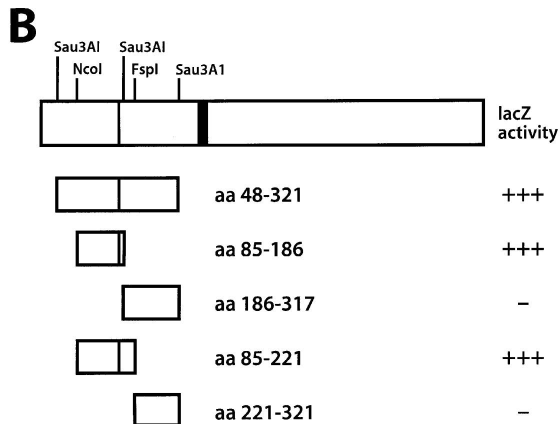

Fig. 3. Two-hybrid interactions between the MSL proteins. Minus and plus signs indicate the absence or presence of activity of a chromosomal *GAL–lacZ* reporter; multiple pluses indicate greater relative activity in side-by-side comparisons. (**A**) Pairwise interaction tests. The full-length proteins or a deletion (MSL2) were fused to the Gal4 DB and AD in the expression plasmids pAS1 and pACT2, and tested for interaction in a mating assay. Informative pairs are in shaded boxes. As a control, the DB–MSL plasmids were mated to the empty pACT2 vector, which expresses the Gal4 AD only. (**B**) Amino acids 85–186 of MSL1 are sufficient for interaction with MSL2 in the two-hybrid system. The MSL1 protein is depicted as a box, with clusters of negatively charged aspartate and glutamate residues indicated by shading. A segment encoding residues 48–321 was selected for interaction with MSL2 from a library of *msl1* fragments. The indicated fusions to the Gal4 AD (small boxes) were used to map the interaction with MSL2 as above. None of the fusions activated *GAL–lacZ* transcription in the absence of DB–MSL2 (1–190).

DB-tagged proteins. A search of protein databases using amino acids 85–186 of MSL1 yielded no significant homologies.

The MSL2 RING finger domain is required for interaction with MSL1

To identify specific MSL2 residues necessary for interaction with MSL1, we developed a modified two-hybrid assay that allows selection against protein interaction. In this 'reverse two-hybrid' scheme (Figure 4), yeast cells in which a mutant DB–MSL2 (1–190) protein does not interact with AD–MSL1 fail to activate transcription of a *GAL–URA3* reporter, and are thereby made resistant to 5-fluoroorotic acid (5-FOA). A secondary screen of *GAL–lacZ* reporter activity and other assays (steps 1–4, Figure 4) then confirm that the loss of interaction is due to a missense mutation in MSL2. Variations on this method have been used previously to map protein interactions

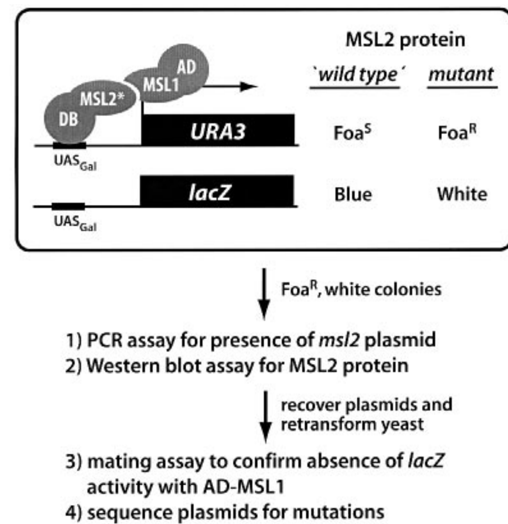

Fig. 4. Scheme for selection of interaction-disruptive mutations in MSL2. Yeast expressing the wild-type AD–MSL1 fusion and a mutagenized DB–MSL2 (1–190) protein (MSL2*) were plated on minimal media containing 5-FOA. Two reporter genes were integrated into the yeast chromosomes. Cells in which interaction of the proteins was preserved ('wild type') activated the *GAL–URA3* reporter and were FOA-sensitive (Foa^S). Cells in which the interaction was disrupted (mutant) failed to activate *GAL–URA3* transcription, were FOA-resistant (Foa^R), and lived to produce white colonies in a secondary screen of *GAL–lacZ* reporter activity. Subsequent assays confirmed that: (i) colonies contained the DB–MSL2 (1–190) plasmid; (ii) an MSL2 fusion protein was made; and (iii) loss of interaction was not due to defects in the MSL1 fusion or in the yeast host cell.

within the yeast Ste5 protein, and to isolate dominant-negative mutations in the human p53 protein (Brachmann *et al.*, 1996; Inouye *et al.*, 1997a).

Libraries of mutations in the DB–MSL2 (1–190) fusion were generated by subcloning the products of mutagenic PCR reactions into the unmutated DB–MSL2 (1–190) plasmid, ensuring that no mutations were made in the Gal4 DB. From the screening of two small libraries, we isolated 19 plasmids that encoded a stable MSL2 fusion protein incapable of interaction with MSL1. While the majority of plasmids carried more than one amino acid substitution, we were able to identify a single interaction-disruptive mutation in all but one case by subcloning and retesting the mutations from seven of the double mutant plasmids. In all, this analysis yielded 12 mutations in 11 different codons that disrupted binding to MSL1, as well as seven mutations which, by themselves, had no effect (Figure 5A).

We noted a striking tendency of the disruptive mutations to cluster about the first zinc-binding site (Z1) of the RING finger (Figure 5). Mutations were found at several conserved positions within the region, including three of the four cysteines thought to coordinate zinc at Z1. Interestingly, two of the three *msl2* mutant alleles sequenced by Zhou *et al.* (1995) also cluster in this region. The $msl2^{\gamma 136}$ and $msl2^{1}$ alleles, which fail to support male viability *in vivo*, carry mutations of residues near Z1 of the RING finger (deletion of V43 and P51L, respectively). To determine whether the encoded proteins would also fail to interact with MSL1, we tested both alleles in our reverse two-hybrid assay (Figure 5A). Both mutations specifically disrupted interaction with MSL1, while

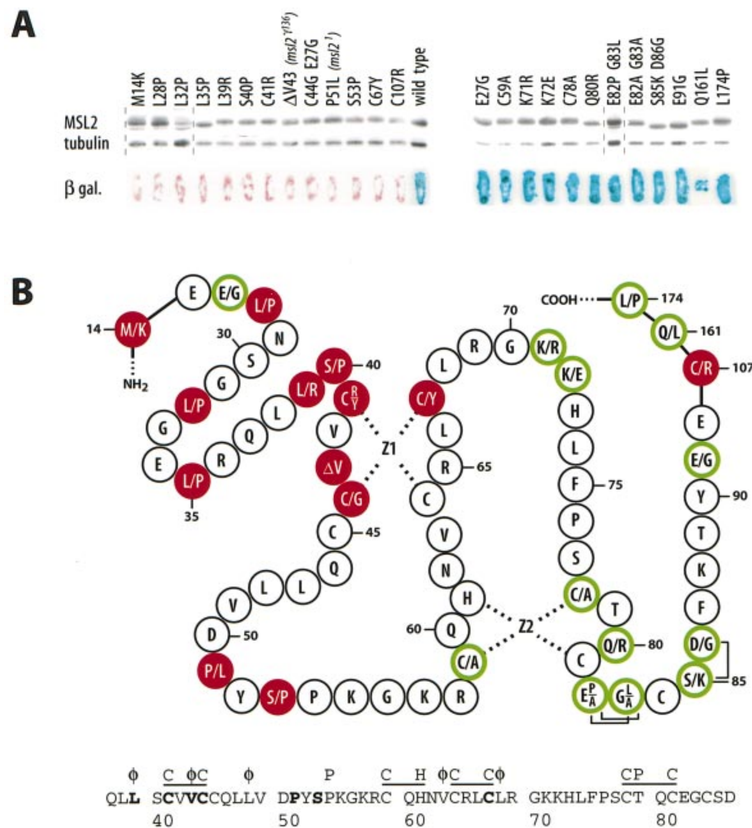

Fig. 5. Results of reverse two-hybrid screening. All mutations were isolated by 5-FOA selection except the fly alleles (ΔV43 and P51L) (Zhou *et al*., 1995) and the site-directed mutations (C59A, C78A, E82P G83L, E82A G83A and S85K D86G) (Lyman *et al*., 1997). (**A**) Western blots and *lacZ* activity assays for interaction-disruptive (left) and non-disruptive mutations (right). Extracts from yeast expressing wild-type MSL1 and the indicated MSL2 mutants were probed for the ~37 kDa MSL2 fusion protein; as a control, identical blots were probed with antibodies to γ-tubulin (~55 kDa). With the exception of the L32P mutant, the various MSL2 mutant proteins were produced at similar levels, regardless of their ability to bind MSL1. After a 24 h incubation in the presence of X-gal, cells in which MSL2–MSL1 interaction is intact are stained blue, while cells in which the interaction is disturbed appear pink due to starvation for adenine. (**B**) Mutations that disrupt interaction of MSL2 and MSL1 cluster about the first zinc-binding site of the MSL2 RING finger. Interaction-disruptive mutations are indicated by red circles, non-disruptive mutations by green rings; the wild-type and mutant residues are separated by a slash; double mutations are indicated by parentheses. A consensus sequence for MSL2-like RING fingers (Zhou *et al*., 1995) is shown beneath the protein, with mutations in conserved positions in bold type (ø = hydrophobic residues).

random point mutations from our PCR mutagenesis did not. In each case, a stable protein was produced in yeast. Therefore, we conclude that one essential function of the RING finger in MSL2 is to mediate interaction with MSL1.

However, further analysis showed that the RING finger is functionally complex. Despite the 'cross-braced' arrangement of the RING finger zinc-binding sites, we found no interaction-disruptive mutations within the second site (Z2). We previously reported that mutations in two of the Z2 cysteines (C59 and C78; Figure 5A) abolish $msl2^+$ function *in vivo* (Lyman *et al*., 1997). When tested for interaction in yeast (Figure 5A), fusion proteins carrying these mutations bound to MSL1 as well as the wild-type MSL2 protein. Thus, we propose that the RING finger domain performs at least two functions in MSL2: the first zinc-binding site is involved in MSL1 binding, while the second site may be required for a separate activity. Unfortunately, analysis of this model *in vivo* is complicated by the fact that non-functional MSL proteins often do not persist in the fly, severely hampering efforts to correlate mutation with the loss or retention of specific protein–protein interactions. Indeed, the protein encoded by the $msl2^1$ allele is not detectable in male larval extracts (Lyman *et al*., 1997), but is stable when expressed in yeast (Figure 5A).

The RING finger domain is conserved in MSL2 of Drosophila virilis

Previous studies have shown that the MSL components of dosage compensation are conserved between *Drosophila melanogaster* and *Drosophila virilis* (Bone and Kuroda, 1996; Marín *et al*., 1996). These species diverged ~60 million years ago (Patterson and Stone, 1952). We used oligonucleotides to the RING finger region of the *D.melanogaster msl2* gene to amplify a portion of the *D.virilis* homolog; this PCR product was then used as a probe to obtain a full-length *msl2* clone from a *D.virilis* genomic library. An alignment of the conceptual translation products of the *D.virilis* and *D.melanogaster* genes (Figure 6A and B) reveals the excellent conservation (74% identity/83% similarity) of the RING finger domain and surrounding region (amino acids 1–115). All but one of the residues found to be important for MSL1 binding by reverse two-hybrid screening are conserved in the *D.virilis* protein; the single exception, S53, is adjacent to a conserved proline (P54) and was itself mutated to proline in the screen. Four cysteine or histidine residues outside the RING domain are also conserved. Although these are not positioned to form a canonical zinc finger structure, mutation of one of the residues (C107) to arginine disrupted the interaction of MSL2 with MSL1.

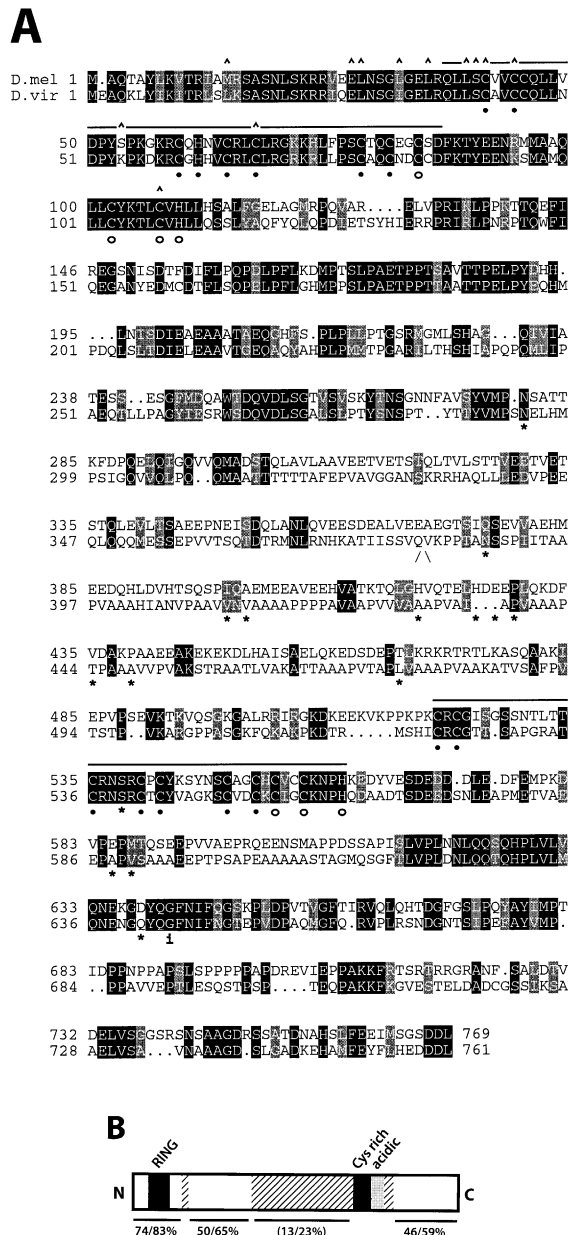

Fig. 6. Alignment of *D.melanogaster* and *D.virilis* MSL2. (**A**) Identical and similar residues are highlighted by black and gray shading, respectively. Bars above the alignment indicate the position of the RING finger and second cysteine-rich domains; caret marks (^) indicate the position of interaction-disruptive mutations in the RING finger. Dots (●) mark the positions of conserved cysteines/histidines; open circles (○) are cysteines/histidines outside the published RING finger and metallothionein alignments. Polymorphisms (*) in the *D.melanogaster* protein, including a four amino acid insertion (/ \), lie mainly within the poorly conserved middle third of the protein. The position of an intron (i) (52 bp in *D.melanogaster*, 69 bp in *D.virilis*) is conserved. (**B**) Conserved domains in the two proteins are shown above a box representing the MSL2 protein. Highly variable gap regions described in the text are indicated by hatching. Amino acid identity/similarity within different regions is indicated by percentages below the protein. These sequence data have been submited to the DDBJ/EMBL/GenBank databases under accession No. AFO79368.

A second cysteine-rich region (amino acids 521–562), which is loosely related to the PHD motif and to metallothioneins (Bashaw and Baker, 1995; Zhou *et al.*, 1995), is conserved at all cysteines and histidines, including three positions not present in the published metallothionein alignment. The conservation of these residues is compatible with our previous report that mutation of C540 and C542 to alanine diminishes, but does not abolish *msl2*$^+$ function *in vivo* (Lyman *et al.*, 1997). The acidic nature of an adjacent region (amino acids 563–592) is also conserved, while a more distal proline-rich region (amino acids 681–701) is incompletely conserved (Zhou *et al.*, 1995).

A remarkable feature of the alignment is the presence of three gap regions having little or no homology. A short gap (amino acids 116–128) separates the RING finger and N-terminus from the rest of the protein. The second gap (amino acids 281–520) corresponds to the middle third of the MSL2 protein. This region contains most of the polymorphisms and length variations present within published *D.melanogaster msl2* sequences; in addition, neither the previously described repeats (Bashaw and Baker, 1995) nor the acidic character of this region of the *D.melanogaster* protein are conserved in the *D.virilis* homolog. Finally, a gap following the second cysteine-rich domain (amino acids 593–614) is conserved in length, but is not conserved in sequence. These data suggest that the functions of the RING finger and other features of MSL2 have been conserved and that these domains may be positioned appropriately in the protein by more or less randomly evolving spacers.

Discussion

Assembly of the MSL complex

We used co-immunoprecipitation, co-fractionation and two-hybrid interaction to assay complex formation by the MSLs. From immunoprecipitations and chromatography of the soluble complex from Schneider cell nuclear extracts, we found that the MSL complex contains MSL1, MSL2 and MSL3, and has an M_r of ~2 MDa. Most of the MLE in Schneider and larval extracts appeared to be uncomplexed, or was removed from the complex during its extraction from chromatin. The minimal set of MSL protein interactions compatible with our results includes binding of MSL2 to MSL1, and of MSL1 to MSL3. These interactions are consistent with known genetic and molecular data, including Western analyses on *msl* mutants which suggested that MSL2 stabilizes MSL1, and that both MSL2 and MSL1 stabilize MSL3 (Palmer *et al.*, 1994; Gorman *et al.*, 1995; Kelley *et al.*, 1995). We previously reported that MSL2 and MSL1 remain co-localized at a subset of X chromosome sites in the absence of the other MSLs (Lyman *et al.*, 1997). Based on the findings of this study, we suggest that MSL2–MSL1 interaction provides a nucleus for assembly of the MSL complex, into which the other proteins may be incorporated by additional stabilizing interactions.

We did not find evidence for direct interaction of MLE with the other MSLs. MLE previously has been shown to have RNA helicase activity (Lee *et al.*, 1997) and is the only one of the MSLs that is sensitive to RNase A for its association with the X chromosome in polytene squashes (Richter *et al.*, 1996). An intriguing possibility is that MLE may be attached to the MSL complex through interaction with the recently described roX RNAs (Amrein and Axel, 1997; Meller *et al.*, 1997). Such a connection would account simultaneously for the co-localization of

MLE and the other MSLs to X chromatin, and the apparent lability of MLE to ammonium sulfate extraction seen in this study. However, MLE could also act catalytically and, therefore, have a more dynamic association with the X chromosome than the other MSLs (for instance, by regulating the assembly of the MSL complex). In either case, the association of MLE with the other MSLs would be preserved in formaldehyde-fixed polytene squashes, but eliminated upon extraction of the MSL complex from X chromatin.

Involvement of the MSL2 RING finger in protein interaction

In this report, we demonstrate that one function of the MSL2 RING finger is to bind a defined region of the MSL1 protein. The RING finger motif, or a variant of it (RING-H2 or $C_2H_2C_4$), has been found in >60 proteins with diverse subcellular localizations and biological functions (Saurin *et al*., 1996). It is not clear whether the RING domain will have a common function in these proteins, as DNA binding (Lovering *et al*., 1993; Kanno *et al*., 1995), RNA binding (Elenbaas *et al*., 1996) and protein interaction (Borden *et al*., 1995) have all been proposed to occur through the RING finger. In most cases, however, the authentic *in vivo* target for the activity has not been identified. We have determined that the MSL2 RING finger is a site of critical protein–protein interactions in the MSL complex, but have not ruled out additional functions for the domain.

The known RING finger ^{1}H-NMR structures have an unusual, 'cross-braced' arrangement in which two zinc atoms are coordinated by interleaved pairs of cysteine or histidine residues (Barlow *et al*., 1994; Borden *et al*., 1995). In our study, a complete RING finger was not a requirement for MSL1 interaction. Rather, we found that residues clustered around the first zinc-binding site (Z1) of the MSL2 RING finger are critical for interaction with MSL1. Mutations in three of the four cysteines thought to coordinate zinc at Z1 abolished binding to MSL1, providing a link between zinc binding and protein interaction at this site. In contrast, mutations in the second zinc-binding site (Z2) did not abolish MSL2–MSL1 interaction, although these residues are required for MSL2 function *in vivo* (Lyman *et al*., 1997). It is possible that a complete RING finger is necessary in *Drosophila* for MSL2–MSL1 interaction, and that the yeast system does not reflect this requirement accurately. However, an interesting alternative possibility is that the two parts of the RING finger do not function as a single structural unit.

Several distinctions can be made between the Z1 and Z2 sites of some well-characterized RING fingers. A comparison of the PML and EHV63 NMR solution structures (Barlow *et al*., 1994; Borden *et al*., 1995) revealed that a highly conserved feature of the RING domain is the topology around the Z1 site, while Z2 is less conserved (Borden *et al*., 1995). Furthermore, at least one RING finger-like protein (Pas10p) has a typical Z1 domain but lacks a discernable Z2 (Kalish *et al*., 1996). Binding of metal ions at Z1 and Z2 is anti-cooperative in spectrographic analyses of the BRCA1 RING domain, with initial filling of the Z1 site being inhibitory to binding at Z2 (Roehm and Berg, 1997). Finally, treatment of the COP1 RING finger with zinc chelators results in preferential removal of one of two bound zinc atoms (von Arnim and Deng, 1993), suggesting different affinities for the two sites.

Evidence that RING finger-mediated protein–protein interaction can be a critical step in multi-subunit complex assembly comes from studies of the yeast RING-H2 protein Ste5. Mutations in the Ste5 RING-H2 domain specifically disrupt interaction with the G_β subunit Ste4p (Inouye *et al*., 1997b), and this interaction appears to be a prerequisite for oligomerization of Ste5p, also through the RING finger. These results suggest that, in addition to providing a surface for interaction with other proteins, the RING finger may in some cases play a regulatory role in an ordered assembly process.

One possibility, based on the previous examples, is that the MSL2 RING finger might serve as a molecular switch to couple MSL1 interaction at Z1 with a separate function of Z2 or other domains in the protein. The MSL1 and MSL2 proteins are never seen independently bound to the X chromosome and, if either is removed, the remaining MSLs fail to bind any sites on the chromosome (Lyman *et al*., 1997). Thus, interaction of MSL1 at Z1 of the MSL2 RING finger could be an important prerequisite for subsequent protein–protein or protein–nucleic acid interactions, allowing specific X chromosome recognition to proceed.

Materials and methods

Preparation of nuclear extracts

Schneider cell nuclear extracts were prepared using a modification of the procedure of Franke *et al.* (1992). All procedures were performed at 0–4°C. In a typical preparation, $2–4\times10^9$ cells (grown in Schneider medium plus 10% fetal calf serum; Gibco-BRL) were harvested by centrifugation at 2500 *g* for 10 min. The cells were resuspended in 15 ml of buffer B [15 mM HEPES Na^+ pH 7.6, 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors (1 mM benzamidine, 10 μM aprotinin and 1 μg/ml each leupeptin, pepstatin, antipain and soybean trypsin inhibitor)] and homogenized with 35 strokes of a type B pestle in a 15 ml Dounce homogenizer. Nuclei were collected by centrifugation of the homogenate at 2500 *g* for 10 min, washed once in ~50 ml of buffer B, and resuspended in 10 ml of the same buffer. The nuclei were pelleted through a cushion of 10 ml of buffer B plus 0.8 M sucrose at 1300 *g* for 10 min and resuspended in 1 ml of buffer B plus 150 mM KCl per initial 5×10^8 cells (~8 ml). Nuclear proteins were extracted by slow addition of one-twelfth volume of 3.9 M ammonium sulfate (final concentration ~0.3 M) with gentle mixing. The extraction was extended over a period of 1 h with additional mixing by inversion, after which insoluble material, including the nuclear membranes and chromatin, was removed by centrifugation at 25 000 *g* for 1 h. Proteins were precipitated from the resulting supernatant by addition of an equal volume of 3.9 M ammonium sulfate over a period of several minutes with intermittent mixing; precipitation was continued for 30 min on ice, after which the mixture was centrifuged at 25 000 *g* for 15 min. The protein pellet was resuspended in 1 ml of buffer C [25 mM HEPES Na^+ pH 7.6, 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 1 mM PMSF and protease inhibitors] per initial 1×10^9 cells, resulting in a protein concentration of 5–10 mg/ml; aliquots of the extract were frozen in dry ice and stored at –80°C.

Immunoprecipitations and column chromatography

Immunoprecipitations were performed essentially as previously described (Kelley *et al*., 1995) except that 200 μg of protein were used and the IP buffer contained 0.1% Tween-20 in both the binding and wash steps. Columns were controlled by a Waters 626 LC system. For sizing of the MSL complex, 250 μl of Schneider nuclear extract were loaded on a Superose 12 HR 10/30 column (Pharmacia) equilibrated in 50 mM Tris pH 8.0, 0.1 M NaCl, at room temperature. The column was run for 1 h at 0.5 ml/min in the same buffer; 0.5 ml fractions were collected on ice,

frozen at –80°C and lyophilized. The dried fractions were resuspended in 100 μl of Laemmli sample buffer, boiled for 5 min and 10 μl were loaded per gel lane. For ion-exchange chromatography, 0.5 ml of nuclear extract was loaded onto a Mono Q HR 5/5 column (Pharmacia) in 50 mM Tris pH 8.0, 60 mM NaCl. After washing with 10 column volumes of the same buffer, the column was run for 30 min at 1 ml/min in the same buffer plus increasing salt (60–600 mM NaCl linear gradient); 1 ml fractions were collected on ice, precipitated by addition of trichloroacetic acid [20% (w/v) final], acetone washed and dried. The dried fractions were resuspended and loaded as for the Superose 12 column. All gels were 7% SDS–PAGE gels. Western blots were performed as described previously (Palmer *et al*., 1993).

Immunostaining

Log phase cells (100 μl) were centrifuged onto slides at 2000 r.p.m. for 5 min in a Shandon Cytospin 3 cytocentrifuge, washed twice in phosphate-buffered saline (PBS), then fixed by incubation in PBS plus 3.7% formaldehyde for 20 min. The fixed cells were washed twice in PBS, then permeabilized by incubation in PBS plus 0.1% Triton X-100 (PBST). Slides were blocked by incubation in PBS plus 3% bovine serum albumin (BSA) for 30 min, and then washed twice more in PBST. Slides were treated with affinity-purified anti-MSL antibodies for 30 min, washed three times in PBST and then incubated with an appropriate Texas Red or fluorescein isothiocyanate (FITC)-conjugated secondary antibody for 30 min. The stained samples were washed three times in PBST, counterstained for DNA with 30 ng/ml Hoechst 33258, and washed three times for 1 min in PBS. Cells were mounted under 80% glycerol plus 2% *n*-propyl gallate and photographed using a Zeiss Axioscope with Kodak Ektachrome 400 film. All wash steps except the final PBS washes were 5 min.

Conventional two-hybrid assays

Complementary DNAs or cDNA–genomic hybrids containing the MSL-coding regions were cloned into the pAS1 vector (*TRP1* marker) (Durfee *et al*., 1993) to create Gal4 DB fusions, and into pACT2 (*LEU2* marker) (Genebank UID U29899) to create Gal4 AD fusions. The DB–MSL2 (1–190) fusion was constructed from a PCR product engineered to have an *Nco*I site spanning codon 1 of MSL2, and stop codons in all three frames after codon 190. The first stop codon of DB–MSL2 (1–190) is due to the alteration of a *Bcl*I site to a *Bam*HI site, resulting in two *Bam*HI sites in the fusion. For mapping the interaction of DB–MSL2 (1–190) with AD–MSL1, we constructed a library of *Sau*3AI restriction fragments from *msl1* fused to the Gal4 AD in pACT (Durfee *et al*., 1993) and pACT2; other MSL1 subclones were made with available restriction sites. Yeast media were as described previously (Rose *et al*., 1990). The yeast two-hybrid hosts for the DB and AD fusions were Y153 (Durfee *et al*., 1993) and Y187 (Harper *et al*., 1993), respectively. Diploid cells expressing the various fusions were assayed for activity of the two-hybrid *lacZ* reporter after transfer to nylon filters (NitroPlus, MSI) (Breeden and Nasmyth, 1985). Filters were incubated for up to 6 h at 30°C in covered Petri dishes, and the interactions in Figure 3 were visible within 1 h.

PCR mutagenesis of MSL2

The error rate of *Taq* polymerase (Perkin-Elmer) was enhanced using manganese chloride and altered dNTP concentrations (Fromant *et al*., 1995). Libraries were constructed from PCR products made under two conditions, using primers spanning the *msl2* start and stop codons (*Nco*I and second *Bam*H1 sites) of the DB–MSL2 (1–190) plasmid. Conditions for the 'GATC' library (16 000 clones complexity) were 0.15 μg of template DNA, 0.56 mM dATP, 0.9 mM dCTP, 0.2 mM dGTP and 1.4 mM dTTP in PCR buffer (3.2 mM $MgCl_2$, 0.5 mM $MnCl_2$, 50 mM KCl, 10 mM Tris pH 8.3 and 2 nmol/μl each primer) for 20 cycles. Conditions for the 'fC' library (3000 clones complexity) were 0.15 μg of template DNA, 0.2 mM dATP, 3.4 mM dCTP, 0.2 mM dGTP and 0.2 mM dTTP in PCR buffer for 16 cycles. In each case, the PCR products from four reactions were cloned into unmutagenized, DB–MSL2 (1–190) plasmid cut with *Nco*I and *Bsp*EI. The libraries were amplified once by passage through bacteria before use.

Reverse two-hybrid screening

The yeast host for the reverse two-hybrid screen (Y166, *MAT***a** *leu2-3, 112, ura3-52, his3Δ200, ade2-101, gal4Δgal80Δ, GAL–lacZ, LYS2::GAL–HIS3, RNR3::GAL–URA3*) was a gift of Stephen Elledge and is derived from Y162 (Durfee *et al*., 1993) by insertion of the *GAL–URA3* reporter. For the reverse two-hybrid screen, Y166 also carried the AD–MSL1 plasmid. MSL2 mutant libraries were introduced into this strain using lithium acetate transformation (Gietz and Woods, 1990). The heat-shocked cells were recovered in liquid SC-Trp-Leu medium for 2 h, washed in sterile water, then plated on SC-Trp-Leu-Ura plus 50 μg/ml uracil and 1 mg/ml 5-FOA. Foa^R colonies were picked as they arose, between 5 and 7 days after plating. All Foa^R colonies were patched to SC-Trp-Leu plates, from which a secondary screen for activation of the *GAL–lacZ* reporter was performed on filters as described above.

For PCR of *msl2* plasmid sequences from $LacZ^–$ (white) colonies, ~1 μl of yeast cells were incubated in 10 μl of SorPZy buffer (1.2 M sorbitol, 0.1 M $NaPO_4$ pH 7.4) (Ling *et al*., 1995) for 20 min and boiled for 10 min, after which 1 μl of the solution was used in a 35-cycle 25 μl PCR. For the Western analysis, 1.5 ml of log phase cells in SC-Trp-Leu were resuspended in ~350 μl of Laemmli sample buffer (on ice) and broken by vigorous shaking with 400 μl of 0.45–0.6 mm glass beads (Sigma) for 10 min at 4°C. The broken cells were boiled for 5 min, and 50 μl were loaded onto 12% SDS–PAGE gels to detect the 37 kDa DB–MSL2 (1–190) fusion protein. Plasmids were recovered into *Escherichia coli* DH5α as described (Hoffman and Winston, 1987) and were sequenced using an ABI 377 automated sequencer. For each mutant, the interaction defect was confirmed by transformation of the plasmid back into yeast (Y153) and mating to Y187 expressing the AD–MSL1 fusion. Double mutations on either side of the first *Bam*HI site of the MSL2 (1–190) plasmid were separated by ligation of a *Bam*HI fragment (encoding residues 50–190 of MSL2) or the rest of the plasmid (encoding residues 1–49) to the opposite, unmutated fragment to generate two single-mutant plasmids. Two independent isolates of each subcloned mutation were then retested for their effects in the two-hybrid *lacZ* activity assay. Approximately 8000 and 14 000 transformants were screened from the GATC and fC mutant libraries (above) (~0.5 and 5 times complexity, respectively). Of these, 43 were Foa^R and $LacZ^–$, 22 still contained *msl2* sequences, and 19 made a detectable fusion protein.

Drosphila virilis MSL2 cloning

A 180 bp probe specific for the RING finger was generated by PCR of *D.virilis* genomic DNA (25 ng of DNA, 56°C annealing, 1.5 mM $MgCl_2$, for 30 cycles) using the primers 5′-CGCCCAAGGGCAAGCGG-<u>GC</u>CCAGCACAACGTGTGCC-3′ and 5′-GAGTGGAGCAGGTGGA-3′. The first primer was used previously for mutation of C59 of the RING finger and contains a 2 bp mismatch (underlined) with the *D.melanogaster* gene. The PCR product was used to screen a genomic DNA library in EMBL3 (gift of Ron Blackman); one positive clone was isolated by high stringency hybridization and was sequenced using dye terminator chemistry on an ABI 377 sequencer.

Acknowledgements

We thank Steve Elledge for the Y166 reverse two-hybrid host and for advice on the two-hybrid system, John Lucchesi for *msl2*[γ136] flies, Ron Blackman for the *D.virilis* genomic library, John Tamkun for anti-BRM antibodies, and Casey Downs for assistance in cloning *D.virilis msl2*. We thank Max Scott for communication of results prior to publication, and Rick Kelley and Steve Elledge for critical reading of the manuscript. This work was supported by the National Institutes of Health grant GM-45744, the Texas Higher Education Advanced Technology Program and the Howard Hughes Medical Institute. K.A.C. is an NIH postdoctoral fellow (grant HG00177-01). M.I.K. is an Associate Investigator of the Howard Hughes Medical Institute.

References

Amrein,H. and Axel,R. (1997) Genes expressed in neurons of adult male *Drosophila*. *Cell*, **88**, 459–469.

Baker,B.S., Gorman,M. and Marín,I. (1994) Dosage compensation in *Drosophila*. *Annu. Rev. Genet.*, **28**, 491–521.

Barlow,P.N., Luisi,B., Milner,A., Elliott,M. and Everett,R. (1994) Structure of the C_3HC_4 domain by ^1H-nuclear magnetic resonance spectroscopy. *J. Mol. Biol.*, **237**, 201–211.

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

Belote,J. and Lucchesi,J.C. (1980) Male-specific mutations of *Drosophila melanogaster*. *Genetics*, **96**, 165–186.

Bone,J.R. and Kuroda,M.I. (1996) Dosage compensation regulatory proteins and the evolution of sex chromosomes in *Drosophila*. *Genetics*, **144**, 705–713.

Bone,J.R., Lavender,J., Richman,R., Palmer,M.J., Turner,B.M. and Kuroda,M.I. (1994) Acetylated histone H4 on the male X chromosome is associated with dosage compensation in *Drosophila*. *Genes Dev.*, **8**, 96–104.

Borden,K.L.B., Boddy,M.N., Lally,J., O'Reilly,N.J., Martin,S., Howe,K., Solomon,E. and Freemont,P.S. (1995) The solution structure of the RING finger domain from the acute promyelocytic leukaemia proto-oncoprotein PML. *EMBO J.*, **14**, 1532–1541.

Brachmann,R.K., Vidal,M. and Boeke,J.D. (1996) Dominant-negative p53 mutations selected in yeast hit cancer hot spots. *Proc. Natl Acad. Sci. USA*, **93**, 4091–4095.

Breeden,L. and Nasmyth,K. (1985) Regulation of the yeast HO gene. *Cold Spring Harbor Symp. Quant. Biol.*, **50**, 643–650.

Brownell,J.E., Zhou,J., Ranalli,T., Kobayashi,R., Edmondson,D.G., Roth,S.Y. and Allis,C.D. (1996) *Tetrahymena* histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. *Cell*, **84**, 843–851.

Cline,T.W. and Meyer,B.J. (1996) Vive la differénce: males versus females in flies and worms. *Annu. Rev. Genet.*, **30**, 637–702.

Durfee,T., Becherer,K., Chen,P.-L., Yeh,H.-H., Yang,Y., Kilburn,A.E., Lee,W.-W. and Elledge,S.J. (1993) The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev.*, **7**, 555–569.

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

Franke,A., DeCamillis,M., Zink,D., Cheng,N., Brock,H.W. and Paro,R. (1992) *Polycomb* and *polyhomeotic* are constituents of a multimeric protein complex in chromatin of *Drosophila melanogaster*. *EMBO J.*, **8**, 2941–2950.

Freemont,P.S. (1993) The RING finger. A novel protein sequence motif related to the zinc finger. *Ann. NY Acad. Sci.*, **684**, 174–192.

Fromant,M., Blanquet,S. and Plateu,P. (1995) Direct random mutagenesis of gene-sized DNA fragments using polymerase chain reaction. *Anal. Biochem.*, **224**, 347–353.

Fukunaga,A., Tanaka,A. and Oishi,A. (1975) *Maleless*, a recessive autosomal mutant of *Drosophila melanogaster* that specifically kills male zygotes. *Genetics*, **81**, 135–141.

Gietz,R.D. and Woods,R. (1990) High efficiency transformation with lithium acetate. In Johnston,J.R. (ed.), Oxford University Press, Oxford.

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

Gu,W., Szauter,P. and Lucchesi,J.C. (1998) Targeting of MOF, a putative histone acetyl transferase, to the X chromosome of *Drosophila melanogaster*. *Dev. Genet.*, **22**, 56–64.

Harper,J.W., Adami,G.R., Wei,N., Keyomarsi,K. and Elledge,S.J. (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G_1 cyclin-dependent kinases. *Cell*, **75**, 805–816.

Hilfiker,A., Hilfiker-Kleiner,D., Pannuti,A. and Lucchesi,J.C. (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.

Hoffman,C.S. and Winston,F. (1987) A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene*, **57**, 267–272.

Inouye,C., Dhillon,N., Durfee,T., Zambrysik,P.C. and Thorner,J. (1997a) Mutational analysis of *STE5* in the yeast *Saccharomyces cerevisiae*: application of a differential interaction trap assay for examining protein–protein interactions. *Genetics*, **147**, 479–492.

Inouye,C., Dhillon,N. and Thorner,J. (1997b) Ste5 RING-H2 domain: role in Ste4-promoted oligomerization for yeast pheromone signalling. *Science*, **278**, 103–106.

Kalish,J.E., Keller,G.-A., Morrell,J.C., Mihalik,S.J., Smith,B., Cregg, J.M. and Gould,S.J. (1996) Characterization of a novel component of the peroxisomal protein import apparatus using fluorescent peroxisomal proteins. *EMBO J.*, **15**, 3275–3285.

Kanno,M., Hasegawa,M., Ishida,A., Isono,K. and Taniguchi,M. (1995) *mel-18*, a Polycomb group-related mammalian gene, encodes a transcriptional negative regulator with tumor suppressive activity. *EMBO J.*, **14**, 5672–5678.

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

Kelley,R.L., Solovyeva,I., Lyman,L.M., Richman,R., Solovyev,V. and Kuroda,M.I. (1995) Expression of MSL-2 causes assembly of dosage compensation regulators on the X chromosomes and female lethality in *Drosophila*. *Cell*, **81**, 867–877.

Koonin,E.V., Zhou,S. and Lucchesi,J.C. (1995) The chromo superfamily: new members, duplication of the chromo domain and possible role in delivering transcription regulators to chromatin. *Nucleic Acids Res.*, **23**, 4229–4233.

Kuo,M.-H., Brownell,J.E., Sobel,R.E., Ranalli,T.A., Cook,R.G., Edmondson,D.G., Roth,S.Y. and Allis,C.D. (1996) Transcription-linked acetylation by Gcn5p of histones H3 and H4 at specific lysines. *Nature*, **383**, 269–272.

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

Lee,C.-G., Chang,K.A., Kuroda,M.I. and Hurwitz,J. (1997) The NTPase/helicase activities of *Drosophila* maleless, an essential factor in dosage compensation. *EMBO J.*, **16**, 2671–2681.

Ling,M., Merante,F. and Robinson,B.H. (1995) A rapid and reliable DNA preparation method for screening a large number of yeast clones by polymerase chain reaction. *Nucleic Acids Res.*, **23**, 4924–4925.

Lovering,R. *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 Manning,J.E. (1987) Gene dosage compensation in *Drosophila melanogaster*. *Adv. Genet.*, **24**, 371–429.

Lucchesi,J.C., Skripsky,T. and Tax,F.E. (1982) A new male-specific mutation of *Drosophila melanogaster*. *Genetics*, **82**, s42.

Lyman,L.M., Copps,K., Rastelli,L., Kelley,R.L. and Kuroda,M.I. (1997) *Drosophila* male-specific lethal-2 protein: structure/function analysis and dependence on MSL-1 for chromosome association. *Genetics*, **147**, 1743–1753.

Marín,I., Franke,A., Bashaw,G. and Baker,B.S. (1996) The dosage compensation system of *Drosophila* is co-opted by newly evolved X chromosomes. *Nature*, **383**, 160–163.

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

Ogryzko,V.V., Schiltz,R.L., Russanova,V., Howard,B.H. and Nakatani,Y. (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell*, **87**, 953–959.

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

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

Patterson,J.T. and Stone,W.S. (1952) *Evolution of the Genus Drosophila*. McMillan, New York.

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

Roehm,P.C. and Berg,J.M. (1997) Sequential metal binding by the RING finger domain of BRCA1. *Biochemistry*, **36**, 10240–10245.

Rose,M.D., Winston,F. and Hieter,P. (1990) *Methods in Yeast Genetics. A Laboratory Course Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Ryner,L.C. and Baker,B.S. (1991) Regulation of doublesex pre-mRNA processing occurs by 3′-splice site activation. *Genes Dev.*, **5**, 2071–2085.

Shattuck-Eidens,D. *et al.* (1995) A collaborative survey of 80 mutations in the BRCA1 breast and ovarian cancer susceptibility gene. Implications for presymptomatic testing and screening. *J. Am. Med. Assoc.*, **273**, 535–541.

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

Turner,B.M., Birley,A.J. and Lavender,J. (1992) Histone H4 isoforms acetylated at specific lysine residues define individual chromosomes and chromatin domains in *Drosophila* polytene nuclei. *Cell*, **69**, 375–384.

Uchida,S., Uyenoyama,T. and Oishi,K. (1981) Studies on the sex-specific lethals of *Drosophila melanogaster*. III. A third chromosome male-specific mutant. *Jap. J. Genet.*, **56**, 523–527.

von Arnim,A.G. and Deng,X.-W. (1993) Ring finger motif of *Arabidopsis thaliana* COP1 defines a new class of zinc-binding domain. *J. Biol. Chem.*, **268**, 19626–19631.

Zhou,S. *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.

Received February 16, 1998; revised and accepted July 16, 1998